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HISTOLOGICAL EFFECTS OF *CITRULLUS COLOCYNTHIS* SEED AQUEOUS EXTRACT ON THYROID GLAND OF ADULT ALBINO RATS

Dr. Sadia Hussain Malik[1*], Dr. Shumaila Sohail[2], Dr. Haseeb Ahmed Awan[3], Dr. Safia Tasawar[4], Dr. Huma Jawad[5], Dr. Sadia Zeeshan[6]

[1*]MBBS, M.Phil, Assistant Professor, Department of Anatomy, Al-Aleem Medical College, Lahore - Pakistan
[2]MBBS, M.Phil, Assistant Professor, Department of Anatomy, FMH College of Medicines and Dentistry, Lahore - Pakistan
[3]MBBS, Senior Demonstrator, Department of Anatomy, Al-Aleem Medical College, Lahore - Pakistan
[4]BDS, M.Phil, Senior Demonstrator, Department of Anatomy, Al-Aleem Medical College, Lahore - Pakistan
[5]MBBS, M.Phil, Assistant Professor, Department of Anatomy, Al-Aleem Medical College, Lahore - Pakistan
[6]MBBS, Postgraduate Trainee, Family Medicine Department, FMH College of Medicines and Dentistry, Lahore - Pakistan

***Corresponding Author:** Dr Sadia Hussain Malik**;**
*****Email: fhareem044@gmail.com

ABSTRACT

Introduction & Objective: The thyroid is the endocrine tissue that controls the basal metabolic rate of the body, regulates the development and growth of many organs of the body. Its active hormones are thyroxine and triiodothyronine. *Citrullus colocynthis*, belongs to melon family of cucurbitaceae. The current study was intended at the effects of *Citrullus colocynthis* seed extract on thyroid gland anatomy as its seeds are extensively used for medicinal purposes but its effects on thyroid gland has never been studied before.

Methodology: An experimental study was carried out with 24 albino rats, divided equally into 4 groups A, B, C and D. **Group A** was control and was given 1ml of distilled water by oral gavage once daily for 28 days. All other groups received *Citrullus colocynthis* seed aqueous extract at different doses by oral gavage once daily for 28 days**. Group B** received 0.5ml/kg; **Group C** was given 1ml/kg while **Group D** was given 2ml/kg. Rats were euthanized on 29th day. Thyroid tissue examined microscopically after staining with H&E and Periodic Acid Schiff for quantitative and qualitative parameters. Results were analyzed by SPSS version 21.0.

Results: *Citrullus colocynthis* induced a statistically significant in the colloid content. On the other hand, a significant decrease was seen in epithelial height of thyroid follicular cells. No inflammation was seen at any magnification in all the groups. No significant change was seen in number of thyroid follicles.

Conclusion: It is our conjecture that flavonoids present in seeds of *Citrullus colocynthis* might be the underlying cause for histological changes in adult albino rats suggestive of hypo-functional state of the thyroid gland.

Keywords: Thyroid, *Citrullus colocynthis*.

INTRODUCTION

Thyroid gland is a vascular organ, located in the anterior region of neck between C5 and T1. It is H-shaped, composed of a pair of lateral lobes. These lateral lobes are joined by the isthmus which lies anterior to trachea.[1] The thyroid follicle is not only the functional but also the structural unit of the thyroid gland. The follicular epithelium (lining epithelium) is the low columnar epithelium or simple cuboidal epithelium. The colloid is an acidophilic gel present in the follicles. Follicles are surrounded by loose connective tissue forming stroma[1] which contains fibroblasts, macrophages and mast cells which are now reported to be involved in development of thyroid cancer.[2]

Two kinds of cells found in the follicular epithelium of thyroid gland:

i. Follicular cells (Thyrocytes)
ii. Parafollicular (C cells)

Follicular cells, also known as principal cells, produce T4 and T3. Different cells have different functional states hence different size and shape. Hyperactive gland exhibits low columnar epithelium, however, hypoactive represents mostly squamous follicular cells. Nucleus is rounded in shape and central in position.[3] At the apical end of follicular cells, typical junctional complexes (JC) are present along with microvilli at its surface while in the basophilic basal area rough-surfaced endoplasmic reticulum (RER) can be easily seen.[3] Parafollicular C cells accounts for only 0.1% of thyroid cells. It is difficult to identify parafollicular cells in the light microscope but can be done easily in electron microscope and by using techniques for immunohistochemistry (IHC) of calcitonin.[1] They help in calcium metabolism as they release a hormone known as calcitonin.[3] Follicles are enclosed by a large network of fenestrated capillaries. Another route to convey the hormones from gland is provided by lymphatic capillaries which are blind ended & present in interfollicular connective tissue.[3]

