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# Research Article
# Acute and Sub-acute Toxicity Studies of a Patented Anti-anxiety Poly Herbal Formulation

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## Abstract

**Background and Objective:** Management of anxiety with synthetic drugs result in diverse systemic side effects. Traditional plant and herbal remedies available come into the limelight for their effective and safe use in anxiety. The present study investigated the acute and sub-acute toxicity studies of a polyherbal combination (PHC), namely PHC3 for the management of anxiety. **Materials and Methods:** The prepared PHC contains the roots of *Withania somnifera* (Ashwagandha), *Hemidesmus indicus*, fruit of *Aegle marmelos* (Bael), pericarp of *Emblica officinalis* (Amla) and fresh juice of aerial parts of *Ocimum sanctum* (Tulsi) in selected ratio. Acute, sub-acute toxicity studies and histopathological study were carried out as per the standard guidelines. **Results:** No mortality was observed in acute toxicity study. In sub-acute toxicity study, no statistically significant ($p>0.05$) difference was observed with respect to feed intake, water intake, body weight changes, biochemical enzymes, biochemical metabolites, electrolytes, hematology and histopathology studies between control and treated rats. **Conclusion:** The developed polyherbal combination was found to be safe in both acute and sub-acute toxicity studies and thus, the tested combination is suitable for further pharmacological and pharmacokinetic studies.

Key words: Polyherbal combination, acute toxicity, sub-acute toxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

In the last two decades, the use of herbal medicines has gained growing popularity and being used worldwide. According to the report by the World Health Organization (WHO), 80% of people rely on herbal medicines as part of their primary health care needs[1]. Herbal formulations have attained wide recognition in comparison to crude plant materials and extracts, due to reduction in dose, convenience and ease of administration. These formulations are popular worldwide as therapeutic agents, in various ailments that impact the quality of life[2].

Anxiety being a normal emotional behavior in humans becomes a physiological disorder characterized by disproportional or persistent fear, unrelated to any kind of risk, leading to aggravation of cardiovascular or psychiatric complications[3]. Plants with very vast medicinal properties are found a plenty among us with a great focus on research aimed at identification and validation of their active principles due to their immense potential in curing multiple ailments and diseases[4,5]. With only a very few effective clinical remedy available for treating the states of anxiety, that results in diverse systemic side effects resulting in developing tolerance against the drug upon chronic use[6]. Traditional plant and herbal remedies available come into the limelight for their effective and safe use in anxiety. Hence, in search for a natural supplement to alter the neuro-transmitters, a polyherbal chewable tablet was developed and patented for the management of anxiety (Patent No. 2308/CHE/2013).

The developed polyherbal formulation contains the roots of *Withania somnifera* (Ashwagandha), *Hemidesmus indicus*, fruit of *Aegle marmelos* (Bael), pericarp of *Emblica officinalis* (Amla) and fresh juice of aerial parts of *Ocimum sanctum* (Tulsi)[7]. The raw materials were standardized according to the WHO guidelines. Except *Ocimum sanctum*, all other plants were shade dried, powdered, exhaustively extracted with various fractions of absolute alcohol and water (60:40) and spray dried, while the fresh juice of *Ocimum sanctum* was lyophilized. The spray dried and lyophilized extracts were mixed in different ratios to arrive at different combinations (PHC1 to PHC15) by bio-guided method.

All the combinations were subjected to preliminary phytochemical investigation and *in vitro* anti-oxidant screening using DPPH scavenging, lipid peroxidation, nitric oxide scavenging and super oxide dismutase activity methods. The $IC_{50}$ concentrations were determined and the best polyherbal combination was selected based on the correlation coefficient calculated using linear regression analysis[7]. The aim of the present study is to perform the acute and sub-acute toxicity studies of the developed polyherbal combination.

## MATERIALS AND METHODS

**Plant collection and authentication:** The roots of *Withania somnifera* (*Solanaceae*), *Hemidesmus indicus* (Asclepiadaceae), fruit of *Aegle marmelos* (Rutaceae), pericarp of *Emblica officinalis* (Euphorbiaceae) were collected in the month of August, 2010 in Tirunelveli district, Tamil Nadu and authenticated by Dr. V. Chelladurai, Research Officer (Retd), Survey of Medicinal Plants Unit, Central Council for Research in Ayurvedic Sciences (CCRAS), Govt. of India. The fresh juice of aerial parts of *Ocimum sanctum* (Labiatae) was collected in the month of September, 2010 in the herbal garden, Sri Ramachandra Institute of Higher Education and Research, Deemed to be University, Chennai, Tamil Nadu. A voucher specimen (No. 11-15) has been deposited in the herbarium of the College of Pharmacy, Sri Ramachandra Institute of Higher Education and Research, Chennai.

