

ORIGINAL CONTRIBUTION

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Agnucastoside C, isolated from *Moringa oleifera* ameliorates thyrotoxicosis and liver abnormalities in female mice

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Abstract

Background: In the present investigation an attempt was made to evaluate the potential of agnucastoside C (ACC), isolated from the leaves of *Moringa oleifera* in ameliorating thyrotoxicosis, hepatic lipid peroxidation (LPO) and hyperglycemia in female mice.

Methods: L-thyroxine (L-T₄) at 0.5 mg/kg/d was administered through intra-peritoneal route for 15 consecutive days to induce thyrotoxicosis and then the effects of three different doses (10, 20 and 30 mg/kg body weight) of the isolated ACC for 21 days were investigated on the changes in serum thyroid hormones, insulin, glucose, different lipids; hepatic lipid peroxidation, enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase, advanced oxidation protein products, reduced glutathione and in lipid peroxides.

Results: Following the administration of L-T₄, serum T₃, T₄, insulin, glucose levels and the tissue LPO were increased with a decrease in serum thyroid stimulating hormone and antioxidative enzymes. However, administration of the test compound to hyperthyroid animals significantly decreased the levels of thyroid hormones, glucose and lipid peroxidation and normalized the concentration of insulin and tissue antioxidants, suggesting its antithyroid, antihyperglycemic and antiperoxidative potential. The T₄-induced adverse effects on liver histology were also abolished. These findings suggest the possible use of the test compound in ameliorating thyrotoxicosis.

Conclusion: ACC exhibited antithyroidic, antihypoglycemic, antioxidative and insulin normalizing activities.

Keywords: Agnucastoside C, Insulin, Thyrotoxicosis, Lipid peroxidation, Mice

Background

Thyrotoxicosis commonly results due to an increase in the secretion of thyroid hormones. Its timely treatment is very much required as its long term effect may cause excessive oxidative damage to liver [1] and can cause hyperglycemia as well [2]. Although conventional drugs such as Neomercazole and Methimazole are commonly prescribed, they often cause side effects. Therefore, now-a-days plant products are preferred that are believed to exhibit no or less adverse effects.

An earlier report from our laboratory indicated that the aqueous leaf extract of *Moringa oleifera* leaves reduces the concentration of thyroid hormones in female animals, indicating its thyroid inhibitory potential [3]. This led us to presume that its active compound may have the potential of regulating thyrotoxicosis and hence the present investigation was planned.

Moringa oleifera belongs to the family of *Moringaceae* and it is considered as an effective therapy for malnutrition, diabetes mellitus and cancer [4]. Its aqueous extract exhibits strong scavenging effect on 2, 2-diphenyl-2-picryl hydrazyl (DPPH) free radicals [5]. According to a recent review, this plant has analgesic, anti-inflammatory, antipyretic, antioxidative, hepatoprotective, gastroprotective, anti-ulcer, cardiovascular,

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antiepileptic, anti-asthmatic, anti-diabetic, anti-urolithiatic, diuretic, local anesthetic, anti-allergic, wound healing, antimicrobial, immunomodulatory, and antidiarrheal properties [6].

Agnucastolide C (7-O-trans-p-coumaroyl-6-O-trans-caffeoyl-8-epiloganic acid), an important iridoid glycoside of *Moringa*, having coumaroyl-caffeoyl-epiloganic acid moiety was recently isolated from its leaves by Panda et al. [7] in our laboratory and was found to be effective in preventing myocardial injury as well as tissue lipid peroxidation. Earlier, iridoid glycosides were also associated with hepato-protective effects against oxidative stress, anti-inflammatory, hypoglycaemic, antioxidative, hypolipidemic, and immunomodulatory activities [8–10]. However, no information till to date is available on the impact of iridoid glycosides in regulating thyroid function. Higher levels of circulating thyroid hormones are known to be associated with oxidative stress in diseased condition. Therefore, the present study was undertaken to investigate the role of Agnucastolide C (ACC) in altering the status of thyroid hormones, lipid peroxidation and the antioxidants in thyrotoxic mice.

As thyrotoxicosis is known to be associated with insulin resistance [11], in this study we also tried to find out the possible linkage between thyroid stimulating hormone (TSH) and insulin resistance (IR) in thyrotoxic mice.