The thyroid saves its secretory product extracellularly making it different from other endocrine glands.[1] The idle storage form of thyroid hormones is thyroglobulin which is the most abundant form of protein in colloid. Thyroglobulin is 660 kilodalton iodinated glycoprotein having atleast 66 tyrosyl residues.[4] Along with thyroglobulin, colloid has many other enzymes and glycoproteins. It is found to be PAS positive and also stains with basic and acidic dyes. Thyroglobulin itself has no hormonal activity rather it is an inactive storage form of T3 and T4 which are released into surrounding fenestrated capillaries when required.[3]

Thyroid hormone is necessary for development of nervous system, and severe neurocognitive impairment occurs in congenital hypothyroidism.[5] Hypothyroidism is a clinical condition resulting from deficiency of thyroid hormone, which when diagnosed can be treated but if untreated proved to be life threatening. The characterization of hypothyroidism depends on reported statistical ranges of the thyroid hormones. Clinical features of hypothyroidism range from asymptomatic to fatal. The most common in adults are fatigue, lethargy, cold intolerance, increase in weight, constipation, and dry skin, but it varies depending on age and sex. The standardized management is levothyroxine (thyroid hormone replacement therapy).[6]

The melon family of cucurbitaceae includes *Citrulluscolocynthis*. The production of cucurbit plant has been increased with the passage of time and largest producers of cucurbit plant are India, China, Russia, America and Iran.[7] It is dispersed over the large areas of deserts all over the world, as well as, in Pakistan. It has both medicinal as well as nutraceutical advantages. For diabetic and cancer patients the *Citrullus colocynthis*'s dried pulp, seeds and root is found to have insulinotropic and anti-hyperglycemic, lipid lowering properties.[8]

Inhibition of gram positive, gram negative bacteria (Staphylococcus aureus, Bacillus cereus, Klebsiella pneumonia, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia,, Pseudomonas aeruginosa and Candida (Candida albicans, Candida glabrata, Candida

kreusei and Candida parapsilosis) is shown by Citrullus colocynthis in vitro study and it was concluded that the extracts of roots, stems and leaves of the plant, both aqueous and acetone diluted extracts, works against all these strains.[9] Tumors, ascites, leucoderma, ulcers, asthma, bronchitis, jaundice, dyspepsia, constipation, anemia, diseases of throat, elephantiasis and pain of joints is one of the extensive lists of the diseases which can be addressed with the help of this plant.[10]

Apart from its useful effects it has many negative effects. When given intra-peritoneally, its seed, fruit have proven to be hepatotoxic by causing inflammation, necrosis of liver cells and harmful effects on the enzymatic activity.[11] Leaves also cause miscarriage if ingested during pregnancy and cause harmful effects on fertility of female rats.[12]

These effects of the plant occur due to different types of chemicals present in it known as phytoconstituents or phytochemicals. Alkaloids, steroids, glycosides, saponosides, terpenoids, coumarinsand flavonoids are the major components of the *Citrullus colocynthis.*[13] They are regarded as antioxidants, anti-inflammatory, antibacterial, antiviral, and anticancer agents.[13]

Citrullus colocynthis is a herb rich in flavonoids. Flavonoids, effective plant-derived naturally occurring composites, have the capability of interfering with thyroid hormone efficacy.[14] The initial study was published in 1950's reporting the anti-thyroid outcome, when it was observed that rats consuming 20 mg of anacardioside and arachidoside, nuts isolated pigments, suffered from goiter.[15] Flavonoids inhibit organification, production as well as secretion of thyroid hormones.[16] Two different doses of *C. colocynthis* fruit extract given for 2, 4 and 8 weeks showed decrease in the serum T4 level.[17] But no study was done on the histological changes on thyroid that occurs in this experiment.