**Acute toxicity study:** The acute toxicity study was carried out as per Organization for Economic Co-operation and Development (OECD) guidelines (427) after prior clearance from the Institutional Animal Ethical Committee (IAEC) No. (IAEC/XIX/SRU/142/2010). The study was carried out in Centre for Toxicology and Developmental Research (CEFT), Sri Ramachandra Institute of Higher Education and Research, Chennai. It is a single dose study for a period of 14 days. Sprague Dawley rats (150-180 g) of either sex (n = 10, male 5, female 5, age 6-8 weeks old) maintained in the Central Animal Facility, Sri Ramachandra Institute of Higher Education, Deemed to be University, Porur, Chennai were used. The animals were housed in a well-ventilated room with 12 h light/12 h dark and temp of 23±2°C with 50-70% relative humidity. The animals had free access to pelleted feed (Nutrilab, Bangalore) of standard compounds containing all macro and micro-nutrients. Aqua guard (on-line water filter cum purifier) water was provided *ad libitum*. The animals were examined at regular intervals by trained personnel for any behavioral abnormalities. The animals were divided into 3 groups viz:

- **Group I:** Vehicle control
- **Group II:** PHC1 (2000 mg $kg^{-1}$)
- **Group III:** PHC3 (2000 mg $kg^{-1}$)

Mortality, clinical observations and body weight changes were recorded[8].

**Sub-acute toxicity study:** The study was approved by the Institutional Animal Ethics Committee of Sri Ramachandra Institute of Higher Education and Research, Deemed to be University, Porur, Chennai (Approval No. IAEC/XIX/RU/142/2010). The study was carried out in Centre for Toxicology and Developmental Research (CEFT), Sri Ramachandra Institute of Higher Education and Research, Chennai. Sprague Dawley rats (150-180 g) of either sex (n = 10, male 5, female 5, age 6-8 weeks old) were used for the study. The female rats were nulliparous and non-pregnant. Animals were obtained from the Centre for Toxicology and Developmental Research (CEFT), Sri Ramachandra Medical College and Research Institute, Deemed to be University, Porur, Chennai, Tamil Nadu. They were kept separately in a room, well ventilated with 100% fresh air. The temperature in the experimental animal room was 22±3°C. Although the relative humidity was at least 30% and preferably not exceed 70% other than during room cleaning, the aim was 50-60%. Lighting was artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets were used with an unlimited supply of drinking water. Sub-acute toxicity study was conducted as per OECD guideline 407. The animals were examined at regular intervals by trained personnel for any behavioral abnormalities[9].

The PHC3 was administered at 3 dose levels viz. 200, 400 and 800 mg $kg^{-1}$ p.o., for 28 days. The animals were observed for mortality, body weights, daily feed intake and the biochemical enzymes and metabolites, hematological analysis, urine analysis includes, specific gravity, pH, protein, ketone bodies, bilirubin, blood cells, nitrites, sugars and urobilinogen and electrolytes such as sodium, potassium and calcium were determined on day 0 and 29.

**Histopathological examination:** On day 90, after the completion of the experiment, all the animals from each group were sacrificed by cervical dislocation under general anesthesia. The adrenal gland, brain, eye cornea, heart, intestine, stomach gland, kidney, thymus, liver, spleen, ovary, testis were collected and fixed in 10% neutral buffered formalin for 48 h, processed for paraffin embedment, sectioned and stained with hematoxylin and eosin for general histopathological examination[10].

**Statistical analysis:** Statistical analysis was performed using GraphPad Prism, 4.03 (San Diego, US). Data were expressed in Mean±SEM. Students t-test was used to compare the mean difference between the vehicle and the drug treated groups. The mean difference was calculated by one-way ANOVA with Dunnett compare all pairs of columns as the *post hoc* test. A probability value less than 0.05 was fixed as the statistical significance criterion.

