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Original Article

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Effect of Ashwagandha (*Withania somnifera*) on Thyroid functions in a Zebra Fish (*Danio rerio*) model

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ABSTRACT

Background: Extract from the roots of Ashwagandha plant has been used through generations for its positive effect as an anti-stress, anti-tumor, anti-arthritic, and neuro-regenerative agent. The compounds have shown considerable effect as a thyroid disrupting agent also. Extremely contradictory findings have been shown in both animal and human studies regarding the effect of Ashwagandha on thyroid levels, and hence it is important to monitor the safety and efficacy in pre-clinical models before we shift to clinical trials for the same.

Objective: To evaluate the thyroid disrupting potential of the Ashwagandha root extract (ARE) on Zebra Fish (*Danio rerio*) model.

Methodology: A total of 105 (7 groups of 15 each) transgenic Zebra fish embryos (tg: mCherry) at 2 days post-fertilization stage were included to study the effect of ARE on the developing thyroid gland. Potassium perchlorate (KOCl), a known thyrotoxic substance was used as a positive control and dimethyl sulfoxide (DMSO) was the vehicle control. The effect on thyroid glands was assessed using a thyroglobulin reporter gene fluorescence assay. Five serial concentrations from 1 mcg/ml to 50 mcg/ml of ARE were used. On day 5 post-fertilization, images were analyzed under a fluorescence microscope for intensity variations.

Result: The result from this study showed no differences in the expression of thyroglobulin reporter gene with Ashwagandha root extract.

Conclusion: The Ashwagandha root extract did not show any effect on thyroid gland in developing zebra fish embryos and can be considered safe with respect to its effects on thyroid gland.

Key Words: *Herbal supplement, Thyroid disrupting compound, Ashwagandha, Thyrotoxicity, Indian Winter Cherry, Indian Ginseng, Thyroid, Thyroid safety*

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1. INTRODUCTION:

Since time immemorial, numerous studies have come up that have shown the potential risk of various natural compounds on the functioning of the endocrine organs. [1,2] Thyroid disrupting compounds (TDCs) specifically alter the functioning of the thyroid glands, interfering with the synthesis, transport, and binding of thyroid hormones. TDC disrupt important physiological processes like energy metabolism, growth, and development. These alterations can have an ever-lasting impact on the nervous, reproductive, cardiovascular, and immune system of our body. [3,4]

Ashwagandha (*Withania somnifera*, family Solanaceae) is commonly known as "Indian Winter cherry" or "Indian Ginseng". The plant has gained importance for its anti-stress, anti-tumour, anti-arthritic and neuro-regenerative effects. [5,6] Studies are still being conducted all around the world to study the clinical impact of Ashwagandha and there still lies hidden potential in this plant yet to be explored. The impact of Ashwagandha on thyroid levels still remain a mystery.

While studies have shown a positive result in improving thyroid levels in subclinical hypothyroid patients, [7] others have shown that thyrotoxicosis could be a potential side effect of long term use of the extract of this plant. [8,9]

Zebra fish (*Danio rerio*) model has shown more than satisfactory results when it comes to studying vertebrate biology. The promising results can be attributed to the fact that it offers external development and optical clarity during embryogenesis that allows for visual analysis of the early development process and high fecundity and short generation time facilitates medium through-put screenings and genetic analysis. [10,11]Zebra fish is a widely used animal model in many biomedical and environmental studies. [12] Zebra fish presents a high genetic homology with humans (over 75% in many proteins functional domains) as well as important parallelisms in organogenesis and functional mechanisms. [13]All of this adds to the role of using Zebra fish model in studying the effect of our test compound in an animal model before we test the role it can play in humans.

The objective of the study was to test the thyroid disrupting potential of our test compound that was a derivative of the root of the Ashwagandha plant by determining their effect in Zebra fish embryos. For the assay, transgenic Zebra fish embryos were used.

2. METHODOLOGY:

2.1. Test Compound:

The test compound was root extract of the Ashwagandha plant that was sourced from Ixoreal Biomed, CA. At the start of the treatment, test substance was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml, instead of 100 mg/ml because there were solubility problems, and the highest concentration tested was adjusted based on the limit of a final 1% DMSO. Further dilutions were prepared from this reference aliquot, also in DMSO, to get x200 working concentrations. The reference aliquot was stored at 2–8 °C until the end of the Study.

2.2. Model:

For the experiment, Transgenic Zebra fish was used (tg: mCherry). The stage at the beginning of the study was 48 hpf (hours post fertilization) and at the end it was 120 hpf (hours post fertilization). 105 transgenic Zebra Fish was used for this study.