Citrullus colocynthis is extensively utilized as an antidiabetic medicine. Its seeds are expected to cause hypothyroidism which had never been investigated before; hence the current study is configured to see the impacts of aqueous abstract of seeds of *Citrullus colocynthis* on the histological aspects of the thyroid gland of adult albino rats.

METHODOLOGY

This study was endorsed by the Ethical Committee of University of Health Sciences, Lahore (UHS) and was conducted under the guidelines of World Medical Association (WMA) declaration of Helsinki. An experimental study was conducted at the Anatomy Department of University of Health Sciences (UHS), Lahore in 2017. After procuring 24 adult Albino male rats from animal house of UHS, each animal was weighed and thoroughly evaluated for any gross morbidity. The healthy rats were 6-8 weeks of age with weight range of 160±10 g was included. Four groups A, B, C and D were made, each containing 6 rats (Table 1). This animal division was carried out randomly and was allocated to 4 groups by using balloting method. Each rat was tagged for identification. The duration of study was 28 days as described by Al-Dujaily et al. (2012).[17]

Table 1: ***Showing experimental grouping of animals and intervention (n=6).***

Groups	Intervention and Dosage	Route of Administration	Duration of Administration	Day of Sacrifice
Group A	Distilled water 1ml	By oral gavage	28days	29th day 24 hours after last dose
Group B	*Citrullus colocynthis* seed aqueous extract stock solution 0.5ml/kg	By oral gavage	28days	29th day 24 hours after last dose
Group C	*Citrullus colocynthis* seed aqueous extract stock solution 1ml/kg	By oral gavage	28days	29th day 24 hours after last dose
Group D	*Citrullus colocynthis* seed aqueous extract stock solution 2ml/kg	By oral gavage	28days	29th day 24 hours after last dose

Dissection and Tissue Sampling

At the time of sacrifice, the rats were anesthetized in plastic container with chloroform-soaked cotton balls and lid of the container was tightly closed for 3-4 minutes. All instruments were sterilized prior to dissection. A midline incision from chin to sternal angle was given, incising skin and muscles followed by bisecting sternum. The thyroid gland was identified in neck close to trachea and then freed from the surrounding connective tissue, separated from trachea, and was placed in a small container with 10% formalin.

Microscopic Parameters

Qualitative:

- Inflammation (present/absent)

Quantitative:

- Mean Epithelial height (µm)
- Mean number of follicles per unit area
- Colloid content (Full, partial, or empty)

Histological techniques:

Hematoxylin & Eosin (H & E) Staining

Hematoxylin & Eosin are the most extensively used stains for histological analysis of tissue specimen. Hematoxylin has the role of nuclear counterstain for emphasizing cytoplasmic components stained by eosin.[18]

Periodic Acid Schiff-Hematoxylin (PAS-H) Staining

The basic histochemical methodology for microscopic visualization of carbohydrates involves PAS (**Periodic Acid Schiff)** staining. Colloid filling the lumen of thyroid follicles is stained by PAS; it is useful for its clear demarcation. The sections were thus stained with PAS procedure for colloid content followed by hematoxylin counterstain for nuclear detail.

Inflammation

Inflammation, if any, was observed at all magnifications in all slides. For this purpose, inflammatory cells (neutrophils, eosinophils, lymphocytes, plasma cells) were observed.

Mean Epithelial Height & Mean number of follicles per unit area:

Leica microscope, (DM 1000), and objective lens 40X with ocular micrometer was used for measuring epithelial height. Five peripheral and five central fields of vision were selected from each animal and in each field 5 randomly non overlapping thyroid follicles were examined thus the follicular epithelium of 50 thyroid follicles per animal, 300 thyroid follicles in 1 group, making a total of 1200 thyroid follicles in all groups were recorded. The mean follicular epithelial height & mean number of follicles per unit area were calculated in each rat and then in each group separately.

Colloid content

Colloid content was best observed with PAS stain in all groups. At 10X, 5 fields of vision were selected from each slide and the number of follicles filled with colloid, partially filled and empty were counted.

Statistical analysis

The data was analyzed using SPSS software version 21. Mean ± SD was assessed for quantitative variables (e.g. mean epithelial height, mean number of follicles per unit area, colloid content). Percentages and frequencies were calculated for qualitative variable (inflammation). Normality of data was checked using Empirical formula for Normal Distribution which explains whether that data is normally distributed or not, when mean, median and mode are all equal. One-way ANOVA was used for comparing means of all four groups. Post-Hoc Tukey test was applied to compare difference of mean among groups. Comparison of qualitative analysis was done by applying Chi-Square test. p-value≤ 0.05 was taken as statistically significant.

