

Acute and chronic toxicity study of *Valeriana wallichii* rhizome hydro-ethanolic extract in Swiss albino mice

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Submitted: 05-09-2015

Revised: 04-10-2015

Published: 10-11-2015

ABSTRACT

Aims and Objective: To evaluate acute and chronic (90 days) oral toxicity of *Valeriana wallichii* rhizome hydroethanolic extract in Swiss albino mice. **Materials and Methods:** *Valeriana wallichii* rhizome was subjected to extraction with Soxhlet apparatus, using ethanol (90%) + water (10%) mixture and dried withrotavapor. Phytochemical fingerprinting of the extract was done with LC/MS (Liquid chromatography–mass spectrometry). Limit Test for acute oral toxicity at 2000 mg/kg body weight was conducted according to OECD guideline no 425. Chronic 90 day oral toxicity study with three different dose groups (200, 600, 1800 mg/kg/body weight/day) with selected in life parameters (physical, behavioural) and post mortem parameters (haematological, biochemical, gross necropsy and histopathological) as per WHO guidelines for testing safety of herbs was conducted. **Results:** Acute toxicity: no signs of abnormality, morbidity or mortality were observed during 14 days of observation. Chronic toxicity: Significant differences between the treated and control groups were observed in the following parameters: Loss of Auditory startle, Aggressiveness (Control > treated), Nasal discharge, Dyspnoea. At necropsy, tracheitis was observed in 3 cases. Results from Photoactometer test indicates dose dependent increase in sedative property. **Conclusion:** From this work it could be concluded that *Valerianawallichii* rhizome hydroethanolic extract didn't exhibit mortality, morbidity or any other neurologic, hematologic or biochemical adverse effects apart from sedation which is extension of their known pharmacological activity, after single oral dose of 2000mg/kg bw (14 days of observation) or after once daily 200mg/kg, 600mg/kg 1800mg/kg oral treatment for 90days in healthy adult Swiss albino mice.

Key words: Valerian, *Valeriana wallichii*, Toxicity, Preclinical toxicity, Swiss albino mice

Access this article online

Website:

<http://nepjol.info/index.php/AJMS>

DOI: 10.3126/ajms.v7i2.13326

E-ISSN: 2091-0576

P-ISSN: 2467-9100

INTRODUCTION

Herbal formulation named Valerian or Tagara is widely marketed worldwide mostly for sedative- hypnotic properties. The formulation include the underground organs of members of the genus *Valeriana* (Valerianaceae), as well as some related genera such as *Nardostachys*, are used in the traditional medicine of many cultures as mild sedatives and tranquilizers and to aid the induction of sleep. *Valerianaofficinalis* is the species most commonly used in northern Europe and retains its official pharmacopoeial status although it is most commonly encountered as

an ingredient of herbal medicines.¹ Indian valerian, *Valeriana wallichii* is commonly known as Tagara, is an herb recommended by Ayurveda and other ethno medicine streams of India for CNS activities like Sedative-hypnotic and anticonvulsant properties.

Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines² published by WHO states that toxicological evaluation of medicinal plants has been neglected since prolonged and apparently uneventful use usually is considered as an evidence of its safety. However, a history of traditional usage is not always a

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reliable guarantee of safety since it was never evaluated scientifically to detect or monitor delayed effects, rare adverse effects, and adverse effects arising on chronic use of food supplements and nutraceuticals. Further many organizations like WHO and ICMR have expressed the urgent need for validation of indigenous medicines as these could be potential alternative to fill in the treatment gap due to lack of sufficient supply and further may help in the discovery of novel active principles and targets.

The widely used herb tagara consists of predominantly dried rhizome, stolon and small portion of root of *Valeriana wallichii* DC, (Fam. Caprifoliaceae): a hairy perennial herb, growing in temperate Himalayas from Kashmir to Bhutan and Khasiahills up to an altitude of 3,000 m.³ These plants have been the subject of considerable research, aiming at establishing the pharmacological basis of the activity with both animal and clinical studies.⁴ *Valeriana wallichii* is classified under family *Caprifoliaceae* but until recent years *Valeriana wallichii* was classified under family *Valerianaceae*. In this work the older classification system is found to be more suitable since the phytopharmacological profile of the plant is better shared by the members of the family *Valerianaceae* than of *Caprifoliaceae*.

