Research Article

Single Dose Oral Acute Toxicity Study in Wistar Albino Rat Primary Safety of Ayabirungarajakarpam(ABK) - A Siddha Drug

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ABSTRACT

The presence of toxic mineral and metal in traditional medicine preparations as well as the contamination of heavy metals in herbal product is subjected to under scientific study. In this juncture, the modern science has revealed that the process of anaerobic heating in preparation of herbomineral drug converts the toxic mega particles of metal into safe and efficacious nano particles or even smaller Pico particles. The objective of the present study is to obtain the primary safety information of Ayabirungarajakarpam (ABK) and also the clarity of its dose and safety for further studies. Healthy young adult female nulliparaous and non pregnant albino wistar rats between 8-12 weeks of age and weighing 150 -175 gm were used for this experiment. In this acute arm of study, a single oral dose of ABK substance at 2000mg/kg/b.w was administered to wistar rats for the study period of 14 days as per the OECD guideline 402. The observation was made for life span of animal, weight changes, battery clinical toxicity signs and gross pathology. No effect on life span of animals during the study period. No test compound ABK related findings were observed at necropsy. All gross observations were normal in nature and no adverse effects occur in related to test substance, and all animal was survived until end of the study. Hence, it is concluded that the single dose of oral administration of ABK substance at 2000mg/kg/b.w did not cause mortality or morbidity, No adverse effect noticed on body weight and gross anatomy of the treated animals. Hence the LD50 of ABK is greater than 2000mg/kg/b.w.

Keywords: Nulliparous, Single dose, life span, Wistar rats, acute toxicity.

INTRODUCTION

The trend of usage of herbal medicine has increased popularity up to 80% of the world population and they rely on herbal medicine for their preventive and curative care[1]. The reason for increasing Acceptability and popularity of herbal medicine is due to the side effect of synthetic drug,[2] and also this demand toward the traditional medicine laid upward trend in the research on herbs and herbal products.[3] In the recent past, the traditional medical system turn their eye towards the in vivo toxicological studies to proof the safety and efficacy of their medical products.[4] Ayabirungarajakarpam is a herbometallic drug had been used as kayakarpam in siddha medicine to treat or preventing anaemia, grey hair and senility.[5] But no proper reports deal with the toxicological profile is found about Ayabirungarjakarpam from past research findings literatures, even though this drug had been used for anaemia in the practice of siddha system of medicine for a long time.

The primary toxicity assessment in higher dose is to identify the adverse effect as well as the determination of limit of toxic exposure level. The presence of toxic metal and mineral in traditional medicine preparation as well as the contamination of heavy metal in herbal products is subjected to under scientific study. Anyhow, the modern science has revealed that the process of anaerobic heating in preparation of herbo-metallic or mineral drug converts the toxic mega particles of metal into safe and efficacious nano particles or even smaller Pico particles[6, 7]. Acute toxicity is the primary effects occurring within a short period of time after oral administration of either single dose of a substance or multiple doses given within a twenty four hour period. The globally harmonized system (GHS) defines acute toxicity as "those adverse effects occurring following oral or dermal administration of a single dose of a substance or multiple doses given within 24 hours or an inhalation exposure of 4 hours [8].

Now a days, increase concern in herbal therapy and demand in consumption of herbal drug products are the global need, approximately 39-65% of population in different part of world use herbal products to improve their health.[9] Generally it is believed they are safe because of their natural origin and long history of application.[10] however the expanding global usage of herbal medicine, the possibility for adverse effect is increasing. Indeed, many side effects have been reported such as contamination of foreign matters and toxic ingredients due to improper purification process and drugs interaction with western medicine.[11] Therefore there is a strong demand of toxicological studies and scientific evidence regarding the safety of herbal product[12].