Methods

Chemicals

L-thyroxine (L-T₄) was purchased from Sigma-Aldrich chemicals (St. Louis, MO, USA), India. While Elman's reagent, m-phosphoric acid, thiobarbituric acid (TBA), sodium dodecyl sulphate, tricarboxylic acid and hydrogen peroxide (H₂O₂) were obtained from E. Merck Ltd., Mumbai; ELISA kits for T₃ and T₄ estimations were purchased from Rapid Diagnostic Pvt. Ltd., Delhi, India. For the estimation of serum glucose and different lipids, specific kits were obtained from Span Diagnostic Limited, India. All other chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

Isolation of Agnucastolide

The test compound, ACC used in the present investigation was isolated earlier in our laboratory and stored at -20 °C [7]. Before using in the experiment, it was dissolved in distilled water and required doses were prepared.

Animals

Swiss albino female mice weighing 25–30 g were selected for the study. Animals were housed in polypropylene cages (43 X 23 X 15 cm) and provided with standard animal feed ad libitum and free access to drinking water.

All the animals were acclimatized for 7 days prior to the start of the experiment.

Experimental design

Thirty five healthy female mice were divided into five groups of seven each and acclimatized for a week. The animals of Group I were given only distilled water (0.1 ml/day/animal) and considered as the control; whereas groups II, III, IV and V were administered with L-T₄ (500 µg /kg, i.p.) for 15 days to induce thyrotoxicosis [12]. Then from the 16th day onwards, animals of group III, IV and V were administered with oral dose of test compound, ACC at 10, 20 and 30 mg/kg respectively for 21 days. After the completion of experiment, animals were kept on fasting and then sacrificed with mild anesthesia; serum was obtained from blood for the estimation of triiodothyronine (T₃) and thyroxine (T₄), insulin, glucose and different lipids. Liver tissue was excised quickly, stored and washed in ice cold phosphate-buffered saline (PBS) and used to estimate the level of lipid peroxidation, superoxide dismutase, catalase and glutathione peroxidase activities, glutathione content, lipid peroxides and advanced oxidation protein products .

Estimation of biochemical parameters

Serum parameters

Estimation of total circulating thyroid hormones (T₃ and T₄) and TSH was performed by enzyme-linked immunosorbent assay (ELISA) kits, while serum total cholesterol (TC), HDL-cholesterol (HDL-C), and triglycerides (TG) were measured using specific biochemical kits as routinely done in our laboratory [12].

Oxidative stress biomarkers

Hepatic lipid peroxidation (LPO) To determine the extent of LPO, thiobarbituric acid substances (TBARS) were measured using an earlier method [13]. For this, a liver homogenate (10%) was prepared in 50-mM phosphate buffer (pH 7.4) and centrifuged at 17,000×g for 30 min at 4 °C. The supernatant (0.2 ml) was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 30% acetic acid (pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid. This reaction mixture was heated for 1 h at 95 °C and then cooled. After cooling, 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 20 min. The absorbance of the pink colored organic layer was measured.

Lipid hydroperoxide (LOOH) For its estimation, the procedure of Jiang et al. [14] was followed. Fox reagent (1 mM xylene orange and 2.5 mM ammonium ferrous sulfate) was dissolved in 250 mM H₂SO₄, and one volume of this reagent was added to 9 volume of methanol

containing 4.4 mM BHT to make final working reagent. A 0.1 ml of the liver tissue supernatant was mixed with 0.9-ml fox reagent and incubated for 30 min at room temperature (RT), following which the absorbance was read at 560 nm. Level of liver LOOH was expressed as nanomoles LOOH formed/mg protein.

Advanced oxidation protein products (AOPP) The concentration of AOPP was measured by spectrophotometry method as described by Witko-Sarsat et al. [15]. Liver tissue samples were homogenized by 50 mM ice-cold Tris-HCl buffer (pH 7.4) and centrifuged at 5000 g for 10 min. Tissue supernatants were then diluted in phosphate buffered saline at a ratio of 1: 5. The sample was mixed with potassium iodide and acetic acid, and the absorbance of the reaction mixture was immediately read at 340 nm. Chloramine was chosen as a standard and the AOPP concentrations were expressed as μmol per mg protein.