## RESULTS

**Acute toxicity study:** In acute toxicity study, the rats were observed for mortality and clinical observations at 0.3, 1, 3 and 4 h post-dose on day 0 and twice daily (morning and afternoon) thereafter for 14 days. Body weights were recorded on day 0, 7 and 14. No mortality and clinical signs such as ataxia, convulsion, exophthalmia, lacrimation, oral/nasal discharges, gait, piloerection and polyuria were observed in all the three groups. No remarkable changes or differences observed in body weight. All the animals were normal in behavior throughout the study.

**Sub-acute toxicity study:** Results of the sub-acute toxicity study of polyherbal combination treated rats are presented in Fig. 1. No mortality was observed during the treatment period either in the control group or in PHC3 treated groups. The treated groups did not show any statistical significance and clinical relevance in feed intake (Fig. 1a), water intake (Fig. 1b), body weight changes (Fig. 1c). Likewise, no statistically significant difference was observed with respect to biochemical enzymes (Fig. 2a-d), biochemical metabolites (Fig. 3a-d), electrolytes (Fig. 4a-d) and hematology (Fig. 5a-c).

**Histopathological study:** The adrenal glands of highest dose treated (800 mg $kg^{-1}$) rats did not show any pathological changes and appears similar to normal groups. The brain of PHC3 treated rats showed no significant pathological changes when compared to control. The heart muscles showed no pathological changes and appears normal when compared to control groups. The cornea of 800 mg $kg^{-1}$ treated rats showed no abnormalities when compared to normal control. The intestine showed normal villi in PHC3 treated rats. No ulceration or necrosis seen in the stomach of treated rats when compared to control. The cortex of the kidney also showed normal glomeruli and nucleated intact cells and were similar in architecture to that of the control group. The thymus of both treated and control groups were found to be normal in architecture. No necrosis seen in 800 mg $kg^{-1}$ treated rats and intact hepatocytes seen in both control and treated rats. The spleen of both normal and treated rats showed no significant pathological changes (Fig. 6, 7).

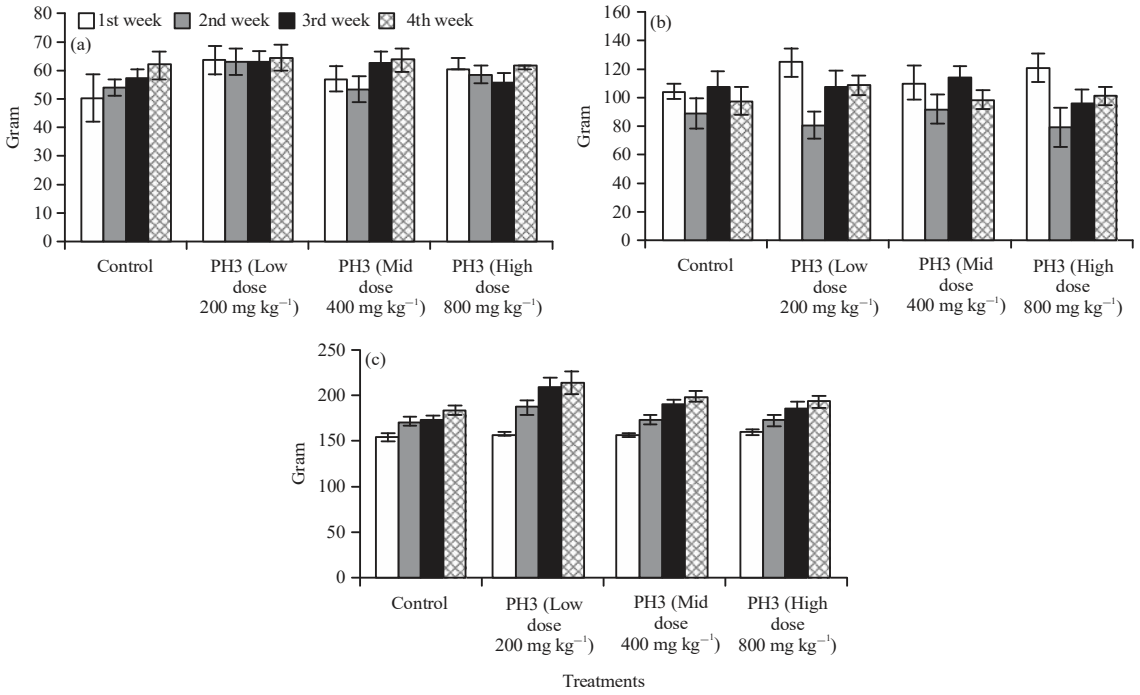


Fig. 1(a-c): Baseline measurements of food, water, body weight at various time intervals (a) Feed intake, (b) Water intake and (c) Body weight

