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Evaluation of subacute toxicity of a polyherbal nootropic formulation in Wistar albino rats

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In ayurvedic system of traditional medicine, 'medhyarasayanas' — decoction of selected plants are used to improve intellect or cognition abilities. Here, we investigated one such polyherbal formulation (PHF) with ingredients *Bacopa monniera*, *Glycyrrhiza glabra*, *Valeriana wallechii* and *Withania somnifera* used as a facilitator of learning, retention and recall. We evaluated the safety of this PHF in animal models. We performed acute oral toxicity test using 2000 mg/kg of the formulation as per OECD guideline 423 and observed for toxicity over 14 days. Thereafter, we divided them into four groups of six animals each and administered Normal saline 5 mL/kg, The PHF (Wilmer®) @500, 1000 and 2000 mg/kg in the respective groups over 28 days. We observed no mortality or physical and behavioural abnormalities in both acute and subacute toxicity study. On the 28th day, animals were sacrificed, blood collected for estimation of haematological and biochemical parameters and histopathological examination of organs was performed. There was significant difference (P <0.05) in Mean ± SEM values of haemoglobin, total cholesterol, total protein, ALT, AST, ALP and serum creatinine in the test groups compared to control as analysed by One-way ANOVA followed by Tukey's multiple comparison test. We observed steatosis and ballooning of hepatocytes, lymphocytic periglomerular infiltrate, eosinophilic hyaline casts and renal tubular coagulation necrosis in histopathology of test groups. Haematologic abnormalities (decrease in haemoglobin concentration), hepatotoxic, nephrotoxic and dyslipidemic effects of the tested PHF were seen in the rats in subacute toxicity study over 28 days, which could be due to the individual plant products, microbial contamination or heavy metals in the formulation in excess of regulatory limits. Hence, it needs further safety evaluation in animals and humans.

Keywords: Ayurvedic, Cognitive, Herbal, Traditional medicine

The process of acquiring, retaining and recalling intellectual knowledge is called cognition. The cognitive processes include learning, memory, comprehension, reasoning, awareness and judgment[1]. Cognitive functions are impaired due to physiological degenerative changes associated with ageing or pathological causes. Cognitive disorders are classified as a category of mental health disorders (dementia, delirium, amnesia, vascular and metabolic changes, drugs, degenerative and neuropsychiatric diseases) that affect memory and problem solving[2,3].

The impairment of cognitive functions can be improved by use of nootropics or cognition enhancers – a heterogenous group of drugs that can facilitate cognitive processes like learning, retention and recall.[4] However, their therapeutic benefit is limited and provide only symptom relief. Thus, there are limitations of conventional allopathic nootropic drugs. Hence, herbal medicine can be a viable option in the treatment of cognitive deficits, especially in developing countries[5,6].

According to the Ayurvedic system of traditional medicine, a few selected plants have long been classified as 'medhyarasayanas', from the Sanskrit words 'medhya', meaning intellect or cognition, and 'rasayana', meaning a therapeutic procedure or preparation that on regular practice will boost nourishment, health, memory, intellect, immunity and hence longevity[6,7]. Wilmer® syrup is a combination of plant materials mentioned under the group Medhyarasayanas, containing plant ingredients of Brahmi (*Bacopa monniera*), Yastimadhu (*Glycyrrhiza glabra*), Tagar (*Valeriana wallechii*) and Ashwagandha (*Withania somnifera*). Various researchers have reported the beneficial effects of active principles of these four plants on cognition[8-14].

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This polyherbal formulation is reported to possess anxiolytic, anti-stress and neuroprotective effect, and used to improve cognitive functions like attention span, learning ability and memory. The formulation is claimed to provide relief from stress, anxiety, fatigue and amnesia and is indicated in the treatment of amnesia (general), attention deficit in children and adults, anxiety neurosis and chronic fatigue syndrome[15].

One of the major drawbacks preventing widespread acceptance of herbal remedies among the scientific community is the absence of evidence-based information about toxicities either inherent to the plant or the combinations used in herbal supplements. Hence, users of these formulations may be facing health risks due to the unknown adverse effects. Despite all such claims there is a lack of research on evaluation of short term and long term toxicity of this formulation. Hence, in this study, we have made an attempt to evaluate the subacute toxicity of this polyherbal formulation (PHF) in Wistar albino rats.

