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RESEARCH ARTICLE

Evaluation of Acute, Subacute and LD_{50} values of Methanolic extract of *Sphaeranthus indicus* leaves in Albino mice

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ABSTRACT:
Sphaeranthus indicus is one of the extremely precious herbs in the Indigenous System of Medicine. The present study was carried out to acute, subacute and LD_{50} values of methanolic extract of *S. indicus* leaves in Swiss mice of both sexes. The acute toxicity studies were conducted oral administration of 1.75, 5.5, 17.5, 55, 175, 550, 2000mg/kg body weight SIME used. Observations were recorded systemically up to 24 h after dose administration for behavior related to nervous system response or autonomic functions. Food and water intake, body weight variations, hematological and biochemical parameters were assessed. In sub acute toxicity treatment there were no significant variation in the body weights and haematological parameters except dose-dependent increase in lymphocyte count was noted in both sexes supported immunostimulant activity. Pathologically, significant protective effect on hepatic, renal functions and decreased cholesterol, triglyceride levels. The results did not show any treatment related abnormalities in terms of hematological and biochemical parameters in sub-acute toxicity. After acute administration, no mortality was recorded in mice treated with the SIME orally at a dose of 1000mg/kg. The LD_{50} values were determined using graphical method; we found a broad therapeutic window and a high therapeutic index value showed that the LD_{50} of the extract is 2480mg/kg. The results suggest that the plant seems to be high margin of drug safety in mice.

KEYWORDS: *Sphaeranthus indicus*, Acute toxicity, Sub acute toxicity, LD_{50} values, Haematological, Therapeutic index.

INTRODUCTION:
Herbal medicines are accepted to be more secure than synthetic medicine. Along these lines, poisonous quality investigations of herbal product don't typically get as much consideration as investigations of allopathic medicine. Be that as it may, some medicinal substances are possibly dangerous and might be unsafe to human wellbeing[1].

Medicinal plants are usually complex mixture of various bioactive compounds and contrasted with single dynamic pharmaceutical medication, phytomedicines may vary in the distinctive component of activity of bioactive constituents, in their dose response relationships and in the combination effect.

The plant *Sphaeranthus indicus* (Linn.) belongs to the family Asteraceae and is known as Mundi in Hindi, Gorakmundi in Gujarati, Sravani in Sanskrit and East Indian Globe Thistle in English is abundantly in the plains all over India, uphill to an attitude of 1500 m in the hills, especially as a weed grow in the rice ground[2]. These herbs and its species are widely distributed in tropical Asia, Africa and Australia.

The aerial parts of plant (mainly leaves and flower) are widely used in traditional medicine for the treatment of various disorders. It is an important indigenous medicinal plant used for the treatment of styptic gastric disorders, skin diseases, antisyphilitic, anthelmintic, glandular swelling, nervine tonic, analgesic, antifungal and laxative properties[3]. The juice of the plant is styptic and diuretic and it is said to be useful against liver and gastric disorders. It is reported that flowers are highly alterative, depurative, cooling tonic and blood purifiers in skin diseases. Dried and powdered leaves of plant are useful in the treatment of chronic skin diseases, urethral