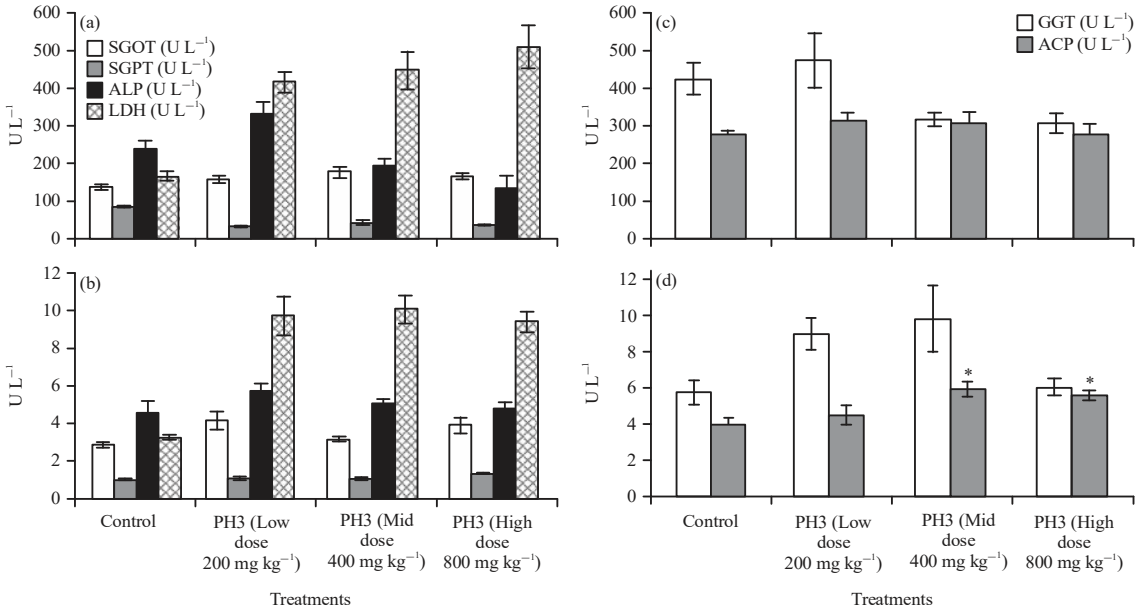


Fig. 2(a-d): Comparison of liver and renal enzymes value of treatment group with control (a) Day 1 value of liver enzymes, (b) Day 29 value of liver enzymes (c) Day 1 value of renal enzymes and (d) Day 29 value of renal enzymes

**Adverse effect level:** The Lowest Observed Adverse Effect level (LOAEL) was not observed and the combination shows only No Observed Adverse Effect Level (NOAEL) at the tested doses. In urine analysis, physical parameters such as bilirubin, nitrite, glucose, blood were totally absent in all the groups of rats on both day 1 and 29. The ketone bodies and urobilinogen were found to be traced in all the groups. The pH of urine in all the groups was in the range of 7.1-8.0 in both