Superoxide dismutase (SOD)

For the study of SOD activity, the method of Marklund and Marklund [16] was followed and was expressed in units per milligram of protein. Pyrogallol auto-oxidation inhibition assay method is commonly used for the determination of activity of SOD, where the rate of auto-oxidation is calculated from the increase in absorbance at 420 nm. DTPA was used as a chelator to prevent the interference of Fe^{++} , Ca^{++} , and Mn^{++} . Tris-HCl buffer, tissue supernatant, and pyrogallol were added in a 3-ml corvette, and the absorbance of the samples was taken at 420 nm at an interval of 30 s.

Catalase (CAT)

For this, the method of Aebi [17] was used that is based on the decomposition of H_2O_2 . In brief, the 50- μl tissue supernatant, 1.0 ml of 50-mM phosphate buffer (pH 7), and 0.1 ml of 30 mM H_2O_2 were added and a decrease in absorbance was measured every 5 s for 30 s at 240 nm. Finally, the CAT activity was expressed as micromoles of H_2O_2 decomposed per minute per milligram of protein.

Reduced glutathione (GSH)

The method of Ellman [18] is often followed for the estimation of GSH content in which 5, 5-dithio-bis-2-nitrobenzoic acid and DTNB reagents are used. GSH reacts with DTNB to produce a yellow-colored 2-nitro-5-mercapto-benzoic acid, and the absorbance is taken at 412 nm. Tissue supernatant (0.5 ml) was pipetted out and precipitated with 2.0 ml of 5% TCA. After centrifugation, 1.0 ml of the supernatant was taken and to it added 0.5 ml of Ellman's reagent and 3.0 ml of phosphate buffer. The yellow color so developed was read at 412 nm and expressed in micromoles of GSH per milligram protein.

Glutathione peroxidase (GPx)

GPx was estimated by the method of Rotruck et al. [19]. Briefly, 0.2 ml of tris buffer (0.4 M, pH 7.0), 0.2 ml of ethylene di-amine-tetra-acetic acid (EDTA), 0.1 ml of sodium azide, and 0.5 ml of liver homogenate were mixed. To this mixture, 0.2 ml of glutathione and 0.1 ml of H_2O_2 were also added. The contents were mixed well and incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was used for the estimation of glutathione following the method of Ellman [19].

Histological study

The liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5- μm serial sections and the sections were stained by haematoxylin and eosin (H&E) to study the histological architecture.

Statistical analysis

Graph Pad Prism 5 was used for statistical calculations (Graph pad Software, San Diego California, USA) and the results of all biological studies were expressed as means \pm S.E.M. One-way analysis of variance (ANOVA), followed by Newman-Keul's test was used to determine the significance level between the 2 groups of animals. A level of $P < 0.05$ was considered statistically significant.

Results

Thyroid hormone levels and antioxidant system

After administration of L- T_4 , the level of serum T_3 and T_4 were significantly elevated ($P < 0.001$) showing the induction of thyrotoxicosis. It also increased both insulin and serum glucose levels as compared to the value of control group ($P < 0.001$ and $P < 0.01$ for insulin and glucose respectively). While following the treatment with ACC in T_4 -induced mice, a significant reduction in serum T_3 , T_4 and in serum glucose concentrations was noticed, serum insulin level was brought back to normal level (Fig. 1). However, out of the 3 different doses, the lowest dose (10 mg/kg) was found to be most effective.

Figure 2c shows the changes in the body weight (b.wt.) of mice by different drug treatment. T_4 treatment caused a significant reduction in b.wt. ($P < 0.01$). However, a significant increase ($P < 0.001$) in b.wt. was observed at lowest dose of ACC treated T_4 -induced mice. Medium dose also caused an increase ($P < 0.05$) in b.wt. as compared to T_4 treated animals, but in the highest dosed group, no significant change was observed as compared to that of T_4 alone treated animals.

We also found significant increase in oxidative markers such as MDA and LOOH ($P < 0.01$, for both) in L- T_4 -treated group as compared to the corresponding value in control animals. However, following ACC