discharges and jaundice[4]. Ethanolic extract of *S. indicus* has been reported for mast cell stabilizing activity[5] and exhibited tremendous antibacterial action against Gram positive as well as Gram negative bacteria[6]. The phytochemical analysis of the plant showed that it contains eudesmanolide type of sesquiterpene possessing immunoprotective[7] and anti-inflammatory actions[8,9] (Heinrich et al., 1998 Jain et al., 2003). It also reported to possess Anxiolytic activity[10] Neuroleptic activity[11], Antioxidant activity[12] activity, Wound healing activity[13] analgesic and antipyretic activity[14], thus based on these characteristics, we believe that *S. indicus* can be a safe nutraceutical for the treatment of various disorders. Although numerous pharmacological studies have been carried out with this herb, there is no experimental evidence on its toxicity. Hence, in the present study, single oral dose toxicity and 28-day sub acute oral dose toxicity studies with were conducted with the SIME in mice. In this study acute, 28-day sub acute oral dose toxicity and LD_{50} values were performed in experimental mice. The Irwin Test is utilized to evaluate the minimum lethal dose of a drug, the portion go for CNS reactions, and the essential consequences for behavior and physiological capacities. Information from the Irwin test is additionally used to survey the dangers related with the utilization of wellbeing pharmacology. The rodents are evaluated for practices explicitly identified with neurotoxicity, for example, seizures and tremor. for practices identified behaviors related nervous system stimulation, for example straub tail, excitation, stereotypes, hopping, hypersensitivity to exterior stimuli and vigorous behavior and depressant activities like sedation, motor in coordination, rolling gait, hyposensitivity to external stimuli, diminished muscle tone, akinesia, catalepsy, and hypothermia[15]. These studies aimed at acquiring safety data for the application of a natural substance-based medicine, including information about the no-observed-adverse effect level and target organs.

MATERIALS AND METHODS:

Plant materials:

The leaves of S. *indicus* were freshly collected from the garden of Garpahra temple Sagar, Madhya Pradesh, India, in the month August 2016 and were authenticated by botanist Dr. P. K. Khare, Professor of Botany, Dr. Harisingh Gour Vishwavidyalaya, Sagar, India. A voucher specimen No. Bot/Her/02/2017 has been deposited in the herbarium of the Department of Botany, Dr. H.S. Gour Vishwavidyalaya, Sagar, India.

Animals and housing:

Healthy Swiss albino mice of both sexes (6-8 weeks old/ 20-25g) were used for the study. All animals were maintained in standard household conditions of temperature, light and humidity were used for study. All animals were stored in standard cage and maintained at $(27\pm2)^0$C under 12 h dark/light cycle. They were feed with standard rodent diet food and water freely available. Animals were fasted overnight prior to drug administration. The Institutional Animal Ethical Committee of BTPC, Sagar, India (0604/IAEC/2017/ 252), approved the study.

Preparation SIME leaf extracts:

The shade-dried leaves of the plant were powdered and subjected to extraction. 70g of the dried leaf powder were extracted with methanol using Soxhlet's apparatus. Solvent was evaporated in rotary evaporator dryness under reduced pressure to obtain the methanol extract (13.88% *w/w*). These extracts were prepared as a fine suspension in 0.2% *v/v* Tween 80 solution and used for the following toxicological studies.

Acute toxicity studies:

The acute oral toxicity test was carried out according to the OECD-425 guidelines (Up and Down Procedure). SIME was administered orally in doses to Swiss mice (n = 6) of female sex selected by random sampling technique were employed in this study. In this method, mice were dosed once at a time, if the animal survived, the dose for the next animal was increased and if the animal died, the dose for the next animal was decreased. The dose progression by a factor of 3.2 as recommended by the guidelines. The animals was fasted for 4 h with free access to water only[16]. The test sample was found safe up to the dose of 2000mg/kg and observation was carried out continuously for the first 4 h and for the next 24 h and for the following 48 h, for any death or changes in general behavior and other physiological activities. The Irwin Test is utilized to evaluate the minimum lethal dose of a drug, the portion go for CNS reactions, and the essential consequences for behavior and physiological capacities. The aftereffects of this test are utilized to anticipate potential therapeutic activity and to choose doses for succeeding tests of efficacy.

Sub acute oral toxicity:

Dose levels group designation: Organization for Economic Co-operation and Development (OECD, 407) guidelines was followed to conduct sub acute oral toxicity study. The dose levels were selected based on the result of a preliminary 4-week repeated oral dose range finding toxicity study. The high dose level was set at 300mg/ kg/day, at which toxicity was expected in this study. The group which served as control received equivalent quantity of vehicle only. A total of 4 male and 4 female mice per group were administered SIME at doses of 150, and 300mg/kg/day.