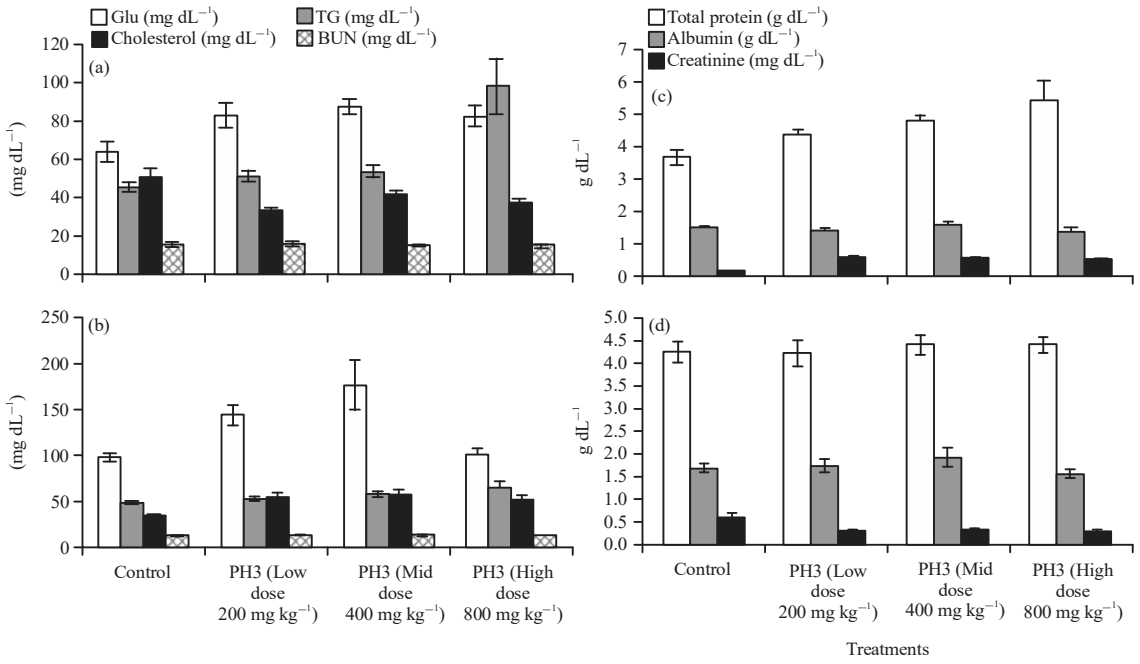


Fig. 3(a-d): Comparison of biochemistry metabolites of treatment group with control (a) Day 1 value of glucose, triglycerides, cholesterol, blood urea nitrogen, (b) Day 29 value of glucose, triglycerides, cholesterol, blood urea nitrogen (c) Day 1 value of total protein, albumin, creatinine and (d) Day 29 value of total protein, albumin, creatinine

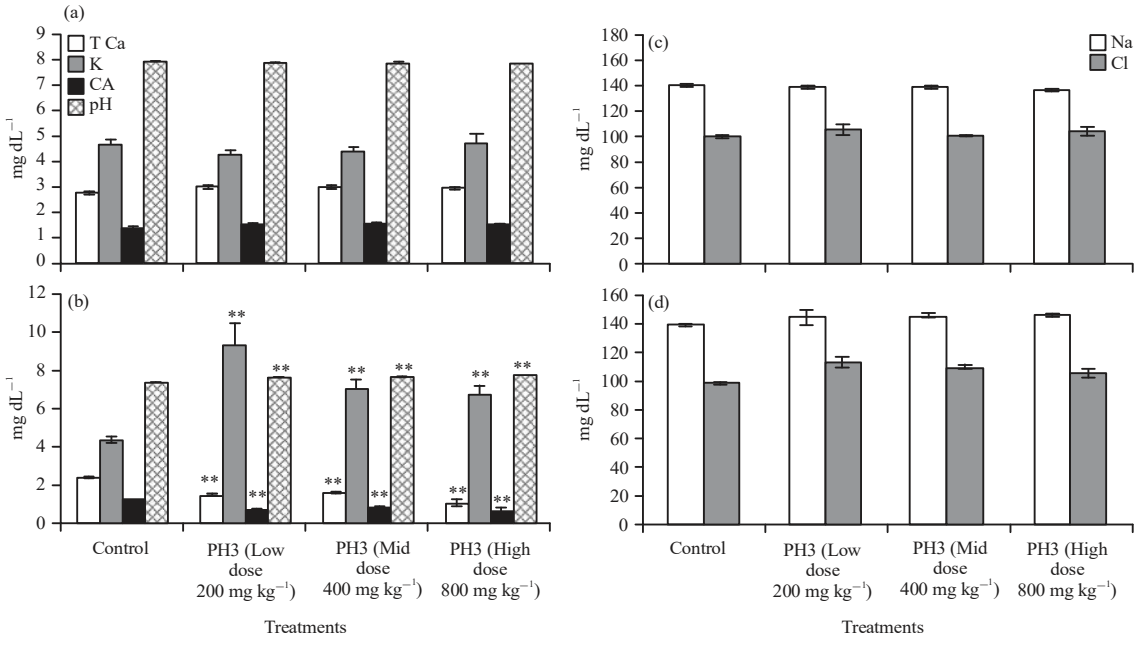


Fig. 4(a-d): Comparison of electrolytes of treatment group with control , (a) Day 1 value of total calcium, potassium, calcium, pH (b) Day 29 value of total calcium, potassium, calcium, pH (c) Day 1 value of sodium, chloride and (d) Day 29 value of sodium, chloride

day 1 and 29. The specific gravity is also un-altered and remains the same in both control and PHC3 treated groups (1.01-1.02). Hence, the selected PHC3 was found to be safe at the tested doses.

