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Research Article

PROTECTIVE EFFECT OF *PUNICA GRANATUM* PEEL EXTRACT AGAINST FIPRONIL INDUCED THYROID TOXICITY IN MALE WISTAR ALBINO RATS

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ABSTRACT: The present study was carried out to check the thyroid toxicity induced by fipronil in male Wistar albino rats and its amelioration with pomegranate peel extract. A total of 24 male Wistar rats were grouped in to 4 groups (Group I, II, III and IV) with 6 animals in each group. Fipronil was administered orally @ 10 mg/kg b. wt. in distilled water for a period of 45 days in group II rats and group IV rats fed with both fipronil (@ 10mg/kg b. wt.) and pomegranate peel extract (@ 200mg/kg b. wt.) for a period of 45 days. Group I and III animals were treated as vehicle (distilled water) and *Punica granatum* (pomegranate) peel extract control respectively. Pooled serum samples were collected at fortnight interval for checking the thyroid hormonal status. Fipronil treated rats showed significant (*$P<0.05$*) decrease in the serum T3 and T4 levels and increased levels of serum TSH. Histopathologically, severe degenerated and disrupted follicular lining epithelium with complete loss of architectural details of thyroid follicles and immunohistochemically, increased expression of BAX antigen was observed in the fipronil treated animals. Co-administration of *Punica granatum* (pomegranate) peel extract along with fipronil resulted in the increased T3, T4 levels and decreased TSH levels compared to fipronil treated group and near to control rats value. Histologically, thyroid gland of rats treated with fipronil along with *Punica granatum* peel extract showed restoration of thyroid architecture with minimal inflammatory changes, normal follicular epithelium and lumen filled with colloidal secretions. Immunohistochemically, ameliorative group showed mild immunoreactivity and decreased expression of BAX antigen. This study highlights the protective role of pomegranate peel extract in reducing fipronil-induced thyroid damage.

Keywords: BAX antigen, Histopathology, Hormonal status, Immunohistochemistry, *Punica granatum*, Thyroid toxicity, Wistar albino rats.

INTRODUCTION

Fipronil is broad-spectrum phenylpyrazole insecticide commonly used in veterinary, agricultural and household purposes. In order to increase the crop yield and to reduce harvest loss, agricultural field workers were forced to use different kinds of pest control methods in fields. One of the common ways is the use of broad-spectrum insecticides. Indiscriminate use of these pesticides results in accidental poisoning in agricultural community [1]. Discussion being going on among the scientific community regarding the extensive use of fipronil and its toxicity in different organisms. Fipronil is reported to be highly toxic to aquatic invertebrates and fishes and moderately toxic to rats and mice [2].

Fipronil acts on central nervous system of insects by inhibiting the GABA-A receptors and glutamate-activated chloride channels. Inhibition of g-aminobutyric acid receptors results in hyperexcitation and death of insects [3]. Fipronil also induce oxidative damage by generating excess free radicals which damages biological membranes and organ toxicity [4]. Other than nervous system, fipronil also cause multisystem impairments in animals which affects hepatic, respiratory, cardiac,

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gastro-intestinal and reproductive systems [5,6,7]. Still, the research on the toxic effects of fipronil on endocrine functions is not widely discussed. So the current study focussed on checking the extend of endocrine disruptive effects of fipronil in Wistar albino rats.

Usage of herbal medicine has been widely recommended in toxicity studies because of their less side effects, easily availability, cheaper rate and long-standing effects. In developed countries also, herbal plant extracts have been in use as complementary and alternative medicines [8,9]. Different varieties of herbal plants have been used in many agro-chemical toxicity studies. In this study, we incorporated the peel extract powder of pomegranate (*Punica granatum*). Because of its easy availability, higher anti-oxidant potential and less side effects, *Punica granatum* has got worldwide recognition. It also contains polyphenolic compounds like catechins, quercetin, anthocyanidin which are responsible for the antioxidant potential of peel extract of pomegranate [10]. Hence, our study focussed on checking the ameliorative effects of *Punica granatum* peel extract against fipronil induced thyroid toxicity.

MATERIALS AND METHODS

Experimental animals, fipronil and pomegranate peel extract procurement

Male Wistar albino rats used for this study was procured from Sri Venkateshwara Enterprises, Bangalore. Acclimatization for the procured rats were provided in a standard polypropylene rat cages for two days and further maintenance given at 25ºC ± 1ºC and a 12:12 hour interval light/ dark cycle for 6 weeks by following all standard laboratory hygienic conditions and *ad libitum* feed and water. Technical-grade fipronil (99% purity; Batch No. FIP92B5266) was sourced from Gharda Chemicals Ltd., Mumbai, while *Punica granatum* peel extract (Product No. Dadim LC23030077) was obtained from Chemiloids Life Science Pvt. Ltd., Vijayawada, Andhra Pradesh.