Body weights:
Food and water intake were recorded daily, whereas, body weight was recorded once in a week throughout. Each mouse was marked with a unique identification number and body weight was measured once a week, and behavior was observed daily during the trial period.

Blood sampling:
On the 28-day, tail vein blood was drawn from the overnight fasted animals. The blood samples were subsequently centrifuged at 4000rpm for 10 min to obtain serum and plasma respectively. The serum and plasma obtained was separated, and used for the subsequent heamatological and biochemical analyses.

Serum isolation and haematology:
Blood was also collected with the anticoagulant coated vials for the analysis of haematological parameters such as haemoglobin (Hb) levels, red blood cell (RBC) count, packed cellular volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and total white blood cell (WBC) count were determined. The differential leukocyte count was performed with percentage of lymphocytes (LY), Monocytes (MO), Granulocytes (GR) and Platelet Count (PLT) using Auto Hematology analyser (Mindray BC-5130).

Serum biochemical parameters:
The blood samples collected without the anticoagulant was centrifuged at 10,000rpm for 10 min at 4°C to separate the serum. The serum was analyzed for biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), cholesterol, triglycerides and creatinine were analysed using Biochemistry analyzer (Mindray BS-240).

Determination of LD_{50} values by graphical method:
To determine the LD_{50} values of methanolic extract of *S. indicus* evaluated, 20 Albino Swiss mice of both sexes were divided into five groups of four mice each. The animals are exposed to different concentrations of SIME, showed no mortality up to 1000mg/kg body weight. Five different doses (1000, 1500, 2000, 3000 and 4000mg/ kg body weight) were employed for SIME. Following administration of the extract, the mice were observed continuously for 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours respectively for appearance of signs of toxicity at different doses. After 24 hours, the number of dead animals in the group was recorded. The data were tabulated. The toxicological effect was assessed on the basis of mortality, which was expressed as an LD_{50} value. In the groups with no dead animals and in the groups with only dead animals, the obtained percentages were corrected using the following formulae:

Correction formula for 0 % dead group = $100 \times (0.25/n)$

Correction formula for 100% dead group = $100 \times [(n - 0.25)/n)]$

Where
n represents the number of animals in the group. After correction, the percentages were converted into probits. The values thus obtained were plotted against log dose. The LD_{50} value was determined by finding the dose that was intersected by probit 5[17].

Statistical analysis:
The values were expressed as mean±SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. P values < 0.05 were considered as significant.

RESULTS:
However, there is a lack of proven scientific studies on the toxicity and safety profile of these treatments in swiss albino mice, the present research was aimed to evaluate the methanolic leaves extract of *S. indicus* for acute, sub-acute and LD_{50} toxicity study to identify the range of dose that could be used for further studies.

Acute toxicity:
In acute toxicity study, oral administration of graded doses (1.75, 5.5, 17.5, 55, 175, 550 and 2000mg/kg body weight p.o.) of the methanol extract of *S. indicus* to mice did not show any signs of adverse reactions and no changes in animals' behaviors during the observation period. Effects of SIME leaves in the primary observation (Irwin) test in the mice are summarized in Tables 1.

Subacute toxicity:
Effects of SIME on body weights:
Body weights were recorded prior to dosing and oral administration of SIME leaves at doses of 150mg/kg and 300mg/kg body weight for 28 days did not produce any mortality in tested animals. The result was shown in Table 2, and the body weight gain of test groups had no statistical difference compared with that of the control group.