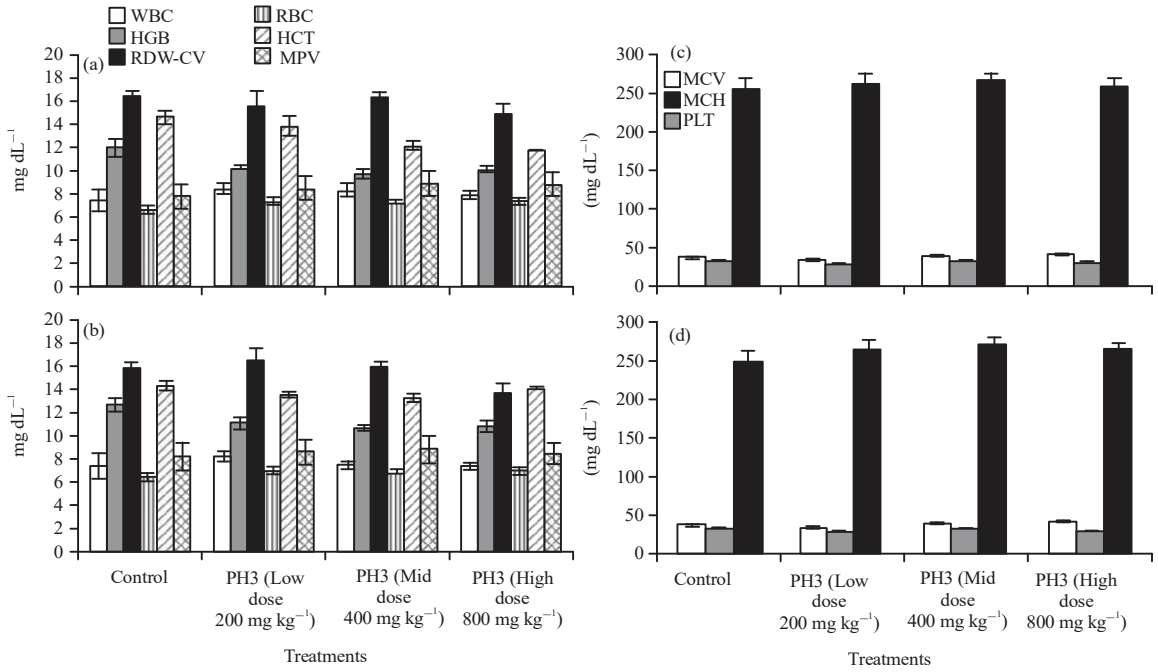


Fig. 5(a-d): Comparison of hematology of treatment group with control (a) Day 1 value of WBC, RBC, HGB, HCT, RDW-CV, MPV (b) Day 29 value of WBC, RBC, HGB, HCT, RDW-CV, MPV (c) Day 1 value of MCV, MCH, PLT and (d) Day 29 value of MCV, MCH, PLT

