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Research Article

# MECHANISTIC ROLE OF VARUNA (*CRATAEVA NURVALA*) EXTRACT ON THYROID GLAND AND ITS HISTOLOGY THROUGH IODOTHYRONINE DEIODINASES

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**ABSTRACT**

**Objective:** *Crataeva nurvala* (CN) is used for its therapeutic effects, but its effect on the thyroid gland in euthyroid conditions and mechanism behind its thyrotropic activity in hypothyroidism is still not explored. This study screened the pharmacological effect of the ethanolic extract of the bark of CN on thyroid hormones, free and total thyroxine ($FT_4$ and $T_4$), triiodothyronine ($T_3$), thyroid-stimulating hormone (TSH) levels, and thyroid histology in normal Swiss albino female mice.

**Methods:** Eighteen animals of 28–33 g were segregated into three groups: Group I treated with vehicle (NOR+VEH), Group II administered CN 400 mg/kg (NOR+CN 400), and Group III given CN 600 mg/kg (NOR+CN 600), for 15 days, per *os* (p.o.). The variation in the $T_4$, $FT_4$, $T_3$, and TSH levels was recorded using ELISA, 24 h after the last dose, and $T_3/T_4$ ratio thus calculated along with the histopathological studies of the thyroid gland.

**Results:** The findings were presented as mean ± standard error of the mean, using one-way ANOVA, followed by Dunnett's post-tests to compare all columns with the control. NOR+CN 600 has shown thyroid protective effect through retaining euthyroid profile, normal $T_3/T_4$ ratio, and near-normal histology. However, NOR+CN 400 had shown the significant decline in $T_3/T_4$ ratio and pathological changes in thyroid histology, in comparison with the control and NOR+VEH group.

**Conclusion:** The higher dose of CN was found to sustain the euthyroid levels through retention of iodothyronine deiodinases activity, facilitating the peripheral conversion of $T_4$ to $T_3$, and in retaining normal histoarchitecture of the thyroid gland in contrary to a lower dose.

**Keywords:** Varuna, Thyroxine, Triiodothyronine, Iodothyronine deiodinase, Euthyroid.

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## INTRODUCTION

*Crataeva nurvala* (CN) commonly known as Varuna is reported to possess various pharmacological activities such as analgesic, anthelmintic, antiarthritic, antibacterial, anticancer, antidiabetic, antidiarrheal, antifertility, anti-hemolytic, anti-snake venom, anti-inflammatory, antimycotic, antioxidant, antiurolithiatic, cardioprotective, hepatoprotective, nephroprotective, neuroprotective, and antimalarial activities. It is also found to be effective in treating urinary tract infections as evident from various *in vivo–in vitro* studies in disease conditions [1-6].

Furthermore, Varuna is a part of various polyherbal Ayurvedic, Siddha, and commercially manufactured formulations, used for certain pharmacological actions such as Asmarihara kasaya (anti-hypertriglyceridemia and hepatoprotective), *Pashanabhedadi Ghrita* (antinephrolithiatic and antioxidant), Vedikara silasathu parpam and Nerunjil kudineer (anti-inflammatory), Himplasia (Himalaya Herbal Healthcare, Bengaluru) used for Benign Prostatic Hyperplasia, and Neeri (Aimil Pharmaceuticals India Ltd., New Delhi) as nephroprotective [7-11].

In a recent study, the bark extract of CN (CN 600 mg/kg) had shown to possess significant thyroid stimulant activity when compared with the standard therapy i.e. levothyroxine in propylthiouracil (PTU) induced hypothyroidism. It showed significant reduction in cholesterol levels and improved thyroid hormone levels, proving its beneficial role in the treatment of hypothyroidism and associated hypercholesterolemia. However, the lower dose (CN 400 mg/kg), despite raising $T_4$ levels, in an erratic manner, raised thyroid stimulating hormone (TSH) also, for which mechanism was not clear [12]. Moreover, its effect in the euthyroid state, when used for other ailments in the form of polyherbal preparations or certain extracts, is still unexplored. For estimating the mechanism, an additional diagnosis like $T_3$ is also required, which was not estimated in previous studies, needed to be estimated.