Table 1: Effects of SIME leaves Behavioral and Physiological Measures (Irwin Test) in mice

Behaviors		0-15 min	30 min	60 min	120 min	24 hr
Excitation	Convulions	-	-	-	-	-
	Tremor	-	-	-	-	-
	Jumping	-	-	-	-	-
	Excitation	-	-	+	+	-
Stereotypy	Head twitches	-	-	-	-	-
	Chewing	-	-	+	-	-
	Sniffing	-	+	+	-	+
	Scraching	-	-	-	-	-
Motor	Catalepsy	-	-	-	-	-
	Motor incoordination	-	-	-	-	-
	Abnormal gait	-	-	-	-	-
ANS	Piloerection	-	-	-	-	-
	Salivation	-	+	-	-	-
	Lacrimation	-	-	-	-	-
	Pupil diameter	↔	↔	↔	↔	↔
Others	Respiration	↔	↔	↔	↔	↔
	Temperature	↔	↔	↔	↔	↔
	Writhing	+	-	-	-	-
	Sedation	-	-	-	-	-

↔: No effect, +: Present, -: Absent

Effect of SIME on heamatological parameters:

The effect of oral administration of SIME leaves for 28 days on haematological parameters of the experimental and control showed no significant difference in the treated groups compared with control are summarized in Tables 3.

Effect of SIME on biochemical parameters:

Results of biochemical studies (Table 4) showed that there was no significant change in the activities of serum ALT, AST and ALP at both doses of SIME in comparison with control group however alterations in serum concentration of creatinine was observed. A slightly decrease difference was observed in the levels of serum total cholesterol and triglyceride level in both female and male mice.

Table 2: Body weights (g) of female and male mice in Sub acute toxicity of the leaves of SIME

S. N.	Treatment	Sex	Body weight (g)				
			Week 0	Week 1	Week 2	Week 3	Week 4
1	Control	Female	20.16 ± 1.29	22.28 ± 1.90	23.65 ± 2.10	25.16 ± 2.34	26.68 ± 3.25
		Male	20.07± 0.64	21.70± 1.14	23.06± 1.44	24.36± 1.54	26.02± 3.04
2	150 mg/kg	Female	21.11 ± 1.12	22.42 ± 1.62	23.58 ± 1.85	25.88 ± 2.12	27.16 ± 3.02
		Male	20.24 ± 0.98	21.64 ± 1.04	23.14 ± 1.12	25.04 ± 1.80	26.56 ± 1.04
3	300 mg/kg	Female	22.14 ± 1.37	23.47 ± 1.34	24.34 ± 2.05	26.08 ± 1.56	27.68 ± 3.66
		Male	21.52 ± 1.42	22.38 ± 0.92	24.02 ± 1.32	25.96 ± 2.86	27.08 ± 3.16

Table 3: Effect of SIME leaves for 28-day treatment on Hematological parameter in mice

S. No.	Parameters	Sex	Control	150 mg/kg	300 mg/kg
1	Hb (g/dL)	Female	14.05±0.25	14.75±0.54	14.80±0.42
		Male	14,47±0.38	14.65±0.12	14.98±0.75
2	RBC (10^6/mm^3)	Female	8.58±0.22	9.03±0.14	9.25±0.36
		Male	8.85±0.42	8.98±0.17	9.31±0.22
3	PCV (%)	Female	44.04±0.93	45.25±0.53	45.65±0.43
		Male	43.65±0.89	44.16±0.90	44.98±0.70
4	MCV (μm^3)	Female	55.10±0.22	56.25±0.90	55.52±0.88
		Male	54.81±0.51	55.38±0.92	55.70±1.04
5	MCH (pg)	Female	15.95±0.06	15.75±0.20	15.90±0.18
		Male	16.08±0.12	16.25±0.43	16.15± .31
6	MCHC (%)	Female	27.98±0.24	28.98±0.24	29.18 ±0.32
		Male	30.23±0.12	30.52±0.27	31.42 ±0.24
7	WBC (10^3/mm^3)	Female	7.02 ± 0.37	7.64±0.22	8.14 ±0.51
		Male	6.96 ± 0.28	7.45±0.35	8.06 ±0.37
8	Neutrophil (%)	Female	22.98±0.72	22.80±0.84	22.56±0.95
		Male	23.76±0.34	23.64±0.38	20.26±0.25
9	Lymphocyte (%)	Female	71.82±1.02	72.42±0.86	72.52±1.32
		Male	69.64±0.54	71.57±0.32	73.84±1.12
10	Monocyte (%)	Female	2.92±0.42	3.21±0.14	3.63±0.10
		Male	3.83±0.24	3.08±0.25	3.90±0.17