2.3. Animal Care:

Adult zebra fish were housed and maintained under standard procedures. [14] Briefly, fish were maintained in 3-liter aquaria heated to 28.5 °C, with about 20 fish per tank and water continuously filtered. Water was maintained at pH 7–7.8, conductivity at 500–800 µS, and O_2 saturation between 80–100%. Water conditions were monitored and regulated conveniently. Fish were kept under a photoperiod of 14/10 hours light/dark. Adults were fed with ground dry pellets and live food as described in the standard protocol at our facility (PNT-BBD-FAC-005).

Embryos were collected following standard operating procedure (PNT-BBD-FAC-022) and raised in E3 media [15] with Ampicillin (100 µg/ml) and Methylene blue (0.0001%) and kept in the incubator at 28.5 °C until they reach the stage for treatment. The stage of development was controlled by visual inspection using the stereoscope.

2.4. Reference Compound:

The reference compound we used is Potassium perchlorate (Molecular weight:138.5) in solid state stored at room temperature. The vehicle used was DMSO.

2.5. Thyroglobulin reporter gene fluorescence assay:

To assess the role of the test compound in Zebra fish, we used a thyroglobulin reported gene fluorescence assay. 15 transgenic embryos (tg: mCherry) was used per condition (5 embryos per well in 24-well plates as shown in figure 1). Five concentrations of the test substance were used. The EC_{10} value for the test compound that had been calculated in a separate experiment was 55.64 µg/ml. Concentrations tested were 50, 25, 10, 3, and 1 µg/ml. The control vehicle was a combination of E3 media + 0.5% DMSO. The positive control was Potassium perchlorate at 300 µM in 0.5% DMSO.

Plate Layout:

- A1, B1, C1: 1 µg/ml
- A2, B2, C2: 3 µg/ml
- A3, B3, C3: 10 µg/ml
- A4, B4, C4: 25 µg/ml
- A5, B5, C5: 50 µg/ml
- D1, D2, D3: Positive control (Potassium perchlorate 300 µM)
- D4, D5, D6: Vehicle control (E3 + 0.5%DMSO)

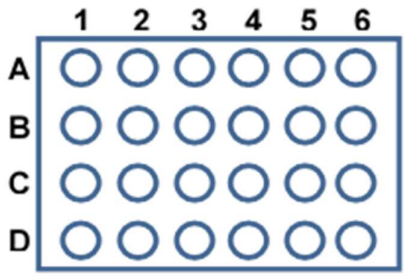

Figure 1: 24-well plate used for inoculation.

2.6. Exposure of the test compound:

At 2 dpf (days post fertilization), 5 unhatched embryos per well were placed in 24-well plates containing 995 µl of E3 medium. A volume of 5 µl of the corresponding working solution (or pure DMSO in the case of the vehicle control group) was added to each well and the content was homogenized by manual shaking of the plates. No precipitation occurred. Embryos treated with Potassium perchlorate 300 µM were used as the positive control. The entire test media volume was replaced at 3 dpf following the same procedure. The exposure plates were incubated at 28.5ºC for 3 days.

2.7. Image Acquisition:

At 5 dpf, 15 embryos per experimental condition were anesthetized in a solution containing 30 µg/ml Tricaine in E3 medium. Following, they were placed in a microscope slide coated with 3% Methylcellulose, and the rostral region was imaged with a DMi8 fluorescence microscope (Leica Microsystems, Danaher Corporation) using a TxRed filter.
Once analyzed, embryos were euthanized and eliminated.

2.8. Image Analysis:

The images were analyzed with ImageJ to assess the fluorescence intensity variations. A signal threshold method was used to define the fluorescence area of interest of each image. The image analysis procedure generated the following parameters related to the fluorescence signal of each image: area, mean intensity, and integrated density (IntDen = mean intensity × selected area).

2.9. Validity criteria, data analysis, and interpretation of the results

2.9.1. VALIDITY CRITERIA

For the test results to be valid, overall survival of embryos in the vehicle control was ≥ 80% until the end of the exposure and the normalized fluorescence integrated density value of the positive control (Potassium perchlorate 300 µM) was at least x1.5 compared to the vehicle control group mean. Since these conditions were met, the assay was not repeated.

2.9.2. DATA ANALYSIS AND INTERPRETATION OF RESULTS

The IntDen value obtained for each image was normalized by dividing it by the mean value of the vehicle control group. The mean, standard deviation (SD), and standard error of the mean (SEM) were calculated and represented for each experimental condition. The normalized mean of each experimental condition was compared to the mean of vehicle control through a two-way ANOVA followed by Dunnett's post-hoc test to identify statistically significant differences. Each experimental condition was considered statistically different from vehicle control if $p \leq 0.05$. The mean and SEM were represented on a bar graph.