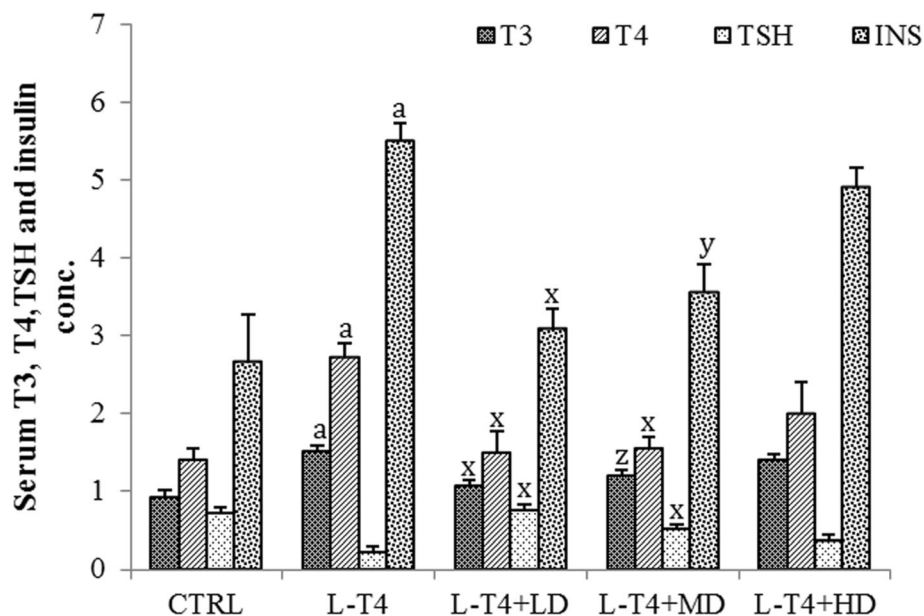


Fig. 1 Effects of ACC on the alterations in serum concentrations of T₃ (ng mL⁻¹), T₄ (ng mL⁻¹ × 10) and TSH (μIU/ml) in L-thyroxine (L-T₄)-induced hyperthyroid mice. Each bar represents the mean ± s.e.m., n = 7. Ctrl, Control; T₃, triiodothyronine; T₄, thyroxine. ^a P < 0.001 as compared to the respective control values. ^x P < 0.001, ^y P < 0.01 and ^z P < 0.05 as compared to the respective values of L-T₄ treated animals

treatment in T₄-induced mice, the low dose of ACC decreased both MDA and LOOH levels. In this group, AOPPs levels were also decreased as compared to the hyperthyroid animals (Fig. 3). Out of the three doses (10, 20, 30 mg/kg/b. wt.), the lowest dose (10 mg/kg), effectively decreased (by 50%) the concentration of MDA. In mice, receiving only L-T₄, there was a decrease in the endogenous enzyme activities such as SOD, CAT and GPx and in non-enzymatic antioxidant, GSH. However, when ACC was administered the levels of these antioxidative enzymes were increased effectively.

Effects on serum lipid profile

A significant decrease in the concentrations of different serum lipids such as TG (*P* < 0.05), TC (*P* < 0.001) and HDL-C (*P* < 0.05) was also observed in T₄-induced rats (Fig. 4). All these alterations indicate induction of thyrotoxicosis. Interestingly, when ACC was administered to thyrotoxic animals, these altered effects were normalized by the low dose.

Histological evaluation

Using Image-Pro Plus 6.0, quantitative analyses of HE stained liver slices were performed. Liver section of control animal showed normal hepatic cells, intact central vein and sinusoidal space. However, in thyrotoxic mouse degeneration of normal hepatic cells, severe centrilobular damage, and inflammatory cells were seen. While ACC administration in thyrotoxic mice at low

dose showed less degeneration of hepatocytes, less inflammatory cells, intact central vein similar to control; at high dose, moderate changes were observed such as distortion of hepatocytes and centrilobular degeneration with more inflammatory cells (Fig. 5). However, ACC treatment at a low dose in T₄-induced mice normalized the hepatocytes and inflammatory cell counts.

Discussion

In this study we evaluated the effects of ACC in T₄-induced thyrotoxic mice. T₄-induced thyrotoxicosis condition was confirmed in mice as the thyroid hormone concentrations and levels of tissue lipid peroxidation were increased with a parallel decrease in body weight. In addition, alterations in different serum lipids and in liver histology were also observed in the same manner as found earlier [12]. However, ACC treatment, particularly at low dose in T₄-induced mice improved the body weight, nearly normalized the thyroid hormones and TSH levels indicating the inhibitory activity of ACC in thyroid hormone synthesis and/or release. Interestingly, when the effects of three different doses were compared, the percentage inhibition in the T₃ concentration was found to be maximum in lowest dosed group (28%), showing the higher inhibitory ability. It also effectively decreased T₄ (by 55%) and normalized the TSH level that is considered as an important parameter because of its highest sensitivity and clinically relevant diagnostic parameter of thyroid disorders [20]. Higher doses of ACC were not found to be effective. Earlier also a study