Hence, this study was framed to evaluate the *per se* effect of CN on thyroid hormone levels, thyroid histology, and its mechanism through estimating thyroid hormones, i.e. $T_4$ (total and free) and $T_3$ levels, TSH, $T_3/T_4$ ratio, and histopathological studies in the normal healthy female mice.

## METHODS

### Animals

Swiss Albino healthy female mice, having age around 3–5 months and body weight 28–33 g, were purchased from "Panacea Biotec Ltd., Lalru (140501), India." Animals were kept in cages made of polypropylene, under specified temperature conditions such as 25±2°C and relative humidity 30–70% with the maintenance of 12-h night and 12-h day cycle. Animals were nourished with standard pellets of food purchased from "Shree Jagdambey Feed Industries" situated in Moga (Punjab), and potable water was supplied on a free basis. The prior approval for the conduct of the study was taken from the Institutional Animal Ethics Committee (IAEC) under Protocol no.: IAEC-CTIPS/2015/VII/0042 (PCL-M) as per the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi.

### Procurement of plant material: CN

The CN bark (3.5 kg) was purchased from Herbal Health Research Consortium Pvt., Ltd. (HHRC), Amritsar, from Lot No. VRN-024 along with Certificate of Analysis (COA) whose A. R. No. was 06/2015/

IH/086, in consistency with Q.S.I.M.P.; 10: 106-108. Certified purchase voucher with voucher no. HHRC/RT/0416/15–16, along with the bark sample, was deposited to the Department of Pharmacology, CT Institute of Pharmaceutical Sciences, for further reference.

**Preparation of extract**

The CN bark extract was prepared through triple maceration strategy [13]. 3.5 kg of CN bark was dried in shade and rendered free of dust. The size reduction was achieved, first by manual crushing, thereafter undergoing an electrical grinding (sieve size #16). Ethanol (1 kg in 3 L) was used as a solvent to macerate the coarse powder at room temperature with periodic shaking. The straining was done by layer muslin fabric in 2 folds, and the marc pressing was done to extricate the solvent. The individual filtrates were obtained, combined, and filtered through Whatman No. 1 paper. This procedure was rehearsed every 3rd day to achieve triple maceration. All the filtrates were stored in light-resistant bottles. The recovery of the solvent was done under vacuum at 37°C and allowed to concentrate to get dark semisolid mass. The yield obtained was 1.37%.

Chosen amount of extract, i.e. 400 mg/kg and 600 mg/kg was used, based on available literature, scientific evidence for its neurological, hepatic and renal safety, therapeutic efficacy, and thyrotropic activity in PTU-induced hypothyroidism to evaluate its mechanism [12]. For administration, suspension using Gum Acacia (1%) and preserved at 2–8°C in light-resistant bottle [14-17].

**Preliminary phytochemical screening**

The qualitative phytochemical estimation of CN ethanolic extract was conducted using tests such as Keller-Killiani test, sodium hydroxide test, lead acetate test, Salkowski test, Lieberman's test, silver nitrate test, and frothing test. Every reagent taken was of analytical grade.

**Experimental procedure**

Mice of 28–33 g were segregated into three groups, i.e. Group I, vehicle treated, and Groups II and III, administered with CN 400 mg/kg BW and CN 600 mg/kg BW for 15 days, orally. The variation in the $T_4$, $FT_4$, T3, and TSH was analyzed on the 15th day. Dosage administration was done every day between 9.00 am and 10.00 am to avoid any circadian alteration.

**Serum preparation**

The blood samples were ensured to be collected after 24 h of the last dose administered, through retro-orbital puncture method. The blood specimen was permitted to get coagulate, and centrifugation for 20 min was carried out thereafter to obtain serum. The serum, thus, obtained was preserved at −2°C−−8°C until examined for biochemical examinations.

