Effect of Ethanolic Extract of *Crataeva nurvala* Bark on Iodothyronine Deiodinases Oxidative Stress and Thyroid Histology in 6-Propyl-2-Thiouracil Induced Hypothyroid Mice Model

Arshvir Kaur^{*1} and Santosh Kumar Verma²

¹Department of Pharmacology, Delhi Institute of Pharmaceutical Sciences and Research, New Delhi, India

²Department of Pharmacology, Faculty of Pharmaceutical Sciences, Motherhood University, Bhagwanpur, Karaundi, Uttarakhand, India

ABSTRACT

In Ayurveda, “Galaganda” (enlarged thyroid gland) occur due to disequilibrium among three doshas i.e. Vata, Pitta and Kapha leading to hormonal imbalance (Ojus). Levothyroxine (LT₄) is the standard replacement therapy in hypothyroidism, clinically that offers a similar life quality, whereas the psychological adverse effects and its pharmacokinetic interactions questions its use. In ancient literature, *Crataeva nurvala* (CN) Buch-Ham, (family, Capparidaceae) or Varuna is known to regulate, tridoshas and other thyroid problems. A preclinical study confirms its thyrotropic and antihyper-chloesteronemic effects but its mechanism via iodothyronine deiodinases, effect on thyroid histopathology and physiological parameters need to be explored. The levels of Total thyroxine (T₄), triiodothyronine (T₃), T₃/T₄ ratio, oxidative stress (reduced glutathione (GSH) and lipid peroxidation) and physiological parameters were studied. The Swiss albino female adult mice were randomly divided into four groups (n=6) i.e. Group I to IV who were made hypothyroid using 6-propyl-2-thiouracil (PTU) for first 15 days and then treated with vehicle only, LT₄ (14.56 µg/kg), CN 400 mg/kg and CN 600 mg/kg (p.o.) for another 15 days respectively. The variation in the biochemical parameters was recorded on Day 30th. Under hypothyroid conditions, T₃/T₄ ratio in comparison with the standard i.e. LT₄, depicts significant iodothyronine deiodinases stimulatory activity of CN extract, with significant consumption of GSH for combating raised MDA levels thus possessing the thyro-protective effect, along with maintaining thyroid histoarchitecture.

KEY WORDS: HYPOTHYROIDISM, LEVOTHYROXINE, CRATAEVA NURVALA, IODOTHYRONINE DEIODINASES, THYROXINE.

INTRODUCTION

Levothyroxine (LT₄) is the standard replacement therapy in hypothyroidism, clinically that offers a similar life quality, whereas the psychological well being is compromised (Garber et al., 2012; Petersen et al., 1990; Saravanan et al., 2002). Also, many physiological and pathological conditions can impair LT₄ absorption such

as patient factors (compliance), certain foods (e.g. Grapes, coffee, soyabean, papaya etc.), age factor, drugs (e.g. Antacids containing aluminium, sucralfate, proton pump inhibitors, rifampicin, antiepileptics, etc.) gastrointestinal diseases (e.g. Helicobacter pylori infection, celiac disease). The overtreatment with LT₄ results in TSH suppression, cardiovascular health risk, high risk of skeletal fracture, especially postmenopausal women, however, short administration of LT₄ does not compromise bone density but possible affective disturbances are seen (Razvi et al., 2012; Schneider et al., 2012; Botella-Carretero et al., 2003).

In Ayurveda, thyroid disorders are discussed under the term “Galaganda” (enlarged thyroid gland). As per Ayurveda, Varuna leaves, stem bark and root bark

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are reported to be used in maintaining equilibrium among three doshas (bodily humours) Vata, Pitta and Kapha, whose imbalance can lead to Ojus (hormonal imbalance), most commonly thyroid disorders (Kaur et al., 2016). *Crataeva nurvala* Buch-Ham (CN), belonging to the family, Capparidaceae, synonymously called as *C. magna* (Lour.) DC., *C. religiosa* Hook. F and Varuna (Khare, 2007). It is reported to possess analgesic, neuroprotective, antiarthritic, anticancer, antidiabetic, cardioprotective, anti-inflammatory, antioxidant, hepatoprotective, nephroprotective activities (Khattar & Wal, 2012). Traditionally its use is reported in treating cancer, paralysis, thyroid problems etc. (Narayana & Subhose, 2005).