11	Eosinophil (%)	Female	1.94±0.08	1.42±0.18	1.24±0.05
		Male	2.47±0.10	1.34±0.04	1.26±0.12
12	RDW (%)	Female	12.32±0.10	12.40±0.06	12.64±0.14
		Male	12.44±0.07	12.68±0.20	12.74±0.18
13	Platelets (10^5/mm^3)	Female	8.53±4.61	9.03±13.35	9.43±14.24
		Male	9.12±7.26	9.98±16.32	10.01±19.70
14	MPV (fL)	Female	6.31±0.20	6.34±0.12	6.54±0.26
		Male	6.32±0.18	6.52±0.45	6.74±0.52

Table 4: Effect of SIME leaves for 28-day treatment on Biochemical analysis in mice

S. No.	Parameters	Sex	Control	150 mg/kg	300 mg/kg
1	Creatinine (mg/dL)	Female	0.64 ± 0.04	0.65 ± 0.05	0.56 ± 0.02
		Male	0.72 ± 0.06	0.67 ± 0.04	0.61 ± 0.07
2	Cholesterol (mg/dL)	Female	62.02 ± 2.32	59.24 ± 1.47	55.48 ± 3.07
		Male	59.33 ± 2.18	56.52 ± 3.13	51.26 ± 1.15
3	Triglycerides (mg/dL)	Female	62.33 ± 5.18	56.30 ± 3.14	50.42 ± 2.52
		Male	56.48 ± 3.11	53.18 ± 1.26	49.86 ± 2.73
4	ALT (IU/L)	Female	41.75±3.14	38.17±1.87	36.13±2.03
		Male	40.06±2.43	37.40±1.54	34.37±2.94
5	AST (IU/L)	Female	72.53±1.34	70.15±2.42	68.95±3.12
		Male	68.37±1.14	67.23±1.14	64.37±4.02
6	ALP (IU/L)	Female	70.30±8.90	69.65 ± 5.28	67.42 ± 3.12
		Male	69.85±4.95	65.46 ± 3.52	62.26 ± 2.24

Determination of the LD_{50} values:

The LD_{50} values were computed by probit analysis with a calculator. After 24 hours, the number of dead animals in the group was recorded. The data were tabulated. The toxicological effect was assessed based on mortality, which was expressed as an LD_{50} value. The values thus obtained were plotted against the log of the dose. (Tables 5, Fig.1). Graphical analysis of the results yielded LD_{50} values 2480mg/kg body weight for SIME.

Table 5: Determination of LD_{50} values for SIME

S. No.	Dose (mg/kg body wt.)	Log dose	% Mortality (after 24 h)	Corrected % mortality	Probit
1	1000	3.00	0	6.25	3.45
2	1500	3.17	0	6.25	3.45
3	2000	3.30	25.0	25.0	4.33
4	3000	3.47	50.0	50.0	5.00
5	4000	3.60	100.0	93.75	6.55

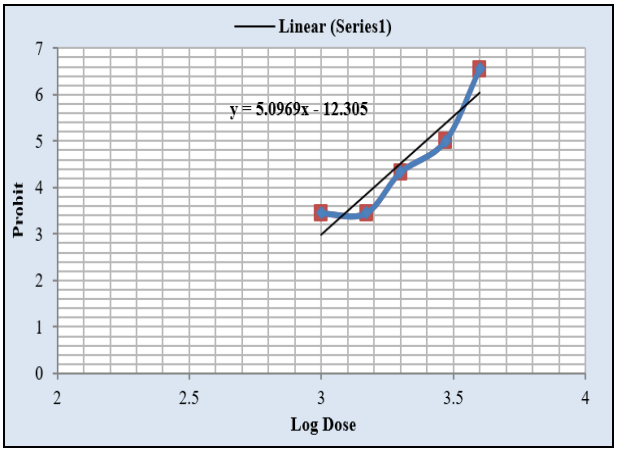

Fig. 1: Determination of LD_{50} value for the extracts of the leaves of SIME administered to mice for 24 h, using graphical method