**Biochemical parameter estimation in serum**

Serum $T_4$, $FT_4$, $T_3$, and TSH were estimated by ELISA as per the instructions given in protocol by ERBA Lachema s.r.o., Czech Republic, and Calbiotech Inc., Austin, CA, at end of the study.

**Histological studies**

The histology of thyroid gland was performed using hematoxylin and eosin staining method [18]. The thyroid glands were removed, rinsed, and immersed in formaldehyde solution. The section was first deparaffinized, and slide was flamed over the burner and repeatedly agitated in xylene for 3–5 min. The section is first hydrated with water and then in decreasing concentration of alcohol, i.e. 100%, 90%, 80%, and 70% for 30–60 s, then washed under tap water, rinsed with distilled water, and drained properly before staining with hematoxylin and eosin solution. The cell nuclei will be viable in blue color, erythrocytes in red color, and muscle and other connective tissue with cytoplasm in shades of pink.

**Statistical analysis**

All the findings are presented as mean ± standard error of the mean (n=6), for serum biochemical parameter estimation, using one-way RM ANOVA followed by Dunnett's post-test to compare all columns with the control group.

## RESULTS

**Preliminary phytochemical screening**

The CN was screened for phytochemical estimation using various qualitative tests and ensured the presence of various phytochemicals such as glycosides, saponins, alkaloids, flavonoids, and terpenoids.

**Effect of CN on thyroid hormones**

*Thyroxine ($T_4$ and $FT_4$)*

Administration of the CN to healthy mice for 15 days, significantly soared $T_4$ (**p<0.01) and $FT_4$ (*p<0.05) levels in NOR+CN 400, whereas no deviation from normal levels, on the significant basis, was observed in NOR+CN 600 when compared to normal control, i.e., NOR+VEH (Figs. 1 and 2).

*Triiodothyronine ($T_3$)*

Administration of extract in healthy mice for 15 days significantly reduced the $T_3$ levels in both the groups, i.e. highly significant decline in NOR+CN 400 (***p<0.001) and significantly NOR+CN 600 (**p<0.01) as compared to NOR+VEH (Fig. 3).

*$T_3/T_4$ ratio*

The calculated $T_3/T_4$ ratio was found to be significantly less in NOR+CN 400 (**p<0.01), with respect to the normal control, whereas this ratio has been found to near normalcy in case of NOR+CN 600 (Fig. 4).

**Effect on TSH**

Administration of CN for 15 days in normal healthy female mice had not significantly altered the TSH levels in any of the test group with respect to normal control, thus depicting the subclinical changes at $T_3$ levels (Fig. 5).

## Histology of thyroid gland

The transverse section (T.S.) of thyroid gland of the normal group (NOR+VEH) showed the appearance of normal structural features such as follicular cells embedded in cuboidal epithelium (*f*), colloidal appearance in follicles with slight variation in size (*co*), parafollicular cells or C-cells clustered in between the follicles (*pf*), and fenestrated capillaries (*ca*) with visible appearance of interlobular connective tissue (*il*) (Fig. 6a), whereas the thyroid gland in NOR+CN 600 appeared to have reduced follicular size (*fr*) with undistinguished columnar epithelium (*ue*), the presence of C-cells (*pf*), few capillaries (*ca*), and large vacuole spaces (*va*) (Fig. 6b). The thyroid gland T.S. of group NOR+CN 600 depicted the bunch of follicles of variable size (*fv*) with cuboidal epithelium and abundant follicular and cluster of C-cells (*pf*) with the presence of colloid in different intensity (*co*) and blood capillaries (*ca*) (Fig. 6c).