Pre-clinically it has been demonstrated that CN bark extract can elevate free thyroxine (FT4) and lower the raised cholesterol levels (Kaur et al. 2017). However the mechanism behind CN thyrotropic activity, mainly via iodothyronine deiodinases, effect on thyroid gland histology and physiological parameters in

hypothyroid mice needs to be explored in comparison with its per se effects in normal healthy mice (Kaur et al. 2018). So, this study is aimed to evaluate the mechanism of thyroprotective activity and effects on thyroid histopathology and physiological parameters in hypothyroid mice, whose thyroid status was altered by using (6-propyl-2-thiouracil) PTU, as an inducing agent, that selectively inhibits iodothyronine deiodinases type I (DIO1) mediated thyroxine (T_4) conversion to Triiodothyronine (T_3) at thyroid gland and at periphery over iodothyronine deiodinases type II (DIO₂) and results in disruption of thyroid histology with physiological changes (Bianco et al. 2002; Moriyama et al., 2007).

MATERIAL AND METHODS

Chemicals: 6-Propyl-2-thiouracil (PTU) was procured from Sigma-Aldrich Chemie GmbH and Levothyroxine Sodium tablets (Eltroxin) were purchased from the local vendor. All other reagents used for analysis, extraction and phytochemical screening were of analytical grade.

Table 1. Biochemical changes in thyroid hormones and oxidative stress parameters in thyroid tissue and serum of PTU induced hypothyroid and CN treated mice.

Groups				
Parameters	PTU+VEH	PTU+STD	PTU+CN 400	PTU+CN 600
Total thyroxine (T_4) ng/ml	91.980±15.466	114.550±14.088 #@@@	36.860±4.738	69.675±11.454 @
Total triiodothyronine (T_3) ng/ml	3.0566±0.315	3.0514±0.112	3.558±0.432	3.343±0.124
T_3/T_4 ratio	0.0359±0.004	0.0281±0.003	0.102±0.015 ###@@@	0.0527±0.009
Thyroid weight (gm)	0.210±0.020	0.160±0.010	0.170±0.020	0.140±0.010#
TBARS (nmol of MDA formed/mg of thyroid tissue)	6.723±1.480	5.120±0.435	8.173±0.880@	6.078±0.740
GSH (Micromol/gram of thyroid tissue)	7.187±0.992	2.333±0.515##	2.707±0.405##	5.087±0.673 @

For serum analysis the values are expressed as mean SEM, (n=5) analyzed using One-way ANOVA followed by Dunnett's Multiple Comparison Test. For tissue parameters the values are expressed as mean SEM, (n=3), analyzed using One-way ANOVA followed by Dunnett's Multiple Comparison Test (#p<0.05 vs PTU+VEH; @p<0.05 vs PTU+STD).

Animals: Adult Swiss Albino female mice (3-5 months; 25-35 gm) were procured from Panacea Biotec Ltd, Lalru India and were housed in polypropylene cage under standard laboratory conditions as per CPCSEA guidelines. They were fed with standard pellet diet (Shree Jagdambey Feed Industries, Moga, Punjab) and water ad libitum. The experimental study was duly approved by the Institutional Animal Ethics Committee (IAEC) [IAEC-CTIPS/2015/VII/0042 (PCL-M)].

Preparation and phytochemical screening of *Crataeva nurvula* plant extract: The dried stem bark of *Crataeva nurvula* (3.5 Kg) was procured from Herbal Health Research Consortium Pvt. Ltd. Amritsar duly certified on basis of description and physico-chemical parameters (Lot No. VRN-024, Certificate of Analysis A. R. No. 06/2015/

IH/086, in compliance with Q.S.I.M.P. Volume 10, Page no. 106-108). It was further authenticated by Singh, Chief Scientist Herbology, Aimil Pharmaceuticals (India) Limited, New Delhi. A voucher (HHRC/RT/0416/15-16) and plant specimen was submitted to Pharmacology department of our college for future reference. The CN bark extract was prepared using repeated maceration technique and screened for presence of phytochemicals as per the method described in (Kaur et al. 2017).

Experimental design: The experimental hypothyroidism was induced in adult female Swiss Albino Mice (25-35 gms) using, 6-Propyl-2-thiouracil (PTU), an anti-thyroid drug (10 mg/kg/day by oral gavage for 15 days) (Panda and Kar, 2005). The experiment was of 37 days including 7 day acclimatization of animals to laboratory

environment. Figure 1 shows that the protocol involved, induction of hypothyroidism for first 15 days using PTU and treatment for the next 15 days with vehicle (VEH) only, standard (STD) drug and test (CN 400 and CN 600) drug respectively).

Figure 1: The Experimental design

Serum triiodothyronine (T_3) and thyroxine (T_4) levels: Blood was withdrawn 24 hours after the last dose on day 30th via retro-orbital method. The blood samples were centrifuged for 20 min (2000 rpm) to separate the serum which was stored at -20°C for further biochemical investigations. The serum T_3 and T_4 levels were determined using the commercially available ELISA thyrokit (ERBA Lachema s.r.o., Czech Republic) on Day 30th. The estimation was based on direct solid phase competitive enzyme immunoassay and the results were expressed in ng/ml.