The scope of the study is to meet the experimental requirement as per WHO/OECD guidelines. The data obtained from the acute study is traditionally a step in classification and labelling as well as to establishing a

dosage. The acute toxicity study may provide initial information on the mode of toxic effect and the incidence of severity of all abnormalities, including behaviour, autonomic and central nervous system toxicity signs, gross necropsy lesions, body and individual organ weight changes and any other toxic effect related to test substance.[13]

The objective of the present study is to obtain the primary safety information of Ayabirungarajakarpam and clarity of its dose and safety for further toxicological and other experimental study.

MATERIAL AND METHODS

Preparation of Ayabirungaraja karpam (ABK)

Procuring raw materials

The raw iron and mandooram were procured from Chennai local market and authenticated by Geochemist. *Wedelia chinensis Osbeck* (Voucher specimen No CARISM 109) was collected from local herbal garden. Thanjavur and *Citrus limon L* (Voucher specimen No CARISM 110) fruit was procured from local market, Thanjavur. Both herbal raw material were authenticated by Dr Ravichandran, Botanist, CARISM, Sastra University, Thanjavur

Preparation of ABK

The Ayabirungarajakarpam contain purified Aya thool (Ferrum) and purified Manduram(ferric oxide)and both purified metal were mixed well in 2:3 ratio and soaked it in Manjal karisalai (Wedelia chinensis) extract in the volume of 1.3L, then the lime juice(*Citrus limon*) 1.3L of volume were added and soaked for complete drying in sun heat Mixed with this juice and kept in sunlight for complete drying. Then the waxy consistency product was transferred to thonnai (herbal cup made with dry leaves of Ficus benhalensis) for complete drying. Finally the dried product was ground it into fine powder to obtain Ayabirungaraja karpam. Even though the ABK is a herbometallic drug it deviates from normal preparation protocol like triturating and calcinations processes of metallic drug.[14, 5]

Animals and husbandry

The five animals were used after 5 days of acclimatization. The animals were allocated standard polypropylene rat cages with stainless steel top grill was used to house the animals. The cages were autoclaved, sieved and sterilized paddy husk was used as the bedding material, animals were housed individually. Bedding materials, cages, grills and water bottles were changed weekly twice. The temperature of the test room was maintained between 22±3°C and relative humidity between 50 to 70% during the experimental period. The experimental room was provided with 12h light and 12h dark lighting condition using automatic timer. Standards rodent pellet feed supplied by M/s ATNT Laboratories, Mumbai, India and Reverse Osmosis (RO) water were provided to the animals ad libitum. All animals were over night fasted about 18 h before dosing and terminal necropsy.

Animal welfare and Regulatory Compliance

The experiment was conducted at the central animal facility, registered (No 817/PO/ReRc/S/04/CPCSEA) for breeding and experiments of animals by the committee for the purpose of control and supervision of experiments on animals, Ministry of forest and Environment, Govt. of India.

Ethical clearance

The study was conducted after approval by the Institutional Ethical Committee, Sastra University, Thanjavur. (No 522/SASTRA/IAEC/RPP).

Preparation of ABK substance

The test substance ABK was suspended in honey water (2:3) and administered as such at the dose of 2000mg/kg body weight.

Study setting and Treatment

Healthy young adult female non- pregnant & nulliparaous albino wistar rats (Rattus norvegicus Sp.) between 8-12 weeks of age and weighing 150 -175 gm were used in this experiment. Rats were randomly assigned to the cages and each animal was identified by tags marked with animal number and dose level was attached to the respective cages. Each animal was identified by following unique animal ID by ear tagging.