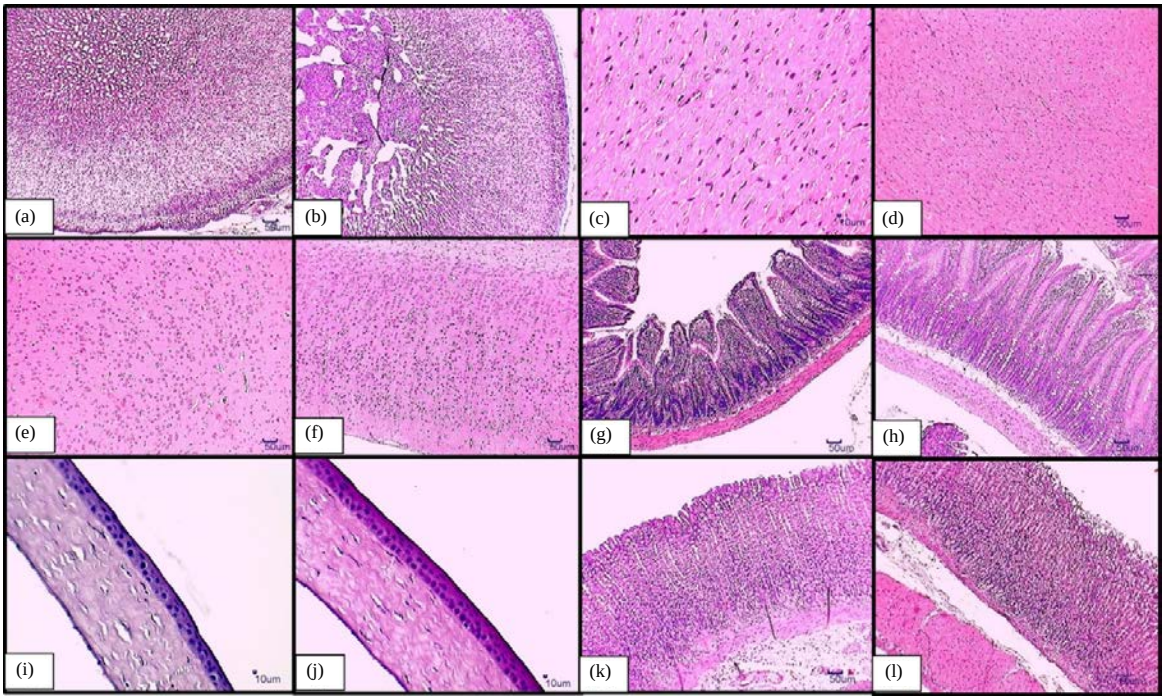


Fig. 6(a-l): Comparison of histopathology of adrenal gland, heart, brain, intestine, eye cornea, stomach glandular of treatment group (800 mg kg$^{-1}$) with control under 10X , (a) Adrenal gland: control, (b) Adrenal gland: treated, (c) Heart: control, (d) Heart: treated, (e) Brain: control, (f) Brain: treated, (g) Intestine: control, (h) Intestine: treated, (i) Eye: control, (j) Eye: treated, (k) Stomach glandular: control and (l) Stomach glandular: treated

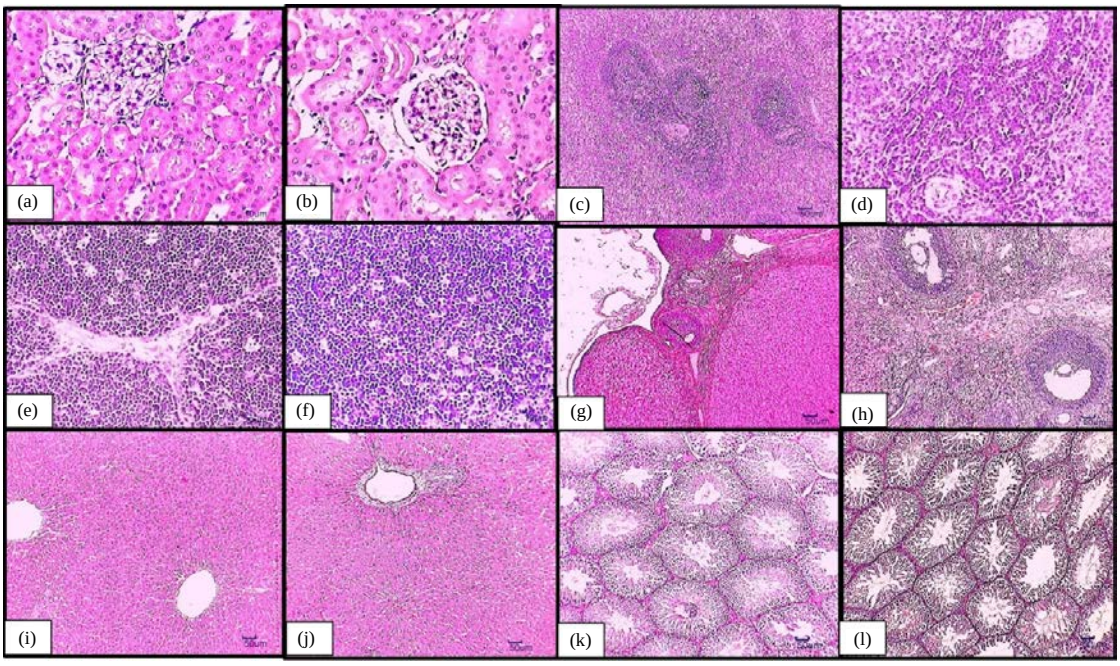


Fig. 7(a-l): Comparison of histopathology of kidney, spleen, thymus, ovary, liver, testis of treatment group (800 mg $kg^{-1}$) with control under 10X, (a) Kidney: control, (b) Kidney: treated, (c) Spleen: control, (d) Spleen: treated, (e) Thymus: control, (f) Thymus: treated, (g) Ovary: control, (h) Ovary: treated, (i) Liver: control, (j) Liver: treated, (k) Testis: control and (l) Testis: treated