Animal ethical committee approval

The Institutional Animal Ethical Committee (IAEC) approval was obtained prior to the commencement of the experiment. (Wide no 281/ go/ ReBi/ S/ 2000/ CPCSEA/ CVSc/ TPTY/ 010/ Veterinary Pathology/ 2023 dated: 08.05. 2023).

Reagents

Primary (BAX) and secondary antibodies for immunohistochemistry were procured from Elabscience Company

Study trial

Study was designed such a way that a total of 24 male Wistar rats were randomly assigned in to four groups with six animals in each group. Group I animals were considered as control group provided them with normal laboratory animal feed and *ad libitum* water. Group II animals were treated with technical grade fipronil (99% pure) @ 10mg/kg b. wt. orally for a period of 45 days of experimental duration. Group III animals served as *Punica granatum* (pomegranate) peel extract control providing them pomegranate peel extract @ 200 mg/kg b. wt. orally for a period of 45 days. Group IV animals were treated with both fipronil and pomegranate peel extract @ 10 mg/kg b. wt. and 200 mg/kg b. wt. orally for checking the ameliorative effect of *Punica granatum* peel extract. Pooled blood samples were collected from all experimental rats at fortnight interval for hormonal assay. Animal sacrifice was done at 6^{th} week (after the end of experimental period) and representative tissue sample of thyroid was collected in 10% neutral buffered formalin for histopathological and immunohistochemical studies.

Hormonal assay

At fortnight intervals, pooled blood samples were collected from all groups directly in to serum vial and allowed to clot for serum separation. This separated serum was centrifuged at 4000 rpm for 20 mins and stored at –20ºC for the estimation of T3, T4 and TSH levels. All hormonal estimations were carried out in Siemens centor 100 automatic biochemical analyzers procured from siemens, Germany using the chemiluminescence immunoassay (CLIA) method.

Histopathology

Detailed post-mortem examination was conducted during sacrifice and representative tissue pieces from thyroid was collected and preserved in 10% neutral buffered formalin for histopathological studies. Dehydration process was done manually by keeping the tissues in different grades of alcohol followed by clearing using xylene. The paraffin infiltration process was carried out by keeping the cleared tissue in paraffin wax bath overnight. Embedding should be completed within 4 to 5 hrs after completion of the paraffin infiltration. Sections of 5-6 (µ) thickness was cut and stained with routine Hematoxylin and Eosin (H&E) stain [11].

Immunohistochemistry

Paraffin sections of thyroid were cut into 3-4 µ thickness and then mounted onto Amino Propyl Ethoxy

Silane (APES) coated slides for overnight incubation at 37ºC. By passing through 2 changes of xylene for 15 minutes each, sections were deparaffinized followed by dipping in 2 changes of absolute alcohol to remove xylene. Under running tap water, the obtained sections were washed for 10 minutes and then rinsed in distilled water for 5 minutes. The sections were kept in a 3:1 (preheated) antigen retrieval solution and kept in a pressure cooker for 3 whistles. Sections were removed from the heat and kept until the retrieval solution was lukewarm (around 45 minutes). Later, sections were kept in distilled water followed by Tris EDTA buffer for 5 minutes each. The slides were kept in the humid chamber and to block the endogenous peroxidase, peroxidase block solution (freshly prepared 3% hydrogen peroxide) was added for 10 min. Washed the sections in Tris EDTA buffer 3 times 5 minutes each. Above these tissue sections, the power block solution was poured and kept for 10 min. Primary antibody (BAX) was added to the thyroid sections, and slides were kept at room temperature for 30 min. Washed the slides in Tris EDTA buffer for 5 minutes each in 3 changes. Super enhancer solution was added and kept for 20 minutes followed by washing in Tris EDTA buffer. A secondary antibody with poly HRP (Horseradish peroxidase) was added and incubated for 20 minutes. Then washed in acid Tris EDTA buffer for 5 minutes each in 3 changes. Working colouring reagent was prepared from Diamino benzidine (DAB) by adding one drop of DAB into 1 ml of the substrate (supplied with kit). The sections were kept in the colouring reagent for 5-8 minutes followed by washing in Tris EDTA and tap water for 2 min each. The staining of sections was done by using Harris haematoxylin for 2 minutes followed by tap water wash for 5 min. Blueing was done by dipping the slides in lithium carbonate solution twice and washing them in tap water. The sections were mounted in DPX after air drying.