Materials and Methods

Drugs

For polyherbal formulation, Syp. Wilmer® (200 mL) containing plant ingredients of *Bacopa monniera*, *Glycyrrhiza glabra*, *Valeriana wallechii* and *Withania somnifera* was obtained from Annapurna Bio Ved Pvt. Ltd., Hyderabad, India.

Animals

Healthy Wistar albino rats 8-10 weeks of age of both sexes weighing between 120-150 g were procured from Kings Institute Guindy, Chennai and kept in the Central Animal House of the institute. The experiments were carried out after a seven-day period of acclimatization of the animals. The animals were cared for and maintained according to the principles of the care & use of animals in the guidelines for laboratory animals by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). They were maintained on their respective diets and water *ad libitum* on a 12 h light/dark cycle in a temperature regulated room (20-25°C) during the experimental procedures.

Acute toxicity test

Healthy Wistar albino rats (8-10 weeks old) of both sexes, weighing 120-150 g and maintained under standard laboratory conditions were used for the acute toxicity test (Fixed dose procedure) according to the OECD guidelines 423. The experimental animals were kept fasting overnight prior to drug administration by oral gavage. Ten animals (five each of male and female) were used and each of them received a single oral-dose of 2000 mg/kg body weight of the polyherbal formulation.

After administration of the drug, food was withheld for a further period of 3-4 h. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days for mortality or any signs of toxicity, like physical changes in skin and fur, eyes, mucus membranes and behavioural changes (sleep, lethargy, altered consciousness as well as food and water intake).

Experimental design

After 14 days, 24 rats were randomly divided by simple random sampling using table of random numbers into four groups of six each, having equal number of males and females and were treated once daily. We administered normal saline 5 mL/kg (Gr. I); and Wilmer® PHF @500, 1000 and 2000 mg/kg in the respective groups (Gr. II-IV) over 28 days. Dose selection was based on the results of acute toxicity study of Wilmer® PHF. Drugs were administered by oral feeding with gavage needle daily between 9-10 a.m. Animals were observed for mortality or any physical or behavioural changes – physical changes in skin and fur, eyes, mucus membranes, lethargy, sleep, altered consciousness, food and water intake for the entire duration of the study (28 days). Body weight of the animals was measured at baseline weekly thereafter. At the end of 28 days, all the animals were sacrificed using Inj. Sodium pentobarbitone 150 mg/kg i.p. after an overnight fast. Prior to dissection, blood samples were collected for haematological and biochemical analysis through cardiac puncture.

Haematological and Biochemical parameters

Haemoglobin, RBC count, WBC count and platelet count were assessed by standard procedures as described by Raghuramulu, *et al.*[16]

Glucose was estimated by Hexokinase method, serum cholesterol by CHOD PAP method, total protein by Biuret method, and albumin by BCG Dye binding method[16]. In liver function tests, alanine transaminase (ALT) was estimated by Modified IFCC/UV kinetic method, aspartate aminotransferase

(AST) by Modified IFCC/UV kinetic method and alkaline phosphatase (ALP) by PNPP-AMP kinetic method. In renal function tests, blood urea nitrogen was estimated by Urease GLDH/UV kinetic method and serum creatinine by Jaffe/kinetic method[16].

Histopathological study

After sacrificing the animals, the vital organs like liver, kidney, spleen, heart, lungs, brain, testes, epididymis, prostate, uterus and ovaries were carefully harvested and weighed immediately without drying. Then the organs were preserved in 10% formalin and sent for histopathological examination as per the method described by Preece[17].

Statistical analysis

We used SPSS Version 22.00 for statistical analysis. Values of the haematological and biochemical parameters were expressed as Mean ± SEM. Statistical significance was analysed using ANOVA followed by Tukey's multiple comparison test for inter-group analysis. Level of significance was kept at P <0.05 at 95% confidence level.

Results

In acute toxicity test with single dose of 2000 mg/kg, there was no mortality and we observed no abnormal physical changes (like changes in skin and fur, eyes and mucus membranes) or behavioural changes (sleep patterns, lethargy, altered consciousness as well as food and water intake) over the 14-day observation period. There were changes in body weight of the animals, with decrease in test groups, but it was not statistically significant.

In subacute toxicity test, all groups of animals displayed no mortality or signs of toxicity over the 28-day period. On estimating haematological and biochemical parameters, we observed Mean ± SEM values in the different groups (Normal saline, 500, 1000 and 2000 mg/kg Wilmer® PHF) with ANOVA and Tukey's test analysis, as shown in Tables 1 and 2.