Table 1: Identification

Animal ID	Sex
10823	Female
10824	Female
10825	Female
10826	Female
10827	Female

The animal allowed for an acclimation period of 5 days and were fasted overnight prior to treatment and feed was made available ad libitum immediately after the treatment. Before commencing the experiment the body weight of the rats were recorded. The test drug Ayabirungarajakarpam substance was administered 2000mg/kg as single dose by oral gavage using an appropriately sized syringe and stainless steel ball-tipped intubation needle. The animals were return to their cages immediately after the drug treatment. The experimental protocol was conducted in accordance with internationally accepted principle for laboratory animal use and care, the Economic Co-operation and Development (OECD) guidelines for the testing of chemicals “Acute dermal “toxicity -402”[15]

Observation of Life span & clinical signs

The mortality and changes on body weight, clinical signs and gross observation were monitored during 14 days after oral administration of ABK substance administered. All abnormal clinical signs were recorded before and after dosing at least twice a day based on the functional observation battery test [16., 17]

Mortality

All animals were observed individually twice every day for mortality. The observation was made first 30 minutes after dosing, periodically during the first 24h with special attention during the first 4h and then daily twice for a period of 14 days.

Body weight

Food intakes of individual animals were recorded for the entire study period. Body weight of each animal was recorded just prior to the test substance treatment day 0, day 7, day 14 using electronic animal weighing balance (Sartorius AG, Germany). In addition, to reduce the individual body weight differences of animals at initial dosing, body weight gain during day 0 –day7, 7-day to 13 and day 0-14 were calculated based on measured body weight at each day.

Toxicity signs

All the animals were observed individually after the drug administration during the entire observation period for the presence of any signs of toxicity including alopecia, catalepsy, chromodacryorrhoea, clonic, coma, convulsion diarrhoea, dullness, excessive grooming, gait, hyperactivity, lacrimation, nasal discharge, nasal irritation, piloerection, polyuria, prostration, repetitive circling, respiratory distress, salivation, scaling, tonic, tremor and urogenital staining.

Gross pathology

Necropsy was performed on all animals at the end of experiment at day 14 after overnight fasting about 18h. Water was not restricted. Animals were asphyxiated by carbon dioxide to do the gross necropsy.

RESULTS AND DISCUSSION

Mortality

No mortality was observed in treated group rats throughout the observation period. At terminal, all of animals 5/5; 100% were survived in higher dose 2000mg/kg body weight.

Table 2: Mortality data

Details of the drug	Number of rats treated	Dose-mg/kg/body weight	Percent of mortality up to 14 days
ABK substance	5	2000 (single dose)	0

Changes in the body weight gains

Body weight changes and gain on day '7'th and day '14'th when compared with day '0' were detected in all five female rats but noticed increase of weight statistically insignificant. The daily feed intake of rats remained unaffected throughout the experimental period.

Table 3: Weekly Mean Body Weight Changes in Rats

Animal ID	Sex	Body Weight (g)		
		Day 0	Day 7	Day 14
10823	Female	173.46	185.64	201.51
10824	Female	163.68	177.67	186.13
10825	Female	163.54	170.32	176.54
10826	Female	157.98	173.72	186.02
10827	Female	156.78	179.9	190.41
Mean		**163.09**	**177.45**	**188.12**
SD		**6.60**	**5.87**	**9.04**

Toxicity clinical signs

No visible signs of toxicity such as changes in respiration, circulation, autonomic and central nervous system, behavioral pattern were observed during the entire observation period.

Gross Pathology

No test compound ABK related findings were observed at necropsy. All gross observations were normal in nature. All rats survived until the end of study.