Statistical analysis: By performing one-way ANOVA, the results were statistically analysed using SPSS software [12].

RESULTS AND DISCUSSION

The mean serum T_3 values were 154, 82.52, 161.80, and 148.28 (ng/dl) in Group I to IV respectively, and are given in Table. 1. There was a significant ($P<0.05$) decrease in serum T_3 values in fipronil treated groups when compared to the control. Whereas significant improvement ($P<0.05$) in T_3 values was observed in *Punica granatum* ameliorated rats (Group IV) when compared to fipronil treated rats. Similarly, a significant decrease in serum T4 values was observed in fipronil treated (Group II) rats when compared to the control group. The mean serum T_4 values in in Group I to IV rats were 9.53, 4.26, 8.43, and 8.5 (µg/dl) respectively, and are given in Table2. Significant improvement ($P<0.05$) in T_4 values was observed in *Punica granatum* ameliorated rats (Group IV) when compared to fipronil treated rats. These observations were in line with [13,14,15] in their work. The decrease in the serum T3 and T4 levels in fipronil treated rats might be due to the induction of hepatic microsomal enzymes by fipronil which results in excessive catabolism and increased clearance of T3 and T4 levels from the body [5]. Fipronil is known to induce hepatic microsomal enzymes, particularly those involved in phase II metabolism, such as UDP-glucuronosyltransferases and sulfotransferases [16]. This enzymatic induction accelerates the glucuronidation and sulfonation of thyroid hormones, which are critical rate-limiting steps in their biliary excretion [17,18]. Consequently, the enhanced metabolism and clearance of T3 and T4 lead to a reduction in their serum concentrations. Hepatic microsomal enzymes play a major role in glucuronidation and sulfonation, which are limiting steps in the biliary excretion of T4 and T3 hormones [19]. Secondly, fipronil disrupts the normal functioning of the hypothalamus-pituitary-thyroid (HPT) axis [20]. It impairs the feedback regulation between thyroid hormone levels and pituitary secretion of thyroid-stimulating hormone (TSH), thereby contributing further to hypothyroxinaemia. This dual effect enhanced peripheral metabolism and impaired central regulation collectively result in significant reductions in serum T3 and T4 levels in fipronil-exposed animals [20].

In contrary to serum T3 and T4 levels, we observed a significant ($P<0.05$) increase in serum TSH values in fipronil treated groups when compared to the control group and a significant decrease in mean TSH values of Group IV rats compared to the fipronil treated rats. The mean serum TSH values in Group I to IV rats were 4.11, 7.93, 4.08, and 5 (µIU/ ml) respectively, and are given in Table 3. This might be due to reduced T4 concentration which will gradually suppress the negative feedback of T4 on TSH secretion, thereby increasing the plasma TSH concentration [13]. This finding was contrary with [21] who noticed declined serum TSH level along with serum T3 and T4 levels in MSG toxicity case.

In *Punica granatum* ameliorated group showed significant improvement in serum T3 and T4 values than fipronil treated group. Similar observation was reported by [21]. This might be due to the scavenging effects of different polyphenolic compounds present in *Punica granatum* such as ellagic acid, punicalagin, gallic acid, and flavonoids [22]. These molecules exhibit strong antioxidative properties by scavenging reactive oxygen species (ROS), reducing oxidative stress in the hypothalamus, pituitary, and thyroid tissues [23]. Oxidative stress plays a critical role in damaging thyrotroph cells of the anterior pituitary and thyrocytes, leading to impaired synthesis and secretion of thyroid hormones [24]. *Punica granatum* components upregulate the expression of nuclear factor erythroid 2–related factor 2 (Nrf2) and its downstream antioxidant response element (ARE)-regulated genes [25]. Nrf2 activation leads to increased transcription of antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase, thereby mitigating ROS-induced cellular apoptosis in endocrine tissues and supporting the functional preservation of the hypothalamic-pituitary-thyroid (HPT) axis [26]. Moreover, polyphenolic compounds in *Punica granatum* may directly influence deiodinase enzymes, particularly type 2 deiodinase (DIO2), which converts T4 into its more active form, T3 in peripheral tissues [27]. Preservation of DIO2 activity ensures proper peripheral activation of thyroid hormones even under toxic conditions.