DISCUSSION:

Herbal medicines are used throughout and developing countries and play a key role in the management of various chronic diseases and in recent times have received a great preference by researchers as alternative source to allopathic pharmaceutical drugs[18]. Sub acute toxicity is repeat-dose study performed to expose any deleterious changes in body weight, hematological and biochemical indices that may arise in the course of repeated administration of a test substance, usually four weeks. In acute toxicity study, mice treated at graded doses of SIME leaves, animals did not show any signs of adverse reactions and no changes in animals' behaviors during monitoring.

In the sub acute toxicity study, the observed no significant difference in the body weights at doses 150 mg/kg and 300mg/kg body weight in both female and male mice during 28-day oral administration of SIME leaves (Table 2) may indicate that the animals were having healthy growth based on their food intake as well as the plant extract. In the subacute toxicity study, the mean body weight and the percentage body weight gain of mice in both SIME treated were similar to those of the control group at the end of week 4. The haematological analysis is very important due to the fact that hematopoeitic system, being one of the most sensitive targets of toxic chemicals, is an important index of physiological and pathological status of animals and human. In this study, the test group of animals did not show any significant deviation in the hematological parameters except lymphocyte count. The prolong administration of SIME appeared to have beneficial effects on their hematopoietic system. A significant and dose-dependent increase in the WBC count was noted in both sexes supported immunostimulant activity[19]. Blood

is the main medium of transport for many nutrients and foreign bodies in the body. Due to this reason, components of the blood such as red blood cells, white blood counts, platelets and haemoglobin are first exposed to significant concentrations of toxic compounds. Damage to the blood cells has an adverse effect on the normal functioning of the body, since the administration of SIME did not cause any significant change in the haematological parameters measured as compared to the controls (Table 3), therefore the plant can be suggested non-toxic. The differential leukocyte count was performed with an optical microscopy after staining. Transaminases such as Aspartate and alanine aminotransferase are distinguished indicator of liver function and used as biomarkers [20]. ALP is most often measured to indicate bile duct obstruction [21]. Liver parameters such as, AST, ALT were within normal range as similar to control group suggesting that sub-acute administration of SIME did not cause deleterious effect on liver functions. The elevated levels of these enzymes are clinically measurable indications of potential risks to normal liver function [22]. In addition, observed a significant protective effect by lowering the serum AST and ALT and ALP (Table 4), might be an indication that SIME may have some hepatoprotective properties.

Creatinine is most often measured to indicators of renal function and any rise in the levels of these parameters indicates a marked renal damage [23]. The results from study however suggest that SIME does not have a negative effect but rather seems to have a renal protective activity. The levels of blood cholesterol and triglyceride significantly reduced in the SIME groups, as compared to the control group, indicating antihyperlipidemic effects. The observed non significant increase in total cholesterol and Triglycerides levels may have occurred this suggests that the extract does not impair lipid metabolism.

CONCLUSION:

In conclusion, the present study provides valuable data on the acute and subacute toxicity profile of the methanol extracts of *S. indicus* leaves in Swiss albino mice. The present investigation demonstrated that the extract at level up to 2000 mg/kg body weight has no harmful effects and considered as non toxic and safe.