## DISCUSSION

Toxicity studies of the polyherbal combination revealed the safety profile in terms of acute and sub-acute studies. In developing countries, most of the population relies on traditional medicines, considering their afford ability, traditional background knowledge on medicinal plants and a belief that they are harmless[11,12]. Many synthetic drugs are known to act on a single molecular target and provide symptomatic relief. The multi-target responses of herbal drugs are proven to be beneficial in chronic conditions such as diabetes, cancer and so forth and also in restoring the health status[13]. Although many natural plant extracts used traditionally have passed the test of time, in terms of toxicity and adverse effects, the safety of the active phytochemicals from these plants must precede their pharmaceutical use. There is a need to assure the safety of herbal formulations in order to acquire their maximum benefits even though these have been proven to be efficacious in pharmacological studies or by clinical evaluation[14,15]. Toxicity studies are considered necessary, especially on drugs that are to be used in chronic conditions.

Anxiety is a normal emotional behavior, however, when it becomes severe and chronic, leads to pathological psychiatric, these components combine to create an unpleasant feeling that is typically associated with uneasiness, fear and worry[16]. Anxiety is a generalized mood condition that occurs without identifiable triggering stimulus, fear is related to the specific behaviors of escape and avoidance, whereas, anxiety is the result of threats that are perceived to be uncontrollable or unavoidable[17]. Although many drugs are available in allopathic medicine to treat anxiety disorders, they produce various systemic side effects of exhibit tolerance up for chronic use. Benzodiazepines are the class of compound used in anxiety and they remain the most commonly prescribed.

In ayurvedic medicine, many plant products have been claimed to be free from side effects and less toxic than synthetic drugs. A new approach has been undertaken to overcome anxiety disorder by formulating a polyherbal formulation as a chewable tablet by using five plants viz., bio-guided combination of the spray dried hydro alcoholic extracts of *Withania somnifera*, *Hemidesmus indicus*, *Aegle marmelos*, *Emblica officinalis* and lyophilized juice of *Ocimum sanctum*.

Among the five plants, *Withania somnifera* is claimed to have immunomodulatory, adaptogenic and anabolic effects along with the ability to improve vital energy[18,19]. Aegle

*marmelos* claimed to possess various therapeutic effects like anti-oxidant, anxi-olytic, anti-diabetic, anti-ulcer, anti-malarial, anti-cancer, anti-fungal, anti-bacterial and anti-viral activities[20], *Emblica officinalis* prevents the retrain stress-induced oxidative stress and balancing the anti-oxidant system, since *Emblica officinalis* is reported as a rich source of vitamin[9] C, *Ocimum sanctum* also claimed to prevent the retrain stress-induced oxidative stress anti-bacterial, anti-fungal, potent natural anti-oxidant[21].

The ingredients present in the formulation are purely phytochemical in origin and contain sterols, triterpenoids, saponins, flavonoids, etc. Since a number of phytoconstituents are present in this formulation, it is decided important to screen the formulation for toxic effect. Since no toxic effects were observed in toxicity study, it could be inferred that the basic principle in the use of crude plant products or polyherbal preparations in traditional medicine, is that the toxic effect of one component is nullified by the protective effect of other components, without inferring with their therapeutic properties.

## CONCLUSION

Polyherbal formulation was prepared for anti-anxiety management. Of the 15 polyherbal combinations prepared (PHC1-PHC15), the best one (PHC3) was selected based on *in vitro* anti-oxidant and $IC_{50}$ values. Both acute toxicity and sub-acute toxicity studies were performed. The PHC3 was found to be non-toxic and suitable for the development of formulation.

## SIGNIFICANCE STATEMENT

This study discovered the acute and sub-acute toxicity profile of a patented polyherbal formulation that can be beneficial for the management of anti-anxiety. The findings of this study greatly help the researchers to provide useful evidence for the development of suitable formulation for its preclinical and clinical evaluation.

## ACKNOWLEDGMENT

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