RESULTS:

Inflammation

Inflammatory cells (neutrophils, eosinophils, lymphocytes, plasma cells) were not noted at any magnification in controls and experimental groups.

Mean Epithelial Height

In group A, B, C and D the mean epithelial height of follicles was 9.47±0.92, 8.49±0.74, 6.62±0.34 and 6.24±0.21 respectively. One way ANOVA showed significant difference in mean epithelial height when compared among groups (p-value< 0.001, Table 2). Post-Hoc Tukey tests revealed significant difference between groups A and C (p-value< 0.001), groups A and D (p-value< 0.001), groups B and D (p-value< 0.001), groups B and C (p-value< 0.001) and no significant difference between groups A and B (p-value =0.061) and between groups C and D (p-value = 0.728, Fig. 1).

Table 2: ***Shows comparison of mean epithelial height (µm) of animals among groups A, B, C and D, using One Way ANOVA (n=6/ group)***

Parameter	Group A Mean±SD	Group B Mean±SD	Group C Mean±SD	Group D Mean±SD	p-value
Epithelial Height (µm)	9.47±0.92	8.49±0.74	6.62±0.34	6.24±0.21	< 0.001

p-value ≤0.05 is considered statistically significant.

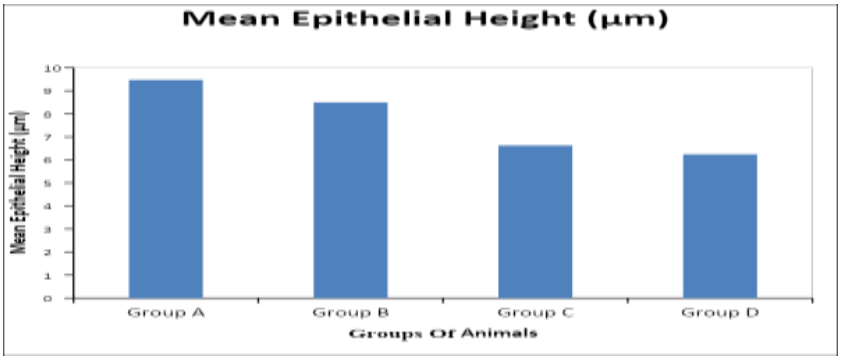

Figure 1: *Bar Chart comparing epithelial height between groups A, B, C and D indicating significant decrease in epithelial height (p < 0.001).*

Number of Follicles

The number of follicles ranged from 5.8- 10.3 follicles with mean of 8.59 ± 1.52/ mm^2 in Group A, 6.2- 10 follicles with mean of 8.30 ± 1.55/ mm^2in Group B, 6.1- 9.9 follicles with mean of 8.07 ± 1.26/ mm^2 in Group C and 6.1- 10.5 follicles with mean of 8.52 ± 1.66/ mm^2 in Group D. However, results were found to be statistically non-significant (p=0.932, Fig. 2).

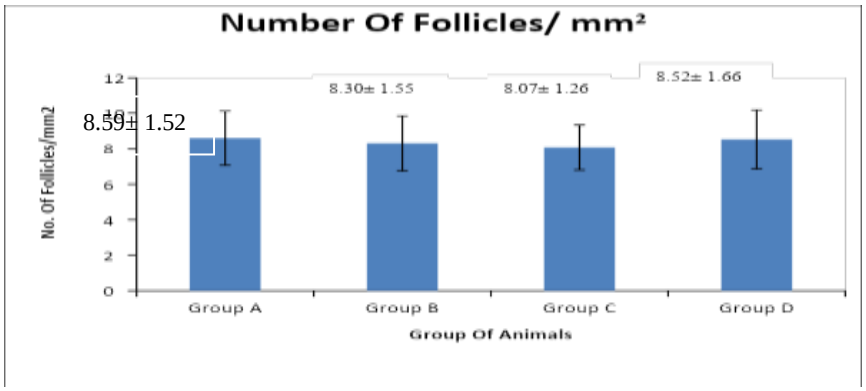

Figure 2: *Bar Chart comparing number of thyroid follicles among groups A, B, C and D.Each bar chart indicating Mean ± SD*