## DISCUSSION

Thyroxine ($T_4$) is the principle prohormone secreted from the thyroid gland. $T_4$ thus produced is metabolically converted into its biologically active form $T_3$, through the process of outer-ring monodeiodination by thioredoxin fold-containing selenoenzymes, known as iodothyronine deiodinases in cytoplasm and nucleus of target/extrathyroidal tissues mainly liver, kidneys, etc. $T_3$ is secreted in small amount by the thyroid gland (13%) and the majority is formed in peripheral tissues through Type I 5'idothyronine monodeiodinase (5'-DI) by peripheral monodeiodination to carry out pro-metabolic, pro-enzymatic, and lipolytic effects [19,20]. Suppression in levels of both $T_4$ and $T_3$ is seen in conditions of hypothyroidism or due to the effect of certain goitrogens like bamboo shoots as a food entity or bark of *Ficus racemosa* Linn. [21,22]. Any change in 5'DI, i.e., inhibition is reflected by a decrease in $T_3$ concentration and $T_3/T_4$ ratio, despite the increase in $T_4$ concentration [23].

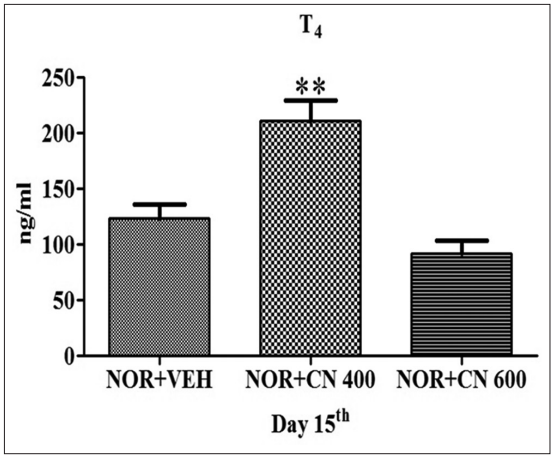


**Fig. 1: Effect of *Crataeva nurvala* on total thyroxine levels (the values are presented as mean ± standard error of the mean (n=6), for serum biochemical parameter estimation, using one-way RM ANOVA followed by Dunnett's post-test to compare all columns with the control group,**p<0.01)**

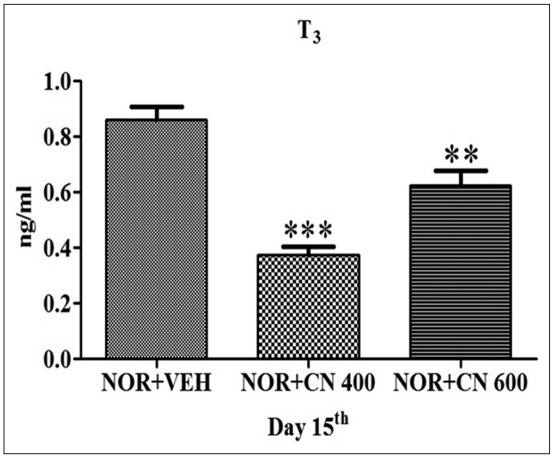


**Fig. 3: Effect of *Crataeva nurvala* on triiodothyronine levels (the values are presented as mean ± standard error of the mean (n=6), for serum biochemical parameter estimation, using one-way RM ANOVA followed by Dunnett's post-test to compare all columns with the control group,**p<0.01 and ***p<0.001)**

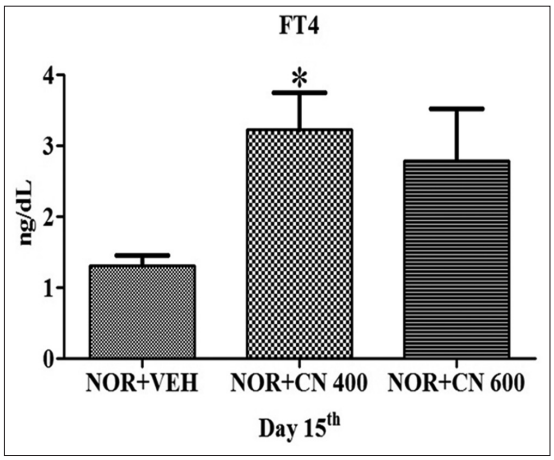


**Fig. 2: Effect of *Crataeva nurvala* on free thyroxine levels (the values are presented as mean ± standard error of the mean (n=6), for serum biochemical parameter estimation, using one-way RM ANOVA followed by Dunnett's post-test to compare all columns with the control group,*p<0.05)**