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CONFLICT OF INTEREST STATEMENT:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES:

1. Palmer ME, et al. Adverse events associated with dietary supplements: an observational study. The Lancet. 2003; 361(9352): 101-106.
2. Gogate VM. Ayurvedic pharmacology and therapeutic uses of medicinal plants (Dravyaganvigyan). Mumbai: Bhartiya Vidya Bhavan. 2000: 112-114.
3. Kirtikar KR, Basu BD. Indian medicinal plants. International Book Distributors, Dehradun.1987: 343.
4. Nadkarni KM. Indian Material Medica, III Edn, Vol I. Popular Prakashan Pvt. Ltd. Bombay. 1976: 1162.
5. Mathew JE., et al. Mast cell stabilizing effects of *Sphaeranthus indicus.* Journal of ethnopharmacology. 2009; 122(2): 394-396.
6. Naqvi BS, et al. Antibacterial activity in fruits and vegetables. Pakistan journal of Pharmacology. 1998; 15:7–11.
7. Shekhani MS, et al. An immunostimulant sesquiterpene glycoside from *Spharanthus indicus.* Phytochemistry. 1990; 29(6): 2573.
8. Heinrich M, et al. Ethnopharmacology of Mexican Asteraceae (Compositae). Annual Review of Pharmacology and Toxicology.1998; 38: 539–565.
9. Jain A, Basal E. Inhibition of Propionibacterium acnes-induced mediators of inflammation by Indian herbs. Phytomedicine. 2003; 10: 34–38.
10. Ambavade SD, et al. Pharmacological evaluation of the extracts of *Sphaeranthus indicus* flowers on anxiolytic activity in mice. Indian Journal of Pharmacology. 2006; 38: 254–259.
11. Mhetre NA, Ambavade SD, Bodhankar SL. Neuroleptic activity of extract of *Sphaeranthus indicus* in mice. Indian Journal of Natural Products. 2006; 22: 24–27.
12. Shirwaikar A, Prabhu KS, Punitha IS. In vitro antioxidant studies of *Sphaeranthus indicus* (Linn) Indian Journal of Experimental Biology. 2006; 44: 993–996.
13. Jha RK, Garud N, Nema RK. Excision and incision wound healing activity of flower head alcoholic extract of *Sphaeranthus indicus* Linn. in albino rats. Global Journal Pharmacology. 2009; 3: 32–37.
14. Nanda BK, et al. Analgesic and Antipyretic activity of whole parts of *Sphaeranthus indicus* Linn. Journal of Chemical and Pharmaceutical Research. 2009; 1: 207–212.
15. Roux, Sylvain, Evelyne Sablé, and Roger D. Porsolt. "Primary observation (Irwin) test in rodents for assessing acute toxicity of a test agent and its effects on behavior and physiological function." Current Protocols in Pharmacology.2004, 27(1):10.
16. Ecobichon, DJ. The Basis of Toxicology Testing. CRC Press, New York. 1997: 43–86.
17. Veerappan, A, et al. Acute and subacute toxicity studies of Aegle marmelos Corr., an Indian medicinal plant. Phytomedicine. 2007; 14(3): 209-215.
18. Vaghasiya YK, Shukla VJ, Chanda SV. Acute oral toxicity study of Pluchea arguta boiss extract in mice. J Pharmacol Toxicol. 2011; 6(2): 113-23.
19. Bothiraja C, et al. Acute and subacute toxicity study of andrographolide bioactive in rodents: Evidence for the medicinal use as an alternative medicine. Comparative Clinical Pathology. 2013; 22(6): 1123-1128.
20. Adeneye AA, Olagunju JA. Preliminary hypoglycemic and hypolipidemic activities of the aqueous seed extract of *Carica papaya* Linn in Wistar rats. Biology and Medicine. 2009; 1(1): 1-10.
21. Witthawaskul P, et al. Acute and subacute toxicities of the saponin mixture isolated from Schefflera leucantha Viguier. Journal of Ethnopharmacology. 2003; 89(1): 115-121.
22. El Hilaly, et al. Acute and chronic toxicological studies of Ajuga iva in experimental animals. Journal of ethnopharmacology. 2004; 91(1): 43-50.
23. Gowda S, et al. Markers of renal function tests. North American journal of medical sciences. 2010; 2(4): 170.