Colloid Content

The number of filled thyroid follicles/mm^2 ranged from 12.2- 14.2 follicles with mean of 13.2±1.09 /mm^2 in group A, 12.8- 14.8 follicles with mean of 13.8 ± 1.09/ mm^2 in group B, 22.8- 24.8 follicles with mean of 23.8 ± 1.09/ mm^2 in group C and 33.8-35.8 follicles with mean of 34.8 ± 1.09/ mm^2 in group D respectively. The number of partially filled thyroid follicles/mm^2 ranged from 4.4- 6.4 follicles with mean of 5.4±1.09 /mm^2 in group A, 5.2- 7.2 follicles with mean of 6.2 ± 1.09/ mm^2 in group B, 6.4- 8.4 follicles with mean of 7.4 ± 1.09/ mm^2 in group C and 8.2- 10.2 follicles with mean of 9.2±1.09/ mm^2 in group D respectively. The number of empty thyroid follicles/ mm^2 ranged from 22.6- 24.6 follicles with mean of 23.6 ± 1.09 /mm^2 in group A, 18.4-20.4 follicles with mean of 19.4 ± 1.09/ mm^2 in group B, 10.2-12.2 follicles with mean of 11.2 ± 1.09/ mm^2 in group C and 6- 8 follicles with mean of 7 ± 1.09/ mm^2 in group D respectively. One way ANOVA showed statistical difference ($p< 0.001$, Table 14). In case of full thyroid follicles, Post-Hoc Tukey's test revealed significant difference among Group A and C ($p< 0.001$), A and D ($p< 0.001$), B and C ($p< 0.001$), B and D ($p< 0.001$), C and D ($p< 0.001$). No statistical difference was observed in Group A and B ($p = 0.779$). Post-Hoc Tukey's test when applied to partially filled thyroid follicles revealed significant difference among Group A and C ($p= 0.023$), A and D ($p< 0.001$), B and D ($p <0.001$), C and D ($p = 0.045$), while no significance was observed among Group A and B ($p = 0.595$), B and C ($p = 0.261$). In empty follicles, Post-Hoc Tukey test revealed significance between all Groups ($p< 0.001$, Fig. 3 and 4).