Major pharmacological actions reported by the research publications involving experiments with *Valeriana wallichii* are sedative, hypnotic,⁵ anxiolytic, anticonvulsant,^{6,7} antidepressant^{8,9} anti-inflammatory¹⁰⁻¹³ analgesic antihelmintic¹¹ antileishmanial¹² insecticidal hypotensive antispasmodic¹³ anti-diarrheal bronchodilatory¹⁴ antipyretic-analgesic¹⁵ antioxidant hepatoprotective¹⁶ neuroprotective¹⁷ and possible molecular targets identified are GABA_A receptor,¹⁸ Glycine receptors, Adenosine, Nitric oxide signalling pathway, potassium channel opening,¹⁹ Anticholinesterase inhibition, modulation of brain monoamine levels inhibition of prostaglandin synthesis.²⁰ Chemical constituents identified or isolated from the herb are 6-methylpigenin, acyl-linarin,²¹ minor iridoids,²² 4-methoxy-8-pentyl-1-naphthoic acid,²³ methyleicosanoate, acevaltrate, isovaleroxyhydroxydidrovaltrate and didrovaltrate,²⁴ podophyllotoxin and pinoselinol, 4'-demethylpodophyllotoxin, β -bisabolol, α -kessyl alcohol, valeranone, bornylisovalerate and linarin-2-O-methylbutyrate.²⁵ Clinical studies on stress management²⁶ and sleep quality are published on this herb.

MATERIALS AND METHODS

Collection, authentication and extraction

Collection: The plant rhizome was collected from M. Subhash Chand and Sons, Karyanamerchants, Ghuhbazar, Paprola –Dist, Himachal Pradesh.176115.

Authentication: The herb was authenticated and a voucher

specimen was deposited (no: R150) at Agarkar Research Institute, Pune, India. **Extraction:** Herbs were cleaned removing foreign materials, ground to coarse powder, packed in a thimble made of muslin cloth, Water/Ethanol extract of *Valeriana wallichii* using exhaustive solvent evaporation technique with Soxhlet apparatus, using ethanol (90%) + water (10%) mixture as solvent was done, separated from solvent using rotavapor. **Fingerprinting:** HPLC-LCMS fingerprinting was carried out on a Shimadzu UFLC-XR (Ultra-Fast Liquid Chromatography) system which is interfaced to a Mass Spectrometry (Make: AB Sciex, Model: API 4000) instrument.²⁷ **Chromatography conditions** - Column: Stainless steel 250 × 4.6 mm, 5 μ m, Solvent: Methanol: 0.5% phosphoric acid (80:20). Flow rate: 1.5 mL/minute. **LCMS Conditions** - Ion Source: Electron Spray Ionization, Mode: Positive Ionization, Mass Scan Range: 100 – 1000 amu, Curtain Gas: 25, Gas 1: 35, Gas 2: 35, Temperature: 500, Ion Spray Voltage: 5500 (Figure 1).

Study design

Acute toxicity study: Acute toxicity was studied in a group of Swiss albino mice (n=6; 3 males and 3 females) by administering 2000 mg/kg body weight of test drug *Valeriana wallichii* rhizome hydroethanolic extract (VWRHEE) orally as per OECD guidelines. The test method 425 was selected from many acute toxicity test methods available in view of the fact that this method provides an option for conducting a limit test at a single dose (2000 mg/kg or 5000 mg/kg) prior to the main test which helps to reduce the number of animals used if the test material is not mortal at the limit dose. Considering the published work on the test herb and long history of use without any report of toxicity the selection of this method is appropriate.