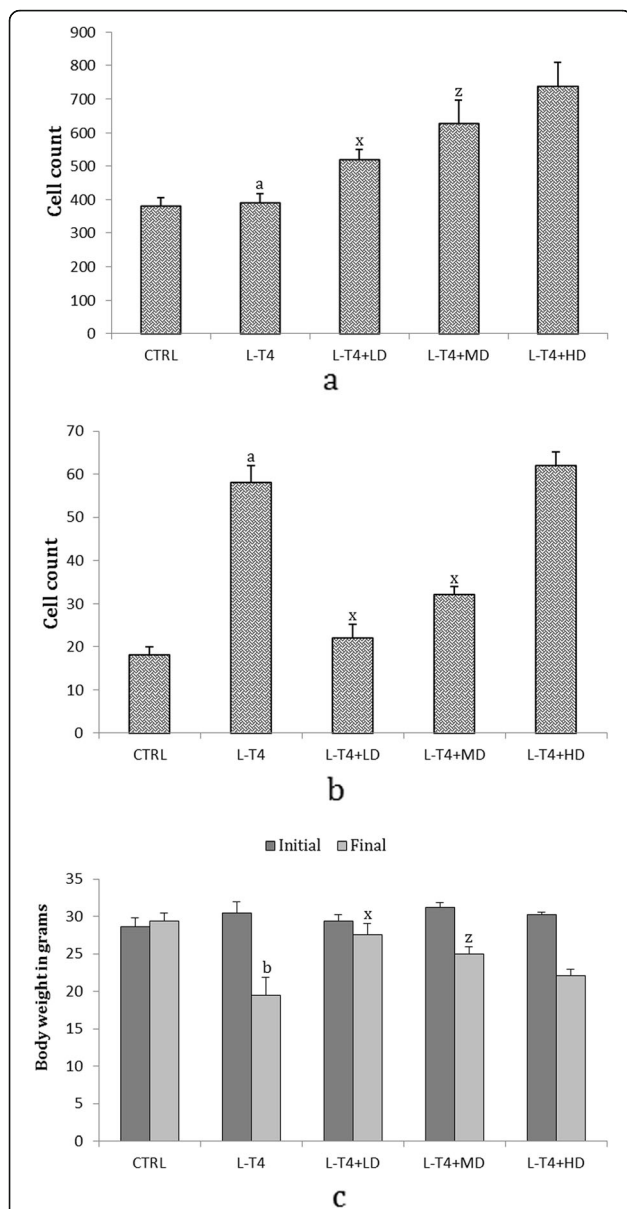


Fig. 2 **a** Effects of ACC on the number of hepatocytes counts in mice with T₄-induced thyrotoxicosis. Each bar represents the mean \pm s.e.m., n = 7. Ctrl, Control; T₃, triiodothyronine; T₄, thyroxine. ^a $P < 0.001$ as compared to the respective control values. ^x $P < 0.001$ and ^z $P < 0.05$ as compared to the respective values of L-T₄ treated animals. **b** Effects of ACC on the number of inflammatory cell counts in mice with T₄-induced thyrotoxicosis. Each bar represents the mean \pm s.e.m., n = 7. Ctrl, Control; T₃, triiodothyronine; T₄, thyroxine. ^a $P < 0.001$ as compared to the respective control values. ^x $P < 0.001$ as compared to the respective values of L-T₄ treated animals. **c** Effects of the test compound, ACC on body weight, in hyperthyroid mice. Each bar represents the mean \pm s.e.m., n = 7. ^b $P < 0.01$ as compared to the respective control values. ^x $P < 0.001$ and ^z $P < 0.05$ as compared to the respective values of L-T₄ treated animals

from our laboratory on cardiac function indicated the toxic effects of ACC, when used at a concentration more than 30 mg/kg [12]. Also in some other drugs/active

compounds similar toxic effect have been reported at higher concentrations [21, 22].