Data interpretation of the results was based on several considerations: the magnitude of the response compared to the vehicle control (fold-change value), the dose-response effect, and the significance of the statistical analysis. The cut-off criteria for the fold-change value were 1.5. Therefore, if a test substance showed a Fold-change (FC) > 1.5 and the effect is dose-responsive, it was concluded that it was having a goitrogenic effect.

3. RESULTS:

Larvae exposed to Potassium perchlorate 300 µM were used as a positive control (Figure 2). As expected, a significant increase in fluorescence intensity in the thyroid gland was detected.

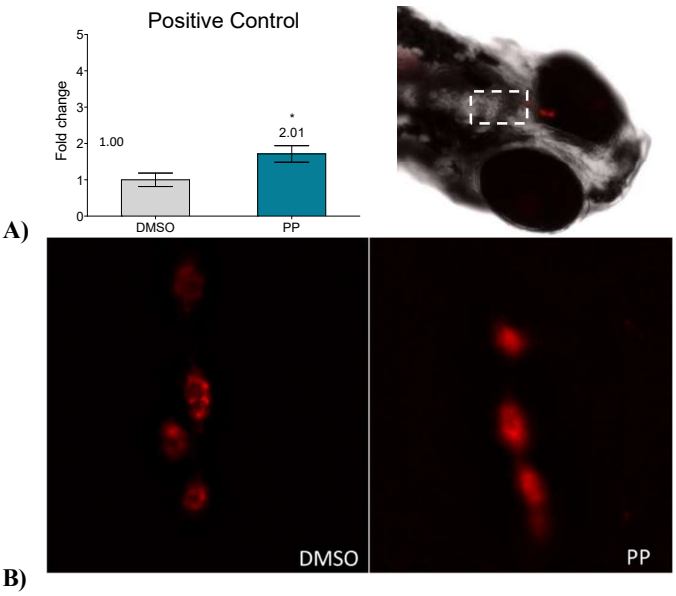

***Figure 2**. **A):** Bar graph of the mean fold-change of IntDen in DMSO 0.5% (vehicle control) and Potassium perchlorate 300 µM (PP, positive control) treated groups of the 3 fluorescence assays. Mean fold-change values are shown in numbers above the bars. Error bars represent the SEM valueabove and below the mean of the data. The dotted boxes indicate the region of interest where fluorescence intensity was quantified. **B):** Representative images of vehicle controltreated larvae (DMSO) and Potassium perchlorate 300 µM (PP, positive control).*

The maximum concentration selected for the subsequent fluorescence assay was around the EC10 value for the test compound.

The fold change in the control group was 1.00 and the fold change in different concentrations of the test compound range from 0.90-1.14. No significant changes in fluorescence intensity were detected after exposure to Ashwagandha extract as compared to the control group, as shown in the graph (Figure 3).