We observed that there was no significant difference in blood glucose and blood urea nitrogen levels between all groups. However, total cholesterol, total protein, AST, ALT, ALP and serum creatinine were significantly different between groups (P <0.05). At the dose of 2000 mg/kg, haemoglobin, total cholesterol, total protein and serum creatinine levels were significantly decreased compared to the control and lower dose test groups. At the dose of 1000 and 2000 mg/kg, ALT levels were significantly increased compared to the control group. Dose dependent significant increase in ALT was seen in 1000 and 2000 mg/kg compared to 500 and in 2000 mg/kg compared to 1000 mg/kg group. AST levels were significantly increased compared to the control at all dose levels and dose dependent increase at different dose levels of PHF was observed. ALP was significantly increased at 2000 mg/kg compared to the control group and dose dependent significant increase was seen at 2000 mg/kg compared to 500 and 1000 mg/kg groups.

Analysis of gross and histological examination of organs

A gross examination of vital organs revealed no abnormalities in both control and test groups. Histopathological evaluation of the brain, lung, spleen, testes, epididymis, prostate gland, uterus and ovaries did not reveal any pathological changes in both control and test groups, except increase in size of the liver in the latter. No abnormalities were observed in liver, heart and kidney in control group.

In histopathological examination, rat liver showed ballooning of hepatocytes, macrovesicular steatosis

Table 1 — Haematological parameters in the respective groups (n=6 in each group)

Group	Haemoglobin (g/dL)	RBC (million/cu. mm)	WBC (/cu. mm)	Platelets (lakhs/cu. mm)
I	15.60±0.64	6.96±0.23	7.22±0.60	6.35±0.23
II	15.65±0.34(ns)	6.61±0.26(ns)	7.02±0.57(ns)	6.25±0.23(ns)
III	15.71±0.57(ns)	6.38±0.22(ns)	8.35±0.44(ns)	6.35±0.40(ns)
IV	13.13±0.70*	6.63±0.34(ns)	8.74±0.63(ns)	6.88±0.22(ns)

[Gr. I, Normal control; Gr. II-IV, Wilmer® PHF @500, 1000 and 2000 mg/kg, respectively. Data expressed as Mean ± SEM. Statistical analysis by one-way ANOVA followed by Tukey's test. Significance P <0.05* and ns = not significant *vs*. control group]

Table 2 — Blood biochemical parameters in the respective groups (n=6 in each group)

Groups	Blood glucose (mg/dL)	Total cholesterol (mg/dL)	Total protein (g/dL)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Blood urea nitrogen (mg/dL)	Serum creatinine (mg/dL)
I	102.66±4.63	97.00±3.17	6.90±0.17	53.16±3.40	29.83±2.15	94.33±2.82	17.83±1.24	0.30±0.05
II	107.00±5.50(ns)	95.67±3.64	6.78±0.21	56.33±2.24	53.00±5.53**	97.00±6.20	20.66±2.02(ns)	0.31±0.04
III	110.16±1.34(ns)	105.66±4.47	6.91±0.20	79.83±3.27**	88.33±3.96**	100.83±3.49	16.50±0.92(ns)	0.38±0.04
IV	118.00±5.88(ns)	137.00±2.82**$^{\$,\%}$	5.86±0.18**$^{\$,\%}$	96.67±2.56**$^{\#,\$,\%}$	139.33±3.13**$^{\#,\$,\%}$	143.16±4.55**$^{\$,\%}$	19.66±1.74(ns)	0.71±0.08**$^{\$,\%}$

[Gr. I, Normal control; Gr. II-IV, Wilmer® PHF @500, 1000 and 2000 mg/kg, respectively. Data expressed as Mean ± SEM. Statistical analysis by one-way ANOVA followed by Tukey's test. Significance at P<0.05*, P<0.01** and ns = not significant *vs*. control group. Significance at P <0.01 ($^{\#}$ between group II and III, $^{\$}$ between III and IV, $^{\%}$ between II and IV)]

(Fig. 1 A and B) and rat kidney showed lymphocytic periglomerular infiltrate, eosinophilic hyaline casts and coagulative necrosis of the renal tubules (Fig. 2 A-C), notably in the 2000 mg/kg group.