Histopathological examination of thyroid gland of group II animals revealed a complete loss of architectural details of thyroid follicles with a lack of colloid and interfollicular blood vessel congestion (Fig: 1A). The majority of fipronil treated rats showed severe degeneration and disrupted follicular lining epithelium with desquamation of epithelial cells into the lumen and variation in the sizes of follicles (Fig: 1B). Few follicles with pale pink colloidal secretions in the lumen, and severe disruption and complete architectural loss of remaining follicles (Fig: 1C) were also noticed in some rats. Similar findings were reported by [14,28]. These alterations might be due to fipronil impaired hypothalamus-pituitary thyroid (HPT) axis,

Table 1: Mean values of serum T3 (ng/dl) in rats of different experimental groups.

Experimental Period (weeks)	GROUP I	GROUP II	GROUP III	GROUP IV
2	152.98	100.30	159.05	154.36
4	153.77	79.03	161.11	151.04
6	155.26	68.25	165.26	139.46
Mean ± SE	154 ± 0.66^{a}	82.52 ± 9.41^{b}	161.80 ± 1.82^{a}	148.28 ± 4.51^{a}

Mean values with different superscripts differ significantly (P < 0.05), ANOVA, S.E – Standard Error

Table 2: Mean values of serum T4 (µg/dl) in rats of different experimental groups.

Experimental Period (weeks)	GROUP I	GROUP II	GROUP III	GROUP IV
2	9.3	6.9	8.2	10
4	9.5	3.8	8.5	8.8
6	9.8	2.1	8.6	6.7
Mean ± SE	9.53 ± 0.14^{a}	4.26 ± 1.40^{b}	8.43 ± 0.12^{a}	8.5 ± 0.96^{a}

Mean values with different superscripts differ significantly (P < 0.05), ANOVA, S.E – Standard Error

Table 3: Mean values of serum TSH (µIU/ ml) in rats of different experimental groups

Experimental Period (weeks)	GROUP I	GROUP II	GROUP III	GROUP IV
2	4.09	7.42	4.11	5.01
4	4.12	7.92	4.08	5.31
6	4.13	8.46	4.06	4.68
Mean ± SE	4.11 ± 0.01^{c}	7.93 ± 0.30^{a}	4.08 ± 0.01^{c}	5 ± 0.18^{b}

Mean values with different superscripts differ significantly (P < 0.05), ANOVA, S.E – Standard Error which reduces the secretion of thyroid hormones like T3, and T4. In addition to hormonal imbalance, free radicals produced by fipronil sulfone cause increased peroxidation of thyroid follicular membrane. These imbalances inhibit the inorganic iodate transfer to the interior of follicles, inhibit colloid conversion into T3 and T4, and various histological alterations in the thyroid gland [13]. Microscopically, similar lesions were observed in ameliorated rats with reduced intensity. Most of the follicles restored their normal architecture with the presence of colloidal secretions inside the follicle, mild congestion, and mild degenerative changes (Fig: 1D). Similar findings were noticed by another author with [21]. It might be due to its free radical scavenging and antioxidant activity [29,30,31] which protects the thyroid gland from oxidative damages induced by fipronil. *Punica granatum* exerts potent anti-inflammatory effects by inhibiting the nuclear factor kappa B (NF-κB) signaling pathway. Polyphenols in the peel inhibit NF-κB activation, leading to a reduction in the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) [32]. This suppression of inflammatory mediators helps maintain the basement membrane integrity of thyroid follicles and prevents inflammatory-mediated destruction of thyroid parenchyma. Restoration of the cellular redox balance and improvement of mitochondrial function support the normal biosynthesis of thyroglobulin and promote the re-secretion of colloidal material into the follicular lumen [32]. The reduced oxidative and inflammatory stress allows thyrocytes to re-differentiate into their normal cuboidal morphology, leading to normalization of thyroid follicular architecture.