Table 4: Daily Feed Intake (g) by Rats

Animal ID	Sex	Days													
		0	1	2	3	4	5	6	7	8	9	10	11	12	13
10823	F	12.94	15.37	14.66	17.08	15.08	16.27	16.64	16.02	13.27	14.60	14.46	16.97	15.47	18.55
10824	F	12.73	18.30	17.59	15.40	17.00	17.32	15.55	13.95	15.76	17.92	13.63	14.24	16.21	14.09
10825	F	14.19	16.70	14.54	17.46	15.46	19.02	17.97	16.59	18.10	17.28	16.89	17.90	18.91	17.09
10826	F	13.06	15.74	14.52	15.35	15.14	14.16	14.97	13.66	15.21	14.46	15.21	14.60	13.80	11.68
10827	F	14.71	16.93	16.01	15.10	14.58	17.36	15.04	16.40	14.78	14.58	13.71	14.62	11.43	13.83
Mean		**13.53**	**16.61**	**15.46**	**16.08**	**15.45**	**16.83**	**16.03**	**15.32**	**15.42**	**15.77**	**14.78**	**15.67**	**15.16**	**15.05**
SD		**0.87**	**1.15**	**1.34**	**1.10**	**0.92**	**1.79**	**1.27**	**1.41**	**1.76**	**1.69**	**1.34**	**1.65**	**2.78**	**2.75**

F- Female; SD – Standard deviation

Table 5: Toxicity signs observed in Female Rats

Observation*	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Appeared Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Found death	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Catalepsy	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Chromodacryorrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Clonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Coma	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Convulsion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Dullness	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Excessive grooming	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Change in Gait	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperactivity	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Lacrimation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal discharge	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal irritation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Piloerection	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Polyuria	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Prostration	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Repetitive circling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Respiratory distress	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salivation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tremor	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Uro-genital staining	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* No. of animals showing the clinical sign / No. of animals per group

Table 6: Gross Pathology

S.No.	Sex	Animal No.	Status of the animal at the time of receipt at necropsy	Lesions observed at necropsy
1	F	10823	Live	NAD
2	F	10824	Live	NAD
3	F	10825	Live	NAD
4	F	10826	Live	NAD
5	F	10827	Live	NAD

F–Female; NAD- No Abnormalities Detected

DISCUSSION

Acute toxicity study is the initial step to provide crucial data from single or brief exposure. Standardized tests are available for oral, dermal and inhalation exposures, the rats are the preferred species for oral, inhalation and dermal testing. Toxicity is the degree to which a substance may be poison or toxin cause harmful effect to human or animals which effect on cell (cytotoxicity),an organ (liver toxicity) or whole organism. All substances are potentially toxic depending on the quantity. Hence, many therapeutic medications are acutely toxic in over dose but it is beneficial in optimal level. Therefore the effect of toxicity depends on type, degree of harm, and amount of a substance or drug[18].

World Health organization recommends investigating the medicinal herb and its product for better understanding of its medicinal properties, effectiveness and safety[19]. Herbal medicine has some sort of popularity and acceptability among the rural and urban area in India, because it is believed to be natural and safe but lack of data available regarding the safety concerned.

The published literatures reported that the possible adverse effect and drug interaction occur during the use of herbal drug.[20] ABK, a siddha herbometallic formulation had been used as haematinic therapeutic potential drug in siddha system of medicine for long time, therefore the safety profile should be established as a guide for management and its application; this will serve to prevent the human subjects from potential toxicity related health risks.

Toxicity studies in animal model are being used to assess potential health of human risk level by addressing the probability of exposure to those particular hazards at certain doses or concentrations[21]. But the centre for drug evaluation and research (CDER) reported that a single dose acute toxicity testing procedures for pharmaceutical substances with fixed safe dose that should not cause adverse event or threaten the life of an animal [22.]

In the present study single dose oral administration of ABK substance in female rats at 2000mg/kg/b.w had showed insignificant body weight gain, no effect on mortality, examined clinical signs such as skin, eye, nasal mucous membrane and autonomic effect like salivation, lacrimation, piloerection, polyuria, urogenital staining, and central nervous system activity like tonic, clonic tremor, drowsiness, gait, convulsion, repetitive circling, the finding of the study did not show any abnormal behaviour and or toxicity signs related to the test drug ABK(table 5). The behavioural, neurological and autonomic parameters was observed in the experiment immediately after the administration of the ABK test substance and then daily once for 14 days, the outcome was within the NOAEL (No observe adverse effect level). No lesions observed at necropsy (table 6). Therefore no acute toxicity was found in rats treated with ABK and the appropriate lethal dose was determined to be higher than 2000mg/kg/b.w.