Discussion

As there was a lack of literature on the safety profile of polyherbal formulations used for cognitive improvement, we performed a subacute toxicity for safety evaluation of the polyherbal formulation. We used Wilmer® PHF, consisting of plant ingredients of Brahmi (*Bacopa monniera*), Yastimadhu (*Glycyrrhiza glabra*), Tagar (*Valeriana wallechii*) and Ashwagandha (*Withania somnifera*) which was available in the market as a nootropic agent. Efficacy of the individual components of this formulation as cognition enhancers have been documented in previous studies. *Bacopa monnieri* (L.) is widely used in traditional medicine as a tonic to improve intelligence and memory. Literature on the cognition enhancing ability of this plant both in animals as well as in humans is widely available[8]. Glycyrrhizic acid which is a major component of licorice, the root of *Glycyrrhiza glabra* L. (Leguminosae), improved cerebral blood flow and prevented impairment of learning and memory displayed in middle-aged C57BL/6 mice[9]. Das *et. al*.[10] have reported the neuroprotective effect of *Valeriana wallechii.* Manchanda *et al*.[11] have reported the nootropic effect of *Withania somnifera* in rats. In a review by Ng *et al*, it was observed that *W. somnifera* extract improved performance on cognitive tasks, executive function, attention and reaction time of the participants in most of the clinical studies[12]. Gayathri, *et al*.[13] have also reported the beneficial effects of *Bacopa monnieri* and *Withania somnifera* against neurodegenerative diseases.

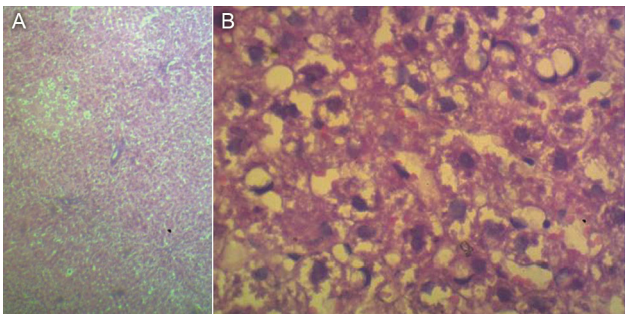

Fig. 1 — H&E stain showing (A) focal ballooning of hepatocytes (Scanner view); and (B) macrovesicular steatosis (High power view) in rat liver of test group (2000 mg/kg Wilmer®)

In the acute oral toxicity test as per OECD guidelines 423, we observed no mortality or any evident physical or behavioural changes in the animals at 2000 mg/kg after observing for 14 days. Thereafter, 24 rats were randomly allocated into four groups of six animals each and orally administered normal saline 5 mL/kg (control) and different doses (500, 1000 and 2000 mg/kg of Wilmer® polyherbal formulation once daily for 28 days. At the end of 28 days, the animals were humanely sacrificed and blood was collected by cardiac puncture for estimating various haematological and biochemical parameters whereas the vital organs were dissected and sent for histopathology.

We observed that there were no significant differences in blood glucose and blood urea nitrogen between the groups. At the dose of 2000 mg/kg of Wilmer® polyherbal formulation, haemoglobin concentration was significantly decreased compared to the control and lower dose test groups, although the values were within normal physiological limits. Total cholesterol was significantly raised beyond normal limits, indicating hypercholesterolemia/dyslipidemia in the 2000 mg/kg group.

Total protein was significantly decreased at 2000 mg/kg but values were within normal physiological limits. At the dose of 1000 and 2000 mg/kg, ALT levels were significantly increased compared to the control group. Dose dependent significant increase in ALT was seen in 1000 and 2000 mg/kg compared to

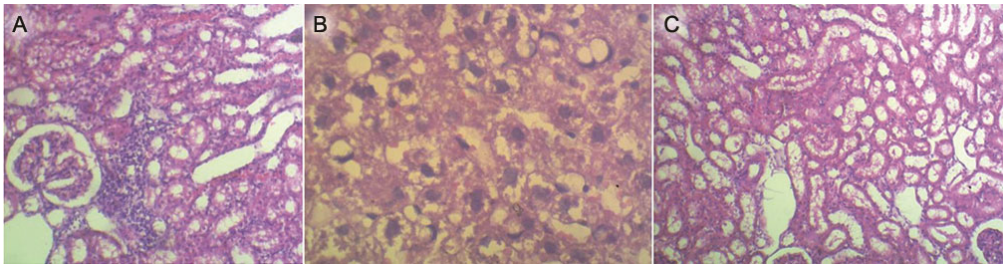

Fig. 2 — H&E stain showing (A) periglomerular lymphocytic infiltrate (Scanner view); (B) eosinophilic hyaline casts (Low power view); and (C) coagulative necrosis of renal tubules (Low power view) in rat kidney of test group (2000 mg/kg Wilmer®)

500 and in 2000 mg/kg compared to 1000 mg/kg group. AST levels were significantly increased compared to the control at all dose levels and dose dependent increase at different dose levels of PHF was observed. ALP was significantly increased at 2000 mg/kg compared to the control group and dose dependent significant increase was seen at 2000 mg/kg compared to 500 and 1000 mg/kg groups.