Thyroid gland oxidative stress parameters: The thyroid gland was perfused with ice-cold 0.85% Sodium chloride solution was isolated, weighed and placed in 1.15% potassium chloride solution (in the ratio of 1 gm wet tissue to 9 ml of 1.15% potassium chloride). It was then homogenized in tissue homogenizer. An aliquot of 10 % w/v homogenate was preserved at -20°C for estimating MDA (TBARS) and reduced glutathione (GSH) levels. TBARS assay values were reported as malondialdehyde (MDA) equivalents, which is a secondary by product of lipid peroxidation. The reaction of a 2-thiobarbituric acid (TBA, chromogenic reagent), with MDA at 25°C at pH 4 gives rise to red colouration, which when extracted in organic layer was determined photometrically at 532 nm (Ohkawa et al. 1979).

To the 10% w/v homogenate, added 5% trichloroacetic acid (TCA), in order to precipitate the protein hydrolysates. The mixture was incubated for 10-15 min at $2-8^{\circ}\text{C}$, and centrifuged (5000 rpm, 10 min). The supernatant was then added to potassium phosphate buffer, 0.1 M (pH 8.0) and 5,5'- dithiobis-2-nitrobenzoic acid (DTNB reagent; 4 mg/ml in KPB, 0.1 M, pH 7.0). Reaction with DTNB reagent gave a yellow colored product that developed rapidly (2 min) and read at 412 nm (Ellman, 1959).

Body weight, food intake and water intake: Parameters like body weight, food intake, and water intake were recorded daily over the study duration and weekly average values were calculated.

Histopathological studies: The thyroid gland was excised, rinsed, and preserved in formaldehyde solution till histopathological studies of thyroid gland were done by hematoxylin and eosin staining (H&E) method (Sayaki and Azure, 1955). The histopathology images depicted the cell nuclei in blue color, blood vessels in red color, and other connective tissue with cytoplasm in shades of pink.

Statistical analysis: The values of biochemical estimation i.e. thyroid hormone ($n=5$) and oxidative stress parameters in thyroid tissue ($n=3$) are expressed as mean $\pm$ SEM, analyzed using One-way ANOVA followed by Dunnett's Multiple Comparison Test. Physiological parameters are expressed as mean $\pm$ SEM ($n=6$), analyzed using Two-way RM ANOVA followed by Bonferroni post test to compare replicate means by row (* $p<0.05$ vs VEH; # $p<0.05$ vs PTU+VEH; @ $p<0.05$ vs PTU+STD).

RESULTS AND DISCUSSION

Serum triiodothyronine (T_3) and thyroxine (T_4) levels: Table 1, shows that serum total T_4 levels were significantly high in standard group in comparison with low and high dose treatment group, at Day 30th. Low dose treatment group had the significantly lower levels of T_4 (# $p<0.05$). There were no significant changes in T_3 levels, however, calculated T_3/T_4 ratio was found to be highest in low dose treatment group followed by high dose treatment group in comparison with standard group.

Studies reveal that fraction of both T_4 and T_3 is bound to mainly two proteins i.e. Thyroxine binding protein (TBG), Thyroxine binding prealbumin (TBPA) and albumin. T_4 is 99.95% bound to plasma proteins and its values are useful for diagnosis of hypothyroidism and hyperthyroidism (Wills & Harvard, 1983). Also, TBG levels are reported to be high in PTU-treated rats than LT4 treated rats that regulate levels of T_4 in serum (Savu et al. 1989). In our study, low levels of T_4 could be attributed to high levels of TBG in serum of treatment group mice. About 13% of T_3 is secreted at thyroidal level but majority is formed by peripheral monodeiodination via Type I 5'-iodothyronine monodeiodinase (DIO1) (Bianco et al. 2002). However, any change in DIO1, i.e., inhibition or stimulation alters T_3 concentration and T_3/T_4 ratio (Visser et al. 1978). So the results indicate that treatment groups showed significant DIO1 stimulatory activity over standard group.