Body weight changes and gain on day '7'th and day '14'th when compared with day '0' were detected in all five female rats but statistically it is insignificant increase, anyhow that infer the administration test substance does not interfere with the growth of the animals.(table 3) The daily feed intake of rats remained unaffected throughout the experimental period. The battery of toxicity signs investigated during the acute oral toxicity study can strengthen the foundation of knowledge of toxicity and safety issues of ABK test substance.

Acute toxicity studies always serve for choosing the test substance for sub-acute or chronic studies and pharmacological activity in animal and human model.[23] The acute toxicity test in which a single dose is used in each animal on one occasion for the determination of LD_{50} or median lethal dose.[24] LD_{50} is defined as the statistically defined dose that when administered in acute toxicity test, is expected to cause death in 50% of the treated animal in a given period.[25] In like that, as per the observation and calculation from the acute oral toxicity, the value of LD_{50} of ABK substance was to be more than 2000mg/kg body weight.

CONCLUSION

It is concluded that the current study of single dose acute oral toxicity of ABK test substance did not cause any mortality, no untoward toxic effect on body weight or body weight changes, and over all normal body weight gain was observed in all the treated wistar albino female rats which is insignificant statistically comparable to initiating therapy. No any adverse signs of toxicity and gross pathological changes observed up to the dose level of 2000mg/kg/b.w, during the study period. The toxicity clinical signs help to calculate "No observed adverse effect level" dose (NOAEL) as per the observation and calculation from the acute oral toxicity (OECD guideline 402), the LD_{50} value of the ABK test substance was to be more than 2000mg/kg/b.w.

Hence, the present acute toxicity study of ABK confirms a fixed safe dose that may not cause untoward effect or threaten the life of the animal. This may be attributed to sub-sufficient absorption of the test substance in the gastro intestinal tract or high first-pass metabolism rate in the liver, by cumulative action of the toxic components would have been converted to their harmless derivatives.

REFERENCES

1. World Health Organization, Traditional Medicine Strategy 2002-2005, Geneva, Switzerland. Available: whqlibdoc.who.int/hq/2002/who_edm_trm_2002.1.pdf.
2. Chan, K.,The role of complementary medicine in Healthcare, Biologist 43, 1996, p 50-51.
3. Pandey M M, Rastogi S and Rawat A K, Indian Herbal Drug for General Healthcare: An Overview, The Internet Journal of Alternative medicine,6(1), 2008, p 1-10.

4. Amster E, Tiwari A, Schenker Mark B. Potential Arsenic Toxicosis is secondary to herbal kelp health supplement, Environmental Health Perspectives; 115(4), 2007, 606-608, DOI:10 .1289/ehp.9495;PMID:17450231.

5. Kuppusamymudhaliyar.K N., Uthamroyan, K S. Siddhavidhiya thirattu, 1st edition, Department of Indian medicine and Homeopathy, Chennai, 1998, p 233-234.

6. Vagbhattacharya S, Samuchchaya, R and Kulkaro K A, Hindi commentary,Puvardha 1/28, Meharchand Laxmandas, New Delhi, India, 1998.

7 Vyas P, A clinical study on the rasayana as a preadjuvant as a radioprotective and chemotherapy in the management of Carcinoma (M S Thesis), I.P.GIT & R.A., Gujarat Ayurved Univrsity, 2005.

8 Wang H, Ye X, Gao Q, Wu C, Qian Y, Luo B, Pharmacovigilence in traditional Chinese Medicine and safety and surveillance, Pharmacoepidemiol Drug safety, 18(5), 2009, 357-361, DOI:10.1002/pds.1725;PMID:19253921

9 Fogden E, Neuberjer J, Alternative medicine and the Liver. Liver Int:23, 2003, 213-220, DOI:10.1034/j.1600-0676.2003.00824.x.;PMID:12895259

10 Ernst E, Risk of herbal medicinal products, Pharmacoepidemial, Drug safe; 13, 2004, 767-771, DOI:10.1002/pds.1014;PMID:15386721

11 Brinker F.N.D, The Toxicology of Botanical Medicines, 3rd edition, Eclectic medical publications, Sandy Oregon; ISBN: 1888483091, 2000, 13-14.