Thyroid hormones have significant effects not only on glucose and lipid metabolism, but also on the development of insulin resistance [23, 24]. Our results also revealed elevated glucose concentrations and higher insulin levels in thyrotoxic mice, which may be due to a decrease in binding of insulin to blood cells and abnormal lipid levels [11]. However, in this study higher insulin concentration in thyrotoxic mice was restored to normal level following the treatment with ACC supporting anti-hyperglycaemic role of ACC. Previously, it has been reported that iridoid glycoside regulates abnormal NF- κ B, cyclooxygenase-2 and inducible nitric oxide synthase (iNOS). COX-2 and iNOS expressions were known to be up-regulated under insulin resistance, obesity, hyperglycemia, and oxidative stress [25, 26]. Therefore, in this study, possibly ACC decreased the insulin resistance and ameliorated the deleterious effects on liver in hyperthyroid animals through the regulation of NF- κ B, COX-2, and iNOS. Thyrotoxicosis is known to be associated with oxidative stress. Some reports suggest that hyperthyroidism is associated with increased production of ROS, enhanced oxidative stress and lipid peroxidation [27, 28]. In traditional medicine, iridoid glycosides from plants have been reported to show a promising protective effect against liver disorders [29]. In our study also we found the higher levels of oxidative markers in the liver of T₄-induced thyrotoxic mice. Interestingly, the anti-peroxidative effect of ACC was shown by all the three doses as evident from the decreased hepatic LPO and increased levels of antioxidative enzymes, namely SOD, CAT and GPx. Decrease in LPO level does suggest the free radical scavenging activity of the test compound. Studies have shown that natural products regulate oxidative stress in the liver by modulating Nrf2-ARE pathway to render hepatoprotective effects [30, 31].

A previous study reported that an iridoide glycoside, named loganin, regulated the protein expressions of Nrf-2 and HO-1 in hepatic tissue, and it protects liver against oxidative stress. Nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor is a key regulator of oxidative stress in numerous cell types including hepatocytes. In fact, Nrf2 plays a crucial role in cellular antioxidant signaling and sensitivity to oxidative stress. In our study we found that the activities of SOD, CAT and GPx were increased as the Nrf2 target genes regulate the antioxidants such as glutathione peroxidases, superoxide dismutase and catalase. Hence, the increase in antioxidant levels by ACC appears to be mediated via Nrf2 activation.

With respect to different serum lipids such as TG, TC and HDL, all three were decreased in T₄-induced thyrotoxic animals as reported earlier [32]. However, following ACC administration along with T₄, aforesaid parameters of lipids were improved. Normal levels of lipids

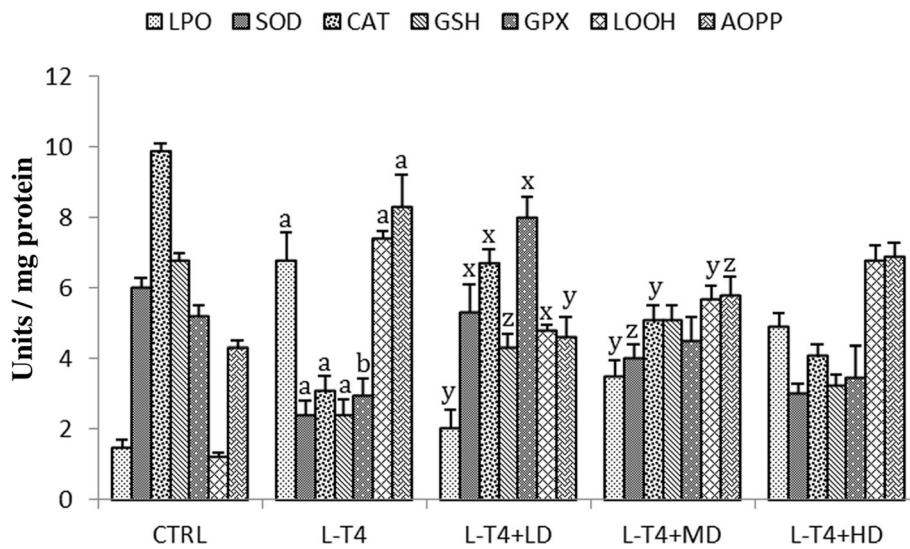


Fig. 3 Effects of ACC on the alterations in hepatic LPO (nm of MDA formed /h /mg protein), SOD (Units / mg protein), CAT (μM of H_2O_2 decomposed per minute per mg protein), GPx (μ moles of GSH oxidized/mg protein) and GSH (μM GSH per mg protein) in liver tissues. Each bar represents the mean \pm s.e.m., $n = 7$. Ctrl, Control; T_3 , triiodothyronine; T_4 , thyroxine. ^a $P < 0.001$ as compared to the respective control values. ^x $P < 0.001$, ^y $P < 0.01$ and ^z $P < 0.5$ as compared to the respective values of L- T_4 treated animals. GLU, glucose; CHOL, cholesterol; HDL, high density lipoprotein and TG, triglyceride

concentrations by ACC clearly indicated its efficacy against thyrotoxicosis.