Serum creatinine levels were significantly increased at 2000 mg/kg compared to the control and lower dose test groups, but within normal physiological limits.

In gross examination of vital organs, no abnormality was seen in the control and test groups, except increase in size of the liver in the latter. In histopathological examination, control group histology was normal, but in the test groups, markedly in 2000 mg/kg group, there was ballooning and macrovesicular steatosis of the hepatocytes in the liver specimens. Periglomerular lymphocytic infiltrates, eosinophilic hyaline casts and renal tubular coagulation necrosis were seen in the kidney specimens at the highest dose level.

Decrease in total protein along with increase in AST, ALT and ALP as well as ballooning and macrovesicular steatosis of the hepatocytes in histopathology are suggestive of hepatotoxicity[18]. Increase in serum creatinine along with periglomerular lymphocytic infiltrates, eosinophilic hyaline casts and renal tubular coagulation necrosis in histological examination are indicators of nephrotoxicity[19].

Kim *et al*.[20] reported that acute and sub-chronic treatment in ICR mice for 120 days with *Glycyrrhiza* extract did not cause any death or adverse effects in Swiss albino mice and also did not induce significant alterations in any of the biochemical, haematological, or histopathological parameters. *Bacopa monnieri* extract at doses of 30, 60, 300 and 1,500 mg/kg given for 270 days did not produce any toxicity in Sprague Dawley rats[21]. LD_{50} of *B. monnieri* aqueous extract was found to be 5000 mg/kg in a previous study as reported in the review of Indian medicinal herbs and formulations for Alzheimer's disease by Mehla, *et al*.[6]. Acute toxicity study of *Valeriana jatamansi* was conducted through oral administration of a single dose of its iridoid fraction (3200 mg/kg body wt.) to adult mice, which did not result in any significant differences in body weights. In the subacute study of *Valeriana jatamansi* conducted over 90 days, low doses (240 mg/kg body wt.), middle doses (960 mg/kg body wt.), and high doses (1,200 mg/kg body wt.) of its iridoid fraction were administered daily to adult rats for 6 days a week. There were no deaths, weight differences or changes in physical appearance or behaviour as well as haematological and biochemical parameters or histopathology[22]. Valerian root used for sleep disorders has been mentioned as a relatively safe drug without any severe toxicities in a systematic review and meta-analysis[23]. However, rise in hepatic enzymes have been reported by others[24,25]. *Withania somnifera* root extract administered orally at a dose of 2000 mg/kg and observed for 14 days produced no physical or behavioural abnormality or any mortality in Wistar rats. Subacute administration once daily for 28 days to rats at 500, 1000 and 2000 mg/kg orally also produced no mortality or toxicity as evidenced by observation of body weight, organ weights and haemato-biochemical parameters[26]. Thus, safety of each of the herbal components of Wilmer® PHF formulation has been separately demonstrated in these toxicity studies. Our observations are in contrast with their results since decrease in haemoglobin concentration and hypercholesterolemia have not been reported as adverse effects of any of the constituents of Wilmer® PHF. Our findings of abnormalities in haemoglobin concentration, hypercholesterolemia, hepatotoxicity and nephrotoxicity cannot be explained by dose and duration as dose levels and duration in three other studies was in fact more than that of our study. Our study doses (500, 1000, 2000 mg/kg) are comparable to those in the quoted studies and duration of administration is lesser than in the first three of those studies and equal to that of the fourth study. Hence, chances of dose and duration playing a role in the results we have obtained are less likely. Although we had randomised the groups, biological variation may still be a factor when comparing the results of our study with those of the above-mentioned studies[27].