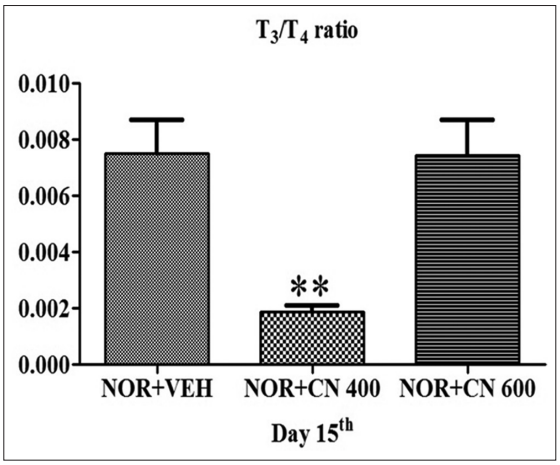


**Fig. 4: Effect of *Crataeva nurvala* on $T_3/T_4$ ratio (the values are presented as mean ± standard error of the mean (n=6), for serum biochemical parameter estimation, using one-way RM ANOVA followed by Dunnett's post-test to compare all columns with the control group,**p<0.01)**

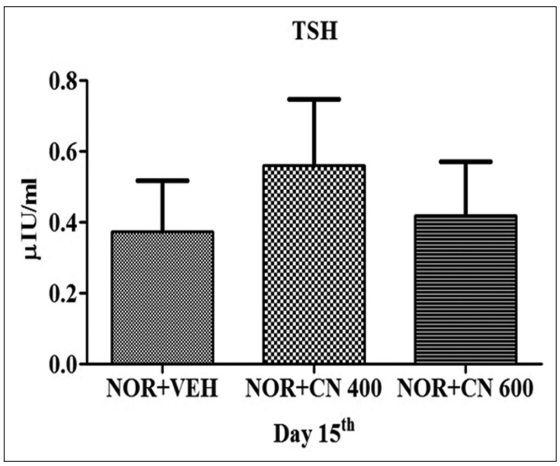


**Fig. 5: Effect of *Crataeva nurvala* on thyroid-stimulating hormone (the values are presented as mean ± standard error of the mean (n=6), for serum biochemical parameter estimation, using one-way RM ANOVA followed by Dunnett's post-test to compare all columns with the control group)**

In this study, administration of the ethanolic extract of CN to healthy mice for 15 days significantly increased the levels of $T_4$ (**p<0.01) and $FT_4$ (*p<0.05) in NOR+CN 400, with a significant reduction in the $T_3$ levels (***p<0.001) (Figs. 1-3). Whereas, in NOR+CN 600, i.e. at the higher dose, the insignificant change was observed in thyroxine levels with significant (**p<0.01) decrease is $T_3$ levels as compared to normal control i.e. NOR+VEH (Figs. 1-3). However, no significant change was observed in TSH levels (Fig. 4). Hence, this extract might have some inhibitory effect on 5'DI, thus affecting the peripheral deiodination, but this 5'DI inhibition is more marked with the lower dose as compared to the higher dose as $T_3/T_4$ ratio in NOR+CN 400 has significantly (**p<0.01) decreased as compared with NOR+CN 600 that retained the levels compared to normal group.