Oxidative stress parameters: Table 1 shows that the weight of thyroid gland was found to be significantly raised in disease control at Day 30th. the level of TBARS in thyroid gland was found to be raised in low dose treatment group with significant decrease in GSH levels of standard and treatment groups in comparison with disease control (@ $p<0.05$). Previous studies in hypothyroid/hypometabolic

state and hyperthyroid/hypermetabolic state concluded that both conditions raised malondialdehyde levels due to altered antioxidant status (generation of H_2O_2 and ROS) and cholesterol profile (Torun et al. 2009; Halliwell and Gutteridge, 1990; Venditti & Di Meo, 2006). Hydrogen peroxide is the main element that is used by thyroid peroxidase (TPO) for thyroid hormone biosynthesis (Weber et al. 2013) whereas PTU significantly inhibit H_2O_2 production and bring perturbances in cholesterol levels, thus acting as powerful antithyroid agent with strong antioxidant activity (Imamure et al. 1986; Kaur et al. 2017) but LT_4 therapy alone is not effective in reducing PTU induced oxidative stress associated with perturbation in lipid profile in preclinical rodent brain studies (Pan et al. 2013; Bhanja & Chainy, 2010). This is in correlation of our study in which disease control group showed lower MDA and raised GSH levels in contrast with standard and treatment groups due to its self antioxidant nature of PTU. Also, this must be discussed on the basis that with the increase in thyroid hormones, there is an increased GSH consumption to combat oxidative stress that can result in its lower levels as seen in treatment groups (Adali et al. 1999).

Body weight, food intake and water intake: Figure 2 shows that the water intake in mice was found to be significantly less in disease control, as compared to standard and treatment groups whereas water intake improved significantly from second week after standard and treatment drug administration. No significant change in food intake was observed in treatment duration. Body weight significantly increased in disease control during two weeks treatment duration whereas it was significantly less in standard group.

Figure 2: Physiological changes in PTU induced hypothyroid and CN treated mice.

Values are expressed as mean SEM (n=7), analyzed using Two-way RM ANOVA followed by Bonferroni post test to compare replicate means by row (* $p < 0.05$ vs VEH; # $p < 0.05$ vs PTU+VEH; @ $p < 0.05$ vs PTU+STD).

Thyroid hormones and pattern of food consumption are interrelated. Higgs et al. 1979 demonstrated that the administration of LT_4 improves the food consumption whereas decrease food intake is observed in hypothyroid conditions with increase in central obesity (unpublished data) (Decuypere et al. 1987; Singh et al. 1971; Bhaskar et al. 2009). However no change in appetite is seen in

our two weeks treatment duration of study. Choi et al. 2002 reported that thyrotropin releasing hormone (TRH) that stimulate thyroid hormone secretion via stimulation of hypothalamic pituitary axis (HPA), increase water consumption in rats whereas the reverse is true in absence of thyroid hormones i.e. hypothyroidism and improvement in water intake is seen in our results of treatment groups and standard group. Varied results are reported regarding body weight change and thyroid conditions i.e. body weight increases with increase in TSH and decreases vice versa (Bray et al. 1976; Fox et al. 2008) and this is evident from our study results.

Histopathology: Figure 3 shows that the disease control group, depicted the scattered appearance and reduction in number of follicles and follicular cells with formation of vacuoles. The epithelial structure was seen lost with reduced colloid volume, however the cluster of C-cells were visible. The standard group had the presence of uniformly distributed follicular cells with cuboidal epithelium, intact cluster of C-cells, capillaries with reduced but visible colloid volume and appearance of vacuolization. The low dose treatment group had the presence of epithelium around follicular cells with some follicles partially filled and some devoid of colloidal material. However, blood capillaries and C-cells are visible with adequate number of follicles. The thyroid gland of high dose treatment group had the bunch of follicles rich in colloidal material with abundant follicular cells, C-Cells and embedded capillaries. However, low degree of vacuolization is also observed.

Figure 3: Histopathological and structural changes in PTU induced hypothyroid and CN treated mice thyroid gland A) PTU+VEH; B) PTU+STD; C) PTU+CN 400 and D) PTU+CN 600 group.

Petrova et al. 2014, demonstrated that the administration of LT_4 or increase in thyroid hormones is characterized by presence of large size follicles, availability of more colloid, reduction in resorptive vacuoles with flattened follicular epithelium. Also Rajab et al. 2015 demonstrated that the supranormal levels of thyroid hormones distort the morphology of gland characterized by distention of lumen of follicles, reduction in thyrocyte height, follicular remodelling (fusion) and thyrocyte death due to lack of trophic effect and cytoprotection offered by

thyroid stimulating hormone. This supports somewhat distorted structure observed in the histology of treatment groups. However, the improved cellular structure is seen in standard and treatment groups as compared to the disease control group.

CONCLUSION

The ethanolic extract of *Crataeva nurvala* is able to raise the thyroidal and extra-thyroidal conversion of T4 to T3 via enhanced activity of DIO1, improved water intake and thyroid gland histology as compared to the standard therapy i.e. LT₄. Genomic level study is required to understand the mechanistic intricacies of the *Crataeva nurvala* in treatment of thyroid disorders.

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