12 Tank J L, Liu By, Ma KW, Traditional Chinese medicine lancet, 372, 2008, p1938-1940, DOI:10.1016/SD140-6736(08)6134-9;PMID:18930523

13 Tahraoui A, Israili Z H, Lyoussi B, Acute and sub – chronic toxicity of a lyophilized aqueous extract of Centaurium erythraea in rodents, J.Ethnopharmacol, 132, 2010, 48-8.DOI:10.1016/j.jep.2010.07.038;PMID:20800671.

14 Thiyagarajan R, Gunapadam Thathu Jeeva Vaguppu, 4th edition,3rd vol;Department of Indian Medicine & Homeopathy,Chennai, 2004, p: 64-150

15 OECD guide line for the testing of chemicals “Acute Dermal toxicity -402, OECD Publishing, Paris. Adopted: 24 Feb 1987.

16 Dourish C T , Gren shaw A J, Effects of drugs on spontaneous motor activity in experimental pharmacology, Humana Press Clifton, 1987, pp; 325 -334, Doi:10.1007/978-159259-461-0_4.

17 Irwin S, Comprehensive observational assessment: A systemic quantitative procedure for assessing the behavioral and physiological stat of the mouse, Psychopharmacology, 13, 1968, 627-640, DOI:10.1007/BF00401402,Online ISSN:1432-2072.

18 Winston D, Maimes S, Adaptogenes: Herbs for strength, stamina and stress relief Rochestr, Vermont: 1st edition, Healing Arts Press:ISBN 9781594771583;2007.

19 Johnson, B A, Market report, Herbal Gram; 40, 1997, p49-50.

20 Aniagu SO, Nwinyi FC, Akumka DD, Ajoku GA and Dzarma, S, Toxicity studied in rats fed nature cure bitters, Afr. J. Biotechnol., 4, 2005, p72-78.

21 Scharer K, The effect of chronic underfeeding on organ weights of rats, how to interpret organ weight changes in cases of marked growth retardation in toxicity tests? Toxicology, 7, 1977, p45-56, PMID: 841584.

22 Guidance for Industry: Single dose acute toxicity testing for pharmaceuticals. Centre for drug evaluation and research (CDER), 1996.

23 Hunter PA, toxic brew we cannot live without micronutrients. Give insight to the therapy between geochemistry and evaluating biology; EMBO Rep, 2008, p915918,DOI:10.1038/sj.embor.7401148;PMID:18174893

24 Rispin A D, Farrar E, Margosches K, Gupta K, Stitzel G, Carr M,Greene W,Meyer and McCall D, Alternative methods for median lethal dose (LD50) test: The up and down procedure for acute oral toxicity. ILAR J., 43, 2002, p 233-243, DOI:10.1093/ILAR.43.4.233;PMID:1239139

25 Oliver J A, Opportunities for using few animals in acute toxicity studies, In; Chemicals testing and Animal welfare, Solna Sweden; The national chemicals Inspectorate, 1, 1986, p 119-142:

26 Schulz V, Hanset R, Tylr V E, and Rational Phytotherapy: A physcian’s Guide to Herbal medicine; Psychology press: London, UK, 2001.

27 Shekelle P G, Morton S C, Suttorp M J, Buscemi N, Friesen C, Challenges in systemic reviews of complementary and alternative medicine topics. Ann. Intern Med. 21, 2005, 1042-47, DOI: 10.7326/0003-4819-142-12__part_2-20056211-0003.

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