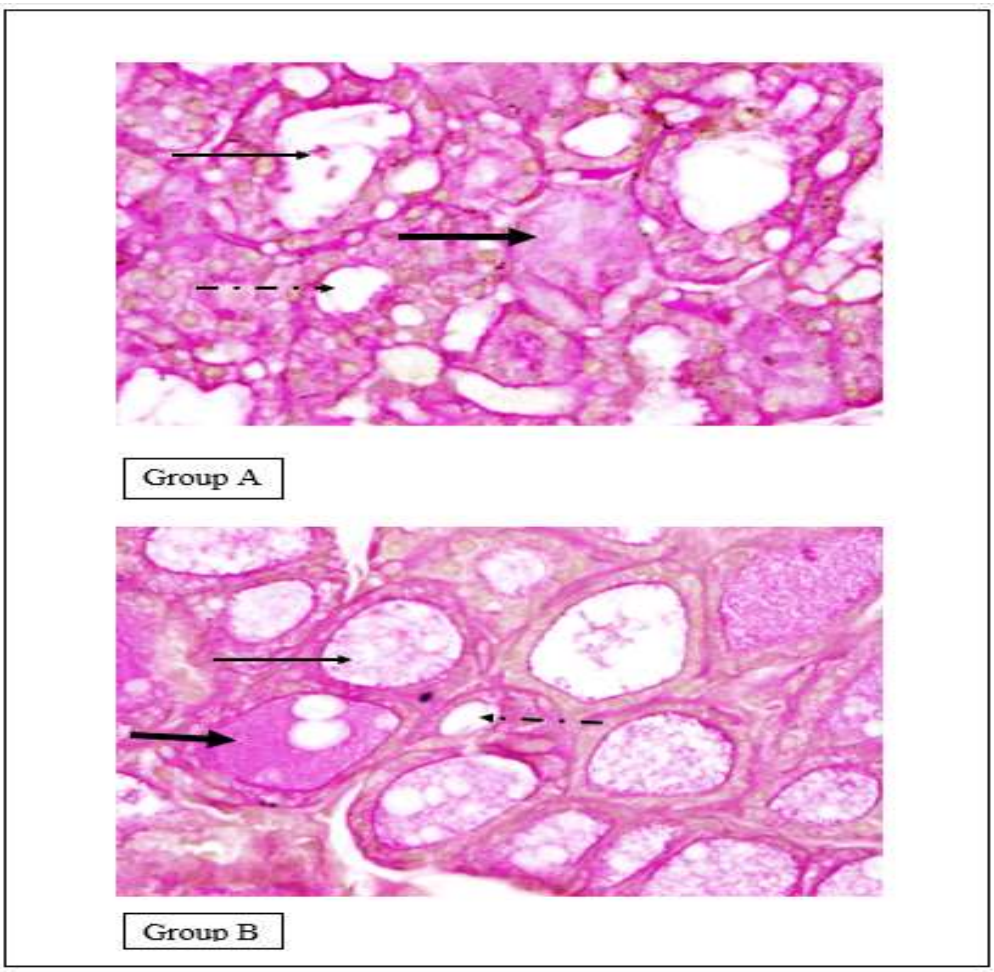

Figure 3: *The single-headed arrows (continuous thick line) indicate the filled thyroid follicles, (continuous thin line) indicate the partially filled thyroid follicles and (dotted line) indicate the empty thyroid follicles on PAS stain X40.*