Chronic toxicity study: Ninety day repeated once daily oral treatment toxicity study with three (200, 600, 1800 mg/kg/body weight/day) different dose groups (Swiss albino mice, 2-3 months old, 25-30 g n=6; 3 males, 3 females), the first group was control [Vehicle - 0.2% Dimethyl sulfoxide], Second group was treated with 200 mg/kg body weight of test drug *Valeriana wallichii* rhizome hydroethanolic extract (VWRHEE) once daily orally, Third group was treated with 600 mg/kg body weight of VWRHEE once daily, Fourth group was treated with 1800 mg/kg body weight of VWRHEE once daily, with selected in life parameters (physical, behavioural) and post mortem parameters (haematological, biochemical, gross necropsy and histo-pathological) as per Research guidelines for evaluating the safety and efficacy of herbal medicines by World health organization. Parameters of the study are detailed below

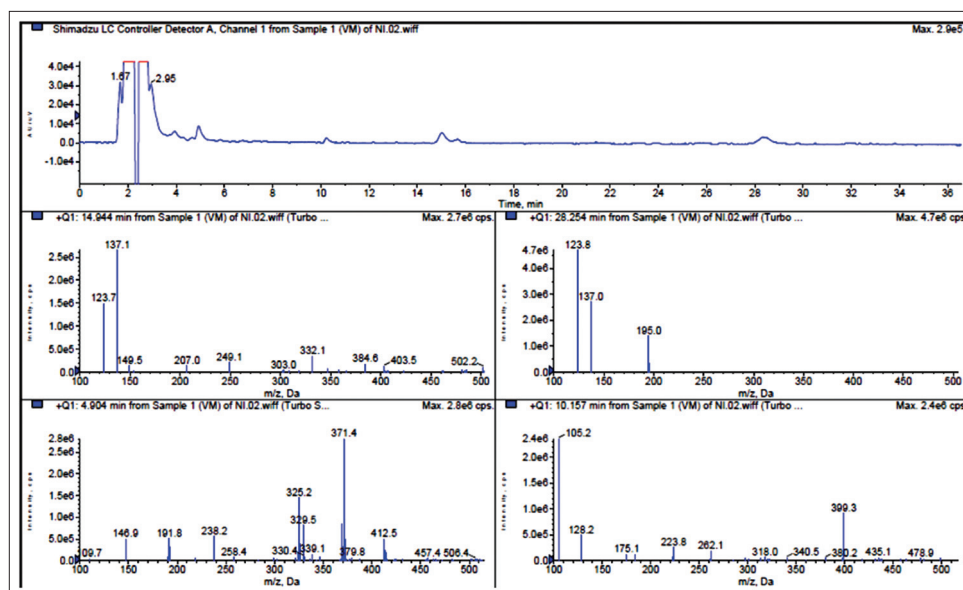


Figure 1: HPLC-LCMS fingerprinting of VwHEE

Observations and examinations

General signs

Changes in appearance & general activity

Appearance: Eye, Nose, Ears, Mouth, Teeth, Fur, Nails, Tail, Genitals, Posture, Faecal consistency, Urine colour.

General activity: Grooming, Photoactometer test, Rota rod test. In photoactometer test 'Percentage reduction in activity' was calculated using following formula.

$$100 \times \frac{(\text{Predose activity} - \text{Postdose activity})}{\text{Predose activity}}$$

Neurological examination tests²⁸

Flexion Reflex: The mouse is picked up and the toes are pinched with forceps. The response is to move the foot away.

Grasping Reflex: The mouse is picked up and the palm is touched with a wire. The response is to grip the wire.

Righting Reflexes: When the mouse is put on its back, it turns over immediately; The animals is held in the lower back; when the body is tilted, its head moves opposite; The mouse is on its back when its head is tilted, its limbs move opposite; When the mouse is dropped, upside down, from 40 cm, it lands right side up.

Placing reactions: Hold the mouse near a table, when the dorsum of the paw contacts the edge of the table, the paw will be placed on the surface of the table.

Equilibrium Tests: The mouse is placed on a bar and should be able to stay there for about 3 minutes.

Corneal Reflex: Hold the mouse, touch the cornea (eye) with a hair - the mouse closes the eye.

Pupillary Reflex: Look at the eye of a mouse with a red light, under a magnifying glass; turn on a white light - the pupil should constrict.

Auditory Startle: When the mouse is quiet on a level surface, give a loud hand clap; it should flex forelimbs and extend hind limbs, arch the body.

Toe

Spreading: Put the mouse on a piece of plexiglass over a mirror; when the plexiglass is tilted, the mouse spreads his toes. **Head Shaking:** When a puff of air is blown through a rubber tube to the ear, the mouse shakes his head. **Conditions for scoring:** The general procedure for each of the tests is to present the test stimulus and observe the reaction. The reaction to the stimulus should be immediate. If the animal produces the correct response, a 1 is scored. If no response or an inappropriate response is emitted, a 0 is used.