These results are in line with the study by Panda and Kar, 1999, done on root extract of *Withania somnifera*, given at the dose 1.4 g/kg to female mice for about 20 days along with the additional parameter observed, i.e., hepatic glucose-6-phosphatase [24]. Tahiliani and Kar 1999 reported similar findings in their study on *Moringa oleifera* leaf extract, which showed approximately 30% reduction in $T_3$, despite marked increased in $T_4$ levels with antiperoxidative effects and is suggested to be used in hyperthyroidism conditions [25]. *Aegle*

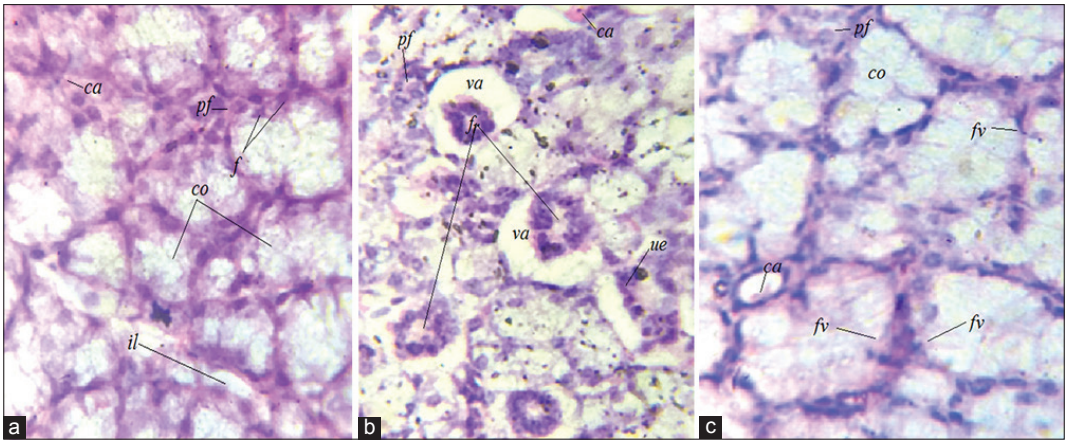


**Fig. 6: Microscopic images of thyroid glands demonstrating the histological changes after the administration of the vehicle, Crataeva nurvala (CN) 400 mg/kg and CN 600 mg/kg for 15 days (a) control, NOR+VEH (b) NOR+CN 400 (c) NOR+CN 600; where *pf*- parafollicular cells or C-cells, f-follicles, co - colloid, *ca* - capillaries, *il* - interlobular connective tissue, *fr* - reduced follicular size, ue - undistinguished columnar epithelium, *va* - vacuoles, and *fv*- variable size follicles. Hematoxylin and eosin staining (×450)**

*marmelos* extract when studied by Kar *et a*l. 2006, it was found that it could decrease $T_3$ concentration by 62% depicting its possibility to be used in the regulation of thyroid disorder like hyperthyroidism [26]. Since our study also revealed that only $T_4$ but not $T_3$ was soared by lower dose, plant might stimulate the synthesis or the release of $T_4$, directly at the glandular level but not through peripheral conversion of $T_4$ to $T_3$ which make it suitable for use in hyperthyroidism, while being higher dose remaining safe to be used for other ailments with respect to thyroid function.

Finding from previous studies using the ethanolic extract of CN in PTU-induced hypothyroidism revealed that CN 600 mg/kg has thyrotropic action as it significantly raised $T_4$ levels (***p<0.001) with a concomitant decrease in TSH (*p<0.05) and associated hypercholesterolemia (***p<0.001). However, the lower dose proved to be less effective in correcting the disorder and seen to increase TSH. This may be attributed to more marked inhibition of 5'DIs by CN 400 mg/kg, thus inhibiting the formation of $T_3$ and rising the TSH.