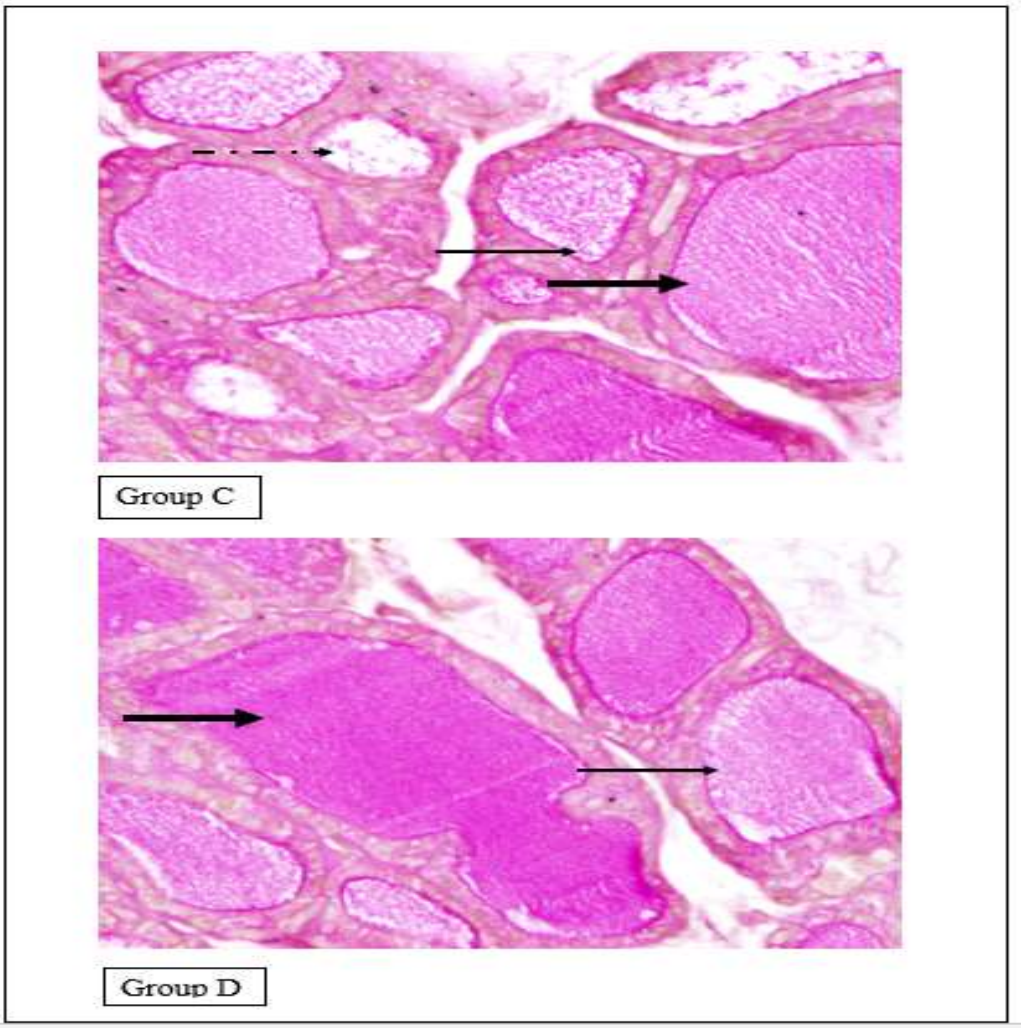

Figure 4: *The single-headed arrows (continuous thick line) indicate the filled thyroid follicles, (continuous thin line) indicate the partially filled thyroid follicles and (dotted line) indicate the empty thyroid follicles on PAS stain X40.*

Discussion

Among qualitative parameters, an increase in colloid content was noted in experimental groups. Histologically, no inflammation was seen. Mean epithelial height decreased pointing towards the classical findings of hypothyroidism.

Among qualitative parameters, inflammation was not observed at any magnification in all groups. The protective nature of *Citrullus colocynthis* against inflammation has been reported. The underlying mechanism discussed is the two-step inhibition of histamine-serotonin and prostaglandin-kinin pathways.[19]

Colloid is a gelatinous material present in thyroid follicles and is the source of thyroid hormone. Our study is in accordance with the earlier study of Chandra (2010) who showed that flavonoids present in catechins and antithyroid drugs like green tea causes blockage of thyroid hormone release that leads to its accumulation in the form of colloid filled follicles. Similar effects were seen in lithium induced hypothyroidism.[20] The underlying mechanism for inhibition of release of thyroid hormones was interference in polymerization of tubulin and blockage of action of TSH on cyclic adenosine monophosphate (cAMP).[20] Inhibition of TSH had an influence on phagocytosis/ pinocytosis of colloid resulting in decreased formation of pseudopodial cytoplasmic extensions and significant ($p < 0.001$) retention of colloid.[21] Increase number of empty follicles represent hyper functioning of thyroid gland.[22] Our study reflected decreased number of empty follicles, thus representing hypofunctioning of thyroid gland.

The present study found a decrease in epithelial height ($p< 0.001$) in groups treated with *Citrullus*

colocynthis which agrees with study performed using lead acetate in rats.[21] The possible mechanism for flattening of epithelium from cuboidal to squamous is increased thyroglobulin in colloid due to hypofunctional state of thyroid gland. The increased colloid content exerts pressure and subsequently impairs the cytoskeleton of epithelial cells lining thyroid follicles.[23] Elevated level of Thyroglobulin impairs the transport of iodide, its organification and epithelial cell growth. Thus, preventing the cells from enhancing their height.[24] Moreover, in hypofunctional thyroid state, there is inhibition of colloid resorption via phagocytosis/pinocytosis causing increased intrafollicular pressure which suppresses the surrounding epithelial cells.[21]

The number of follicles assessed in the thyroid gland didn't follow any ascending or descending set and was found to be statistically non-significant. Nevertheless, the number of thyroid follicles was documented to be increased when treated with green tea and clomiphene citrate.[25]

So, all the above characteristics are the potential cause for the appearance of hypothyroidism in adult albino rats in the present study. Histological both qualitative and quantitative showed a good correlation with each other. From the foregoing discussion it is clear that *Citrullus colocynthis* extract is anti-thyroid and its use as a herbal drug should be monitored, if the long term use of the plant is intended for human use.

Like every study there were few limitations, in our study raw seeds were used instead of extracting its phytoconstituent, study neither included any marker for vascular congestion or immunohistochemistry due to budget and time constraint. More work is needed to explore the effects on thyroid gland by extracting the flavonoids from seeds of *Citrullus colocynthis*. Ultrastructural microscopic support can further reinforce the study.

Conclusion

Considering the parameters like epithelial height and colloid content specified that *Citrullus colocynthis* administration has significant anti-thyroid effects on cellular level.

Authors Contribution:

Dr. Sadia Hussain Malik: Study Concept & Design
Dr. Shumaila Sohail: Manuscript Writing
Dr. Haseeb Ahmed Awan: Data Collection
Dr. Safia Tasawar: Data Collection
Dr. Huma Jawad: Manuscript Revision
Dr. Sadia Zeeshan: Data Analysis

Disclosure/Conflict of Interest: "none to declare".

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