With respect to the alterations in the histological structure of liver, positive changes were observed in ACC treated animals. It has been reported that, in thyrotoxic state the number of inflammatory cells and hepatocytes counts were increased [12]. In this study, we observed that ACC improved the liver histology as well as the hepatocytes and inflammatory cell counts that

were reduced in thyrotoxic mice. This improvement could be explained by the attenuation of oxidation and inflammatory response by the liver through regulation of the transcription factor NF- κB [33]. Cytokines play critical pathological role in liver necrosis, increase vascular permeability and mediate inflammatory cell activation and apoptosis of hepatocytes [34]. NF- κB is a transcription factor which is known to be involved in controlling the expression of various genes including, TNF- α , IL-1 β and

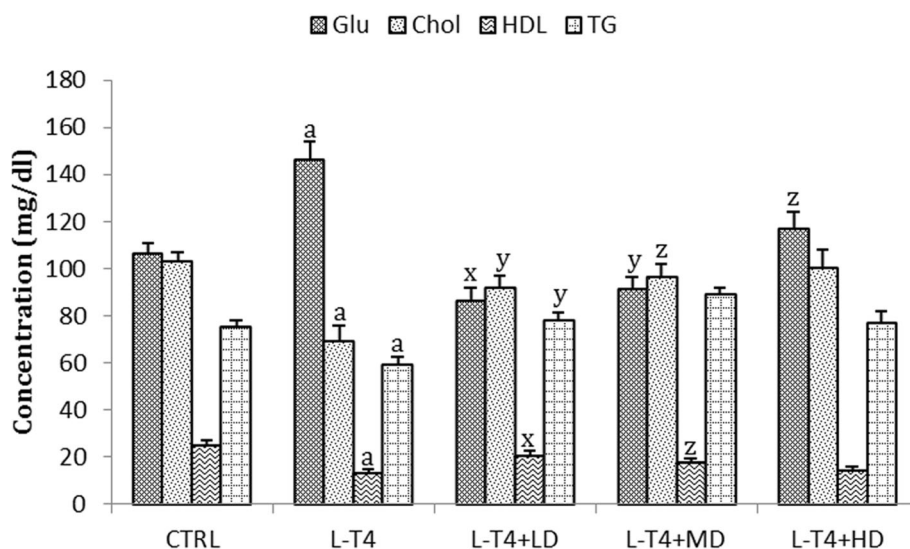


Fig. 4 Effects of ACC extract on the alterations in serum glucose and different lipids. Each bar represents the mean \pm s.e.m., $n = 7$. Ctrl, Control; L- T_4 , L-thyroxine. ^a $P < 0.001$ as compared to the respective control values. ^x $P < 0.001$, ^y $P < 0.01$ and ^z $P < 0.5$ as compared to the respective values of L- T_4 treated animals. GLU, glucose; CHOL, cholesterol; HDL, high density lipoprotein and TG, triglyceride