Structurally, the thyroid gland in rodents is similar to that of humans, except the difference in size of the follicles that are comparatively small and are surrounded by the cuboidal epithelium [27,28]. Petrova *et al.* 2014 demonstrated that the administration of l-thyroxine or availability of thyroid hormones is characterized by the presence of large size follicles, availability of more colloid, reduction in resorptive vacuoles with flattened follicular epithelium as it is evident from the images of normal (NOR+VEH), and the group administered with CN 600 mg/kg, where no or less vacuolization was present and follicular size was large with flattened cuboidal epithelium, colloidal appearance in follicles, slightly variable in size, parafollicular cells, or C-cells in between the follicles, fenestrated capillaries with visible appearance of interlobular connective tissue [29]. Ali Rajab *et al.* 2015 demonstrated that the supranormal levels of thyroid hormones or conditions resembling hyperthyroidism distort the morphology of the gland characterized by distention of lumen of follicles, reduction in thyrocyte height, follicular remodeling (fusion), and thyrocyte death due to lack of trophic effect and cytoprotection offered by TSH [30]. NOR+CN 400 (Fig. 6b) depicted the hypotrophic follicles i.e., reduced in size and undistinguished epithelium, with presence of C-cells, capillaries, and vacuoles as compared to NOR+VEH group (Fig. 6 a).

NOR+CN 600 (Fig. 6c) depict the bunch of follicles with fused epithelium and abundant follicular and a cluster of C-cells with the presence of colloid in different intensity. However, blood capillaries and C-cells are visible with a number of follicles.

The thyroid gland comprises of acini or follicles that are spherical bodies that selectively absorb iodine in the form of iodide ions, $I^-$ from the blood circulation for the production of the thyroid hormones, and also for its adequate storage in thyroglobulin (Tg). 25% of the body's $I^-$ ions are in the thyroid gland. Follicles contain a region called the follicular lumen, containing colloid comprising of a protein, Tg that serves as the reservoir of materials for the thyroid hormone production and, to a lesser extent, also acts as a reservoir for already synthesized thyroid hormones. The follicles are lined by multiple cuboidal cells, whose size varies depending on age and locality [31,32]. The size of the follicles and the follicular lumen is found to be reduced in NOR+CN 400 group, thus indicating the depletion of colloid accompanied by vacuole formation, whereas normal follicular size with no vacuolization was seen in NOR+CN 600 (Fig. 6a and b). "C cells" or parafollicular cells which secrete calcitonin were found in abundance, scattered within the follicular cells and in the spaces between the spherical follicles in NOR+VEH and NOR+CN 600; however, they were poorly localized in NOR+CN 400. As the acini size has decreased in NOR+CN 400, the acinar epithelium appears more cuboidal as compared to NOR+VEH and NOR+CN 600, in which with the increased size of follicles and the epithelial lining becomes flatter or low cuboidal.

As per the findings of this study, CN 400 mg/kg was found to be beneficial to be used in hyperthyroidism, as evident from raised $T_4$ and reduced $T_3$ and $T_3/T_4$ ratio, whereas CN 600 mg/kg was able to maintain euthyroid state in *per se* or hypothyroid mice, compared to the normal group. For future studies, CN extract must be studied extensively for its effect on peripheral organs also such as determination of a additional parameter for thyroid function and glucose-6-phosphatase (G-6-Pase) activity in liver tissues as carbohydrate metabolism is also influenced by thyroid hormones and moreover the anti-peroxidative effects in relation to thyroid disorders [33]. However, before human therapy, further investigations are required such as the direct measurement of 5'DI using specific radioimmunoassay for more confirmation.

## CONCLUSION

The ethanolic extract of CN is thyrotropic, stimulatory at glandular level but possesses the 5'DIs inhibitory activity in a dose-specific manner. Lower dose, i.e. CN 400 mg/kg is suitable to be used in hyperthyroidism, whereas higher dose, i.e. CN 600 mg/kg is found to be effective in hypothyroidism, in maintaining euthyroid levels and in retaining normal histoarchitecture of the thyroid gland as evident from preclinical studies.

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**AUTHOR'S CONTRIBUTIONS**

All the authors have significantly contributed to the concept, design, definition of intellectual content, literature research, the conduct of the study, manuscript editing, preparation, and review.

**CONFLICTS OF INTEREST STATEMENT**

The authors mentioned in this paper do not have any personal or financial relationship with any other person or organization that can influence the content of the paper.

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