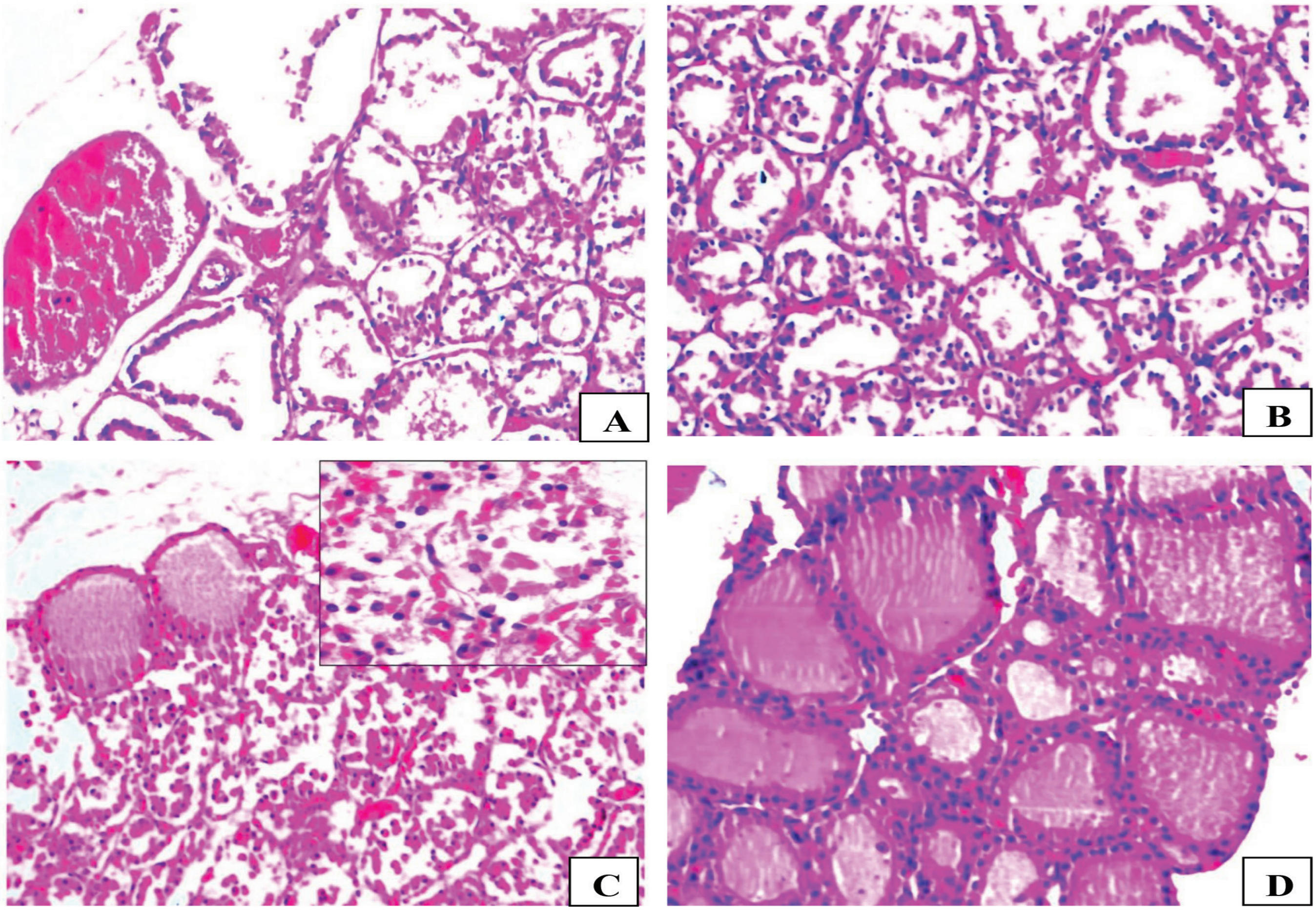

Fig. 1. Histopathological changes of thyroid gland in different experimental groups: (A) Complete loss of architectural details of thyroid follicles with a lack of colloid and interfollicular blood vessel congestion in Group II rats. H & E: x 100. **(B) Severe degeneration and desquamation of thyroid follicles with desquamated cellular debris inside the lumen and variation in the size of follicles in Group II rats.** H & E: x 100. **(C) Severe disruption and complete architectural loss of follicles in Group II rats.** H& E: x 100 (Insight microphotograph x 400). **(D) Restoration of normal architecture with presence of colloidal secretions inside the follicle, mild congestion, and mild degenerative changes in Group IV rats.** H & E: x 100.