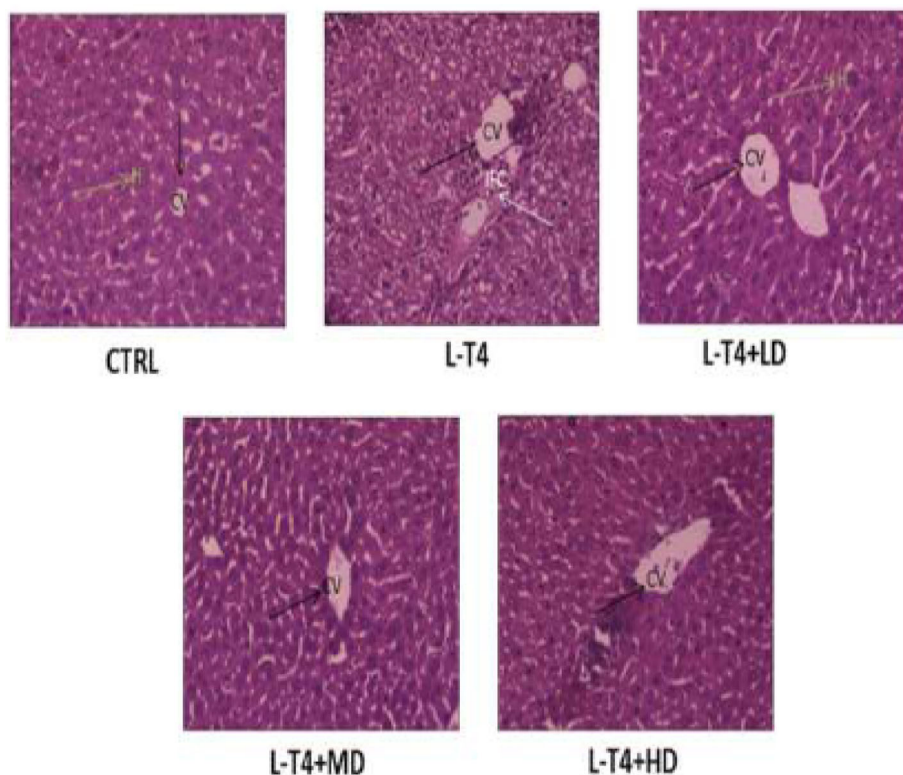


Fig. 5 Effects of ACC on the liver histology. While control (CTRL) animal showed normal liver architecture, in L-T4 induced mice severe centrilobular damage and cell necrosis were seen. L-T4 + low dose of ACC showed almost normal histological features. In hyperthyroid animals, treatment with medium dose of ACC exhibited moderate liver damage and its high-dose administration showed cellular and central vein damage

cyclooxygenase-2 [35]. Therefore, the hepatoprotective effects of ACC could be due to antioxidant defense through the Nrf2 pathway and an anti-inflammation effect by inhibiting NF- κ B. The other possible mechanism of action of ACC in ameliorating hyperthyroidism induced liver damage could be through peroxisome proliferator activated receptor gamma (PPAR γ) coactivator 1 α (PGC-1 α) [36], which is a potent stimulator of mitochondrial biogenesis and respiration. As a transcription coactivator it has also been shown to regulate genes of the ROS detoxifying systems and thus plays a significant role in protection against oxidative stress. Therefore, it is quite possible that the beneficial effects of ACC in this study might have been mediated through activation of PPAR- α [37].

Conclusion

Our results suggest that low dose of ACC effectively ameliorated thyrotoxic condition by decreasing the clinically most relevant thyroid hormones, T₃ and T₄ and normalizing the TSH and inhibiting the tissue LPO with a parallel increase in the antioxidant enzymes. Furthermore, histological improvement by ACC also demonstrated the hepato-protective effects of the test compound. This may be the first report on the antithyroid and hepatoprotective potential of ACC, isolated from *Moringa oleifera*. It is

further suggested that the thyroid induced diabetic problems can also be overcome by the ACC treatment. However, further study will be helpful to establish the molecular mechanism of action of this active compound, ACC.

Abbreviations

ACC: Agnucastolide C; ANOVA: Analysis of variance; AOPPs: Advanced oxidation protein products; B.wt: Body weight; CAT: Catalase; GPx: Glutathione peroxidase; GSH: Glutathione content; H₂O₂: Hydrogen peroxide; HDL-C: HDL-cholesterol; IR: Insulin resistance; LOOH: Lipid hydroperoxide; LPO: Lipid peroxidation; L-T4: L-thyroxine; SOD: Superoxide dismutase; T3: Triiodothyronine; TBA: Thiobarbituric acid; TBARS: Thiobarbituric acid substances; TC: Total cholesterol; TG: Triglycerides; TSH: Thyroid stimulating hormone

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Authors' contributions

Isolation of the active compound and data interpretation was done by Dr. S Panda and Ms. L. Sunhre wrote the manuscript, performed all of the experiments in the laboratory and worked for the graphical representation and statistical analysis. Dr. Kar designed the experiment, analyzed and interpreted the data as well as edited the manuscript. The final manuscript has been approved by all the authors.

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University grant commission, New Delhi, India, provided the contingency for the research experiment.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The experimental design was approved by Institutional Animal Ethical Committee (Registration No.779/PO/Re/S/03/ CPCSEA) and the study was performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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