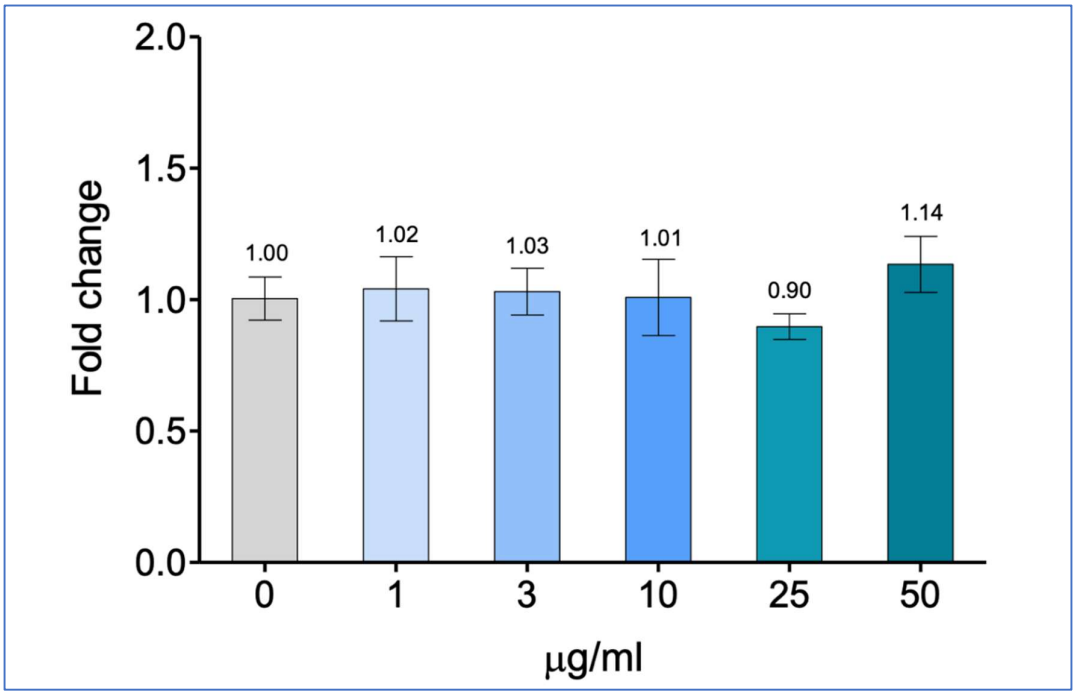

***Figure 3.** Bar graph showing mean fluorescence FC after exposure to test compound. Mean FC values are shown in numbers above the bars. Error bars represent the SEM value above and below the mean of the data.*

4. DISCUSSION:

The efficacy and safety of Ashwagandha extract, specially from the roots of the plant is well-established in Ayurveda, Unani, and Siddha systems of medicine. Chemo-assessment of Ashwagandha roots revealed that its primary ingredients are alkaloids and steroidal lactones. Withanolides, a group of steroidal lactones, is thought to be responsible for the pharmacological effects of Ashwagandha roots. [16] The pharmacological benefit of Ashwagandha has been established mostly through Reverse Pharmacology. The analysis of the chemical structure displayed multiple positions that can interact with target proteins. [17] However, further evidence is required to understand the exact molecular mechanism by which the pharmacological effects take place. Though recent times have shown a lot of backlash to the use of Ashwagandha because of a case series of liver toxicity reported in Iceland and US Drug Induced Liver Injury Network, [18] that led the Food and Drug Administration issuing a warning against the use of Ashwagandha products. However, in depth analysis of the case series shows that most of these patients were using other supplements that could have been the cause of toxicity, or the formulation was made not made out of just pure root extract and had leaves or whole plant, or was not taken in the recommended dose. [19]

Before we explore the vast therapeutic potential of the roots, it is important to establish the safety profile of the extract in humans. Studies have been conducted that have shown that the extract is safe from toxicological effects in a 28-days study in rats. [20] When the focus was shifted to evaluate the effect of Ashwagandha in thyroid hormone levels in adult male and female mice, it was shown to increase the values. [21,22] However, two human studies show contradictory results. [23, 24] First was a randomized, placebo-controlled study conducted on healthy volunteers by Verma N. et al (2021). The authors established the safety of Ashwagandha extract based on haematological and biochemical parameters (Liver function tests, thyroid profile) that were evaluated. [23] Second was a study by Chengappa RK. Et al (2014) in patients with bipolar disorders where Ashwagandha extract was given to improve cognition. In this study, one patient having subclinical hypothyroidism was reversed, and there were subtle changes in serum T4 levels. [24] In view of this finding, it was important to establish the safety profile and the effect of these drugs on thyroid levels in humans. To establish that, we need to first evaluate this in pre-clinical models that will act as a guide to move to studies on humans on a larger scale.

The objective of this study was to evaluate the thyroid disrupting potential of the extract of Ashwagandha plant. For this purpose, the EC_{10} value of the test substance was calculated from a previously conducted study and selected as the maximum dose for the thyroid disrupting assay. Potassium perchlorate was used as a positive control because it has shown effect in blocking uptake of iodide by the hyperactive thyroid and thus reduce thyroid hormone production. [25] Afterward, the fluorescence assay was carried out exposing embryos to 5 concentrations of the test substance and fluorescence images of the thyroid gland of 15 embryos per condition were taken. The test compound showed similar levels to the vehicle control, and no statistically significant differences, indicating safety of the extract to thyroid gland in the body even if there is no treatment-related goitrogenic effect. The use of Zebra fish for this seems to be a reasonable approach to understand if there is any potential for severe effect to thyroid levels in our body that can arise with the use of this extract. Our study shows no goitrogenic potential, and has shown results similar to the vehicle control indicating that the drug is safe for the thyroid gland. This study helps pave the way for more clinical trials to be conducted with larger sample sizes to see if we can explore the potential of this drug to have an impact on the thyroid level in human subjects.

5. CONCLUSION:

Ashwagandha root extract failed to show any effects on thyroid function in the Zebra fish model and can be considered safe for use with regards to its effect on thyroid function.

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