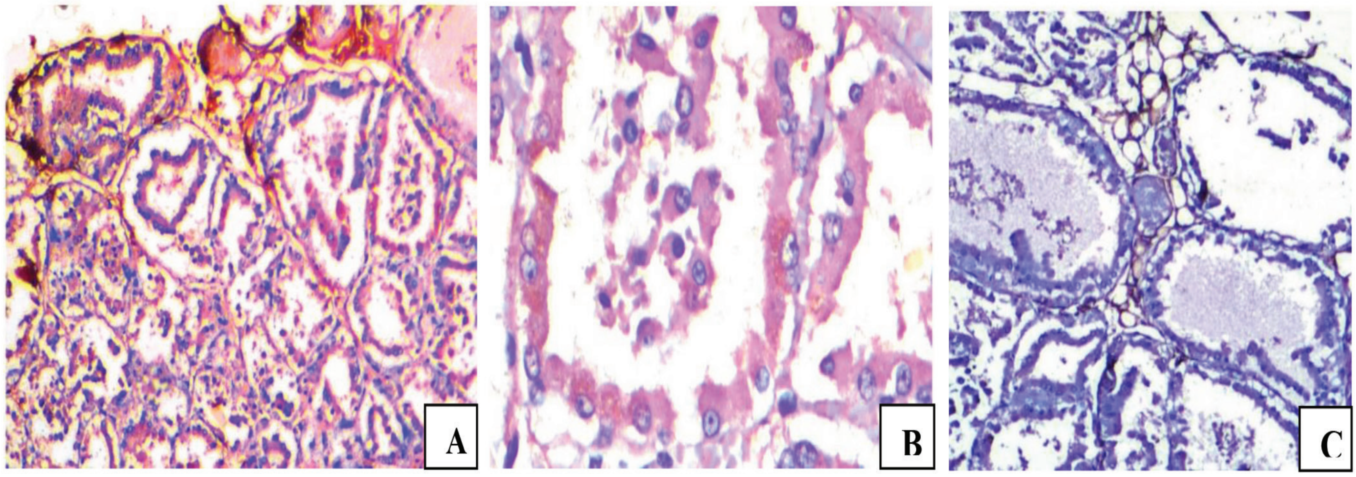

Fig. 2. Immunohistochemistry with BAX marker to check the apoptosis level: (A) Increased reaction in lining epithelium of thyroid follicles of Group II animals. BAX marker x 100; **(B) Increased reaction in lining epithelium of thyroid follicles in Group II animals.** BAX marker x 400; **(C) Negative immunoreactivity of thyroid follicles in Group IV rats.** BAX marker x 100.

Immunohistochemistry was done for the detection of apoptotic changes in thyroid by using monoclonal antibodies against BAX antigen in which brown colour develops indicates the presence of antigen. Immunohistochemically, BAX antigen was detected in the lining epithelium of thyroid follicles (Fig 2A, B) of fipronil treated rats (Group II). The similar observations were made by [33,34,35]. The findings might be due to fipronil induced alteration in the mitochondrial membrane integrity and inhibition of energy transport chain. In addition to decreased ATP production, it also activates the apoptotic enzymes like caspases 3 and 7 which initiates cell damage [2]. *Punica granatum* ameliorated rats (Group IV) showed mild immunoreactivity and decreased expression of BAX antigen (Fig 2C).This might be due to presence of ellagic acid and gallic acid present in pomegranate peel extract which exhibit antioxidant properties and powerful free radical scavengers which supresses lipid peroxidation and apoptotic damage of tissues by restoring the antioxidant enzyme activities like catalase, peroxidase and superoxide dismutases [36,37]. Punicalagin and ellagic acid downregulate the expression of the pro-apoptotic protein BAX while upregulating the anti-apoptotic protein Bcl-2, thereby improving the Bcl-2/BAX ratio [38]. This alteration in the expression of apoptotic regulators prevents mitochondrial outer membrane permeabilization and inhibits the downstream caspase cascade, ultimately reducing thyrocyte apoptosis and preserving the structural integrity of the follicular epithelial lining [21].

CONCLUSION

The present study was caried out to check the effects of fipronil toxicity in thyroid gland of Wistar albino rats and to check the ameliorative effect of *Punica granatum* peel extract in it. The observations made in this study indicates that fipronil @ 10 mg/kg b. wt. /day orally for a period of 6 weeks induces toxic effects on thyroid gland in rats due to accumulation of fipronil metabolites in these organs and associated oxidative damage. Alterations of thyroid hormones indicating that fipronil has an endocrine disruptive action. Co-administration of *Punica granatum* peel extract along with fipronil resulted in a significant improvement of hormonal status and restored histologic structures of thyroid gland near to normal which clearly supports the ameliorative potential of *Punica granatum* (pomegranate) peel extract for fipronil induced endocrine disruptions.

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