However, it should be noted that hepatotoxicity and nephrotoxicity of these medicinal plants have been reported in other studies. A comprehensive review of hepatotoxicity of traditional Ayurvedic herbs has implicated all the constituents of Wilmer®, except *Glycirrhiza glabra*[28], but Nazari *et al*.[29] mentioned about the hepatotoxic effects of *Glycirrhiza glabra* in their review of the plant. Many herbal preparations are also known to have nephrotoxic potential[30,31],

although the specific components of Wilmer® PHF apart from *Glycirrhiza glabra* have not been reported as nephrotoxic in previous studies. *Glycyrrhiza glabra* can cause severe hypokalaemia which can lead to acute kidney injury. Chronic hypokalaemic nephropathy secondary to its long-term consumption has been reported. It also has the ability to affect water retention, blood pressure and serum levels of electrolytes[32-34].

In the drug development cycle of a promising new allopathic molecule or formulation, toxicity testing is conducted in the preclinical testing phase before human trials. The aim of toxicity testing is to provide evidence of the safety of a product in animal models before it goes for further clinical development. The chosen animal models mimic the toxicities which can be produced in humans. There are different types of toxicity testing, according to duration of administration — Acute toxicity study which has single dose administration or multiple doses over 24 h followed by 14-day observation period, sub-acute study with daily administration over 28 days, subchronic study which lasts from 90 to 180 days and chronic study in which daily drug administration lasts from 180 to 730 days. The duration of administration of the test product to animals is determined by the anticipated duration of its clinical use in humans.

Herbal formulations are often marketed with unsubstantiated claims of non-toxicity, leading to their acceptance as safe medicines for use by the public[35]. According to WHO, the absence of any reported or documented side effects is not an absolute assurance of safety for herbal medicines. In fact, with respect to safety assessment of a traditional medicine, WHO recommends the use of toxicity testing in animal models[36]. The polyherbal formulation used here (Wilmer®) contains plant products with evidence of safety of the individual herbal components in other toxicity studies, but the formulation as a whole is observed to cause haematological abnormalities (decrease in haemoglobin concentration), hypercholesterolemia, hepatotoxicity and nephrotoxicity. In this context, it should be noted that besides toxicity of the main constituents, contamination of the preparation by heavy metals or microorganisms is also possible. Thus, the presence of metals and metalloids in the formulation at concentrations above acceptable regulatory standards can also cause the afore-mentioned toxic effects on the animals. Such reports have been described by researchers evaluating the safety of traditional medicine formulations[35]. As part of a cluster investigation of lead poisoning cases in the US, a study by Mikulsky, *et al*.[37] reported that approximately 50% of formulations containing mercury, 36% of samples containing lead and 39% of samples containing arsenic had high concentrations of those metals that exceeded the recommended daily intake values for pharmaceutical impurities over a thousand times. In another study, over 30% of 247 herbal preparation samples tested in a toxicological laboratory had high heavy metals content, bacterial contamination or presence of toxic organic substances[38]. Another report reviewed the extent of the problem of heavy metal contamination of traditional Ayurvedic or Chinese medicine products and nutraceuticals[39].

Strengths of our study are the measurement of standard haematological and biochemical parameters as well as histopathological study for evaluating toxicity on major organs following administration of Wilmer® polyherbal formulation as per OECD guidelines. Toxicity evaluation of polyherbal formulations ensures their safe use which are not only consumed as medicines but also as wellness products. One limitation here in this study is that we could have included measurement of electrolytes which would have corroborated the findings of nephrotoxicity.

Conclusion

The polyherbal formulation (Wilmer®) containing plant ingredients of *Bacopa monniera*, *Glycyrrhiza glabra*, *Valeriana wallechii* and *Withania somnifera* caused no mortality or any abnormal physical changes (like changes in skin and fur, eyes and mucus membranes) or behavioural changes (sleep patterns, lethargy, altered consciousness as well as food and water intake) on acute toxicity study after oral administration of a single dose of 2000 mg/kg to Wistar rats and observed over 14 days. Similar observations were noted at doses of 500, 1000 and 2000 mg/kg orally administered to the animals over a 28-day period (subacute toxicity). But it showed decrease in haemoglobin concentration, hypercholesterolemia, hepatotoxicity and nephrotoxicity in the rats. Thus, the polyherbal formulation studied above has multiple safety concerns which need further evaluation in both animals and humans before it can be prescribed as a relatively safe nootropic drug.

Ethical statement

The study was undertaken after the approval by the Institutional Animal Ethics Committee vide certificate of approval 05/IAEC/MG/04/2014. The study was conducted according to the principles of care and use of animals in the guidelines for laboratory animals by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Animal Welfare Division, Govt. of India.

Conflict of Interest

Authors declare no competing interests.

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