Body weight: Before the start of drug administration, once a week thereafter.

Haematological & Blood chemistry examinations &

Hepatic function tests: Blood samples were taken immediately after Euthanasia, before autopsy. Total WBC, Total RBC, Lymphocytes (%), Neutrophil (%), Platelet count, Haemoglobin, Blood glucose, SGPT, SGOT (Mindray BC-2800Vet). **Autopsy:** After Euthanasia (Ketamine overdose-350 mg/kg/ip)²⁹ of animals or if found dead. Macroscopic examination of organs and tissues and Histo-pathological examinations are to be done only if changes are found on gross or macroscopic examination of their organs and tissues of these animals or if the highest dose group reveal significant changes. **Euthanasia:** Immediately for Moribund animals and for others at the end of the study (90 days) Ketamine over dose (350 mg/kg/ip).

Ethical approval

The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC), Yenepoya University and care of laboratory animals was taken as per Committee

for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

RESULTS

Acute toxicity test

At the single oral limit dose of 2000 mg/kg, in a group of six mice, no signs of abnormality, morbidity or mortality were observed during 14 days of observation.

Chronic toxicity study

Significant differences between the treated and control groups were observed in the following parameters: Loss of Auditory startle (alarm reaction), Aggressiveness (Control > treated), Nasal discharge, Dyspnoea (Figure 2: Chronic toxicity - Physical examination and behavioural observations). There were 6 deaths during the study, but there was no significant difference in mortality rate between groups. At necropsy, tracheitis was observed in 3 cases. Histo-pathological analysis of liver and kidney samples did not show any sign of toxicity in any of the tested animals.

Results from Photoactometer test indicates dose dependent increase in sedative property without motor incoordination as indicated by all animals passing rota rod test (Figure 3).

VwRHEE was not found to be toxic at the dose ranges during the period of 90 days as per physical examination, behavioural observation, biochemical, haematological (Table 1), gross necropsy and Histo-pathological reports. The drugs were administered daily for 90 days, the animals were observed daily for any untoward effect, few animals showed loss of auditory startle (alarm reaction), aggressiveness, and nasal discharge. It was also observed that aggressiveness of some animals in the control group. Dyspnoea were also observed in the last two weeks.

DISCUSSION

'Valerian' the herbal formulation of *Valeriana wallichii* rhizome is one of the most widely used herb all over

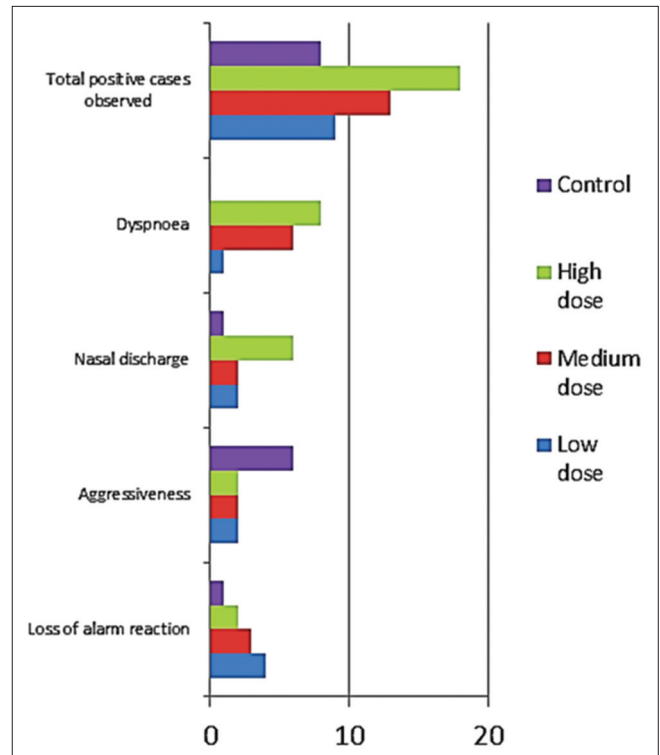


Figure 2: Chronic toxicity - Physical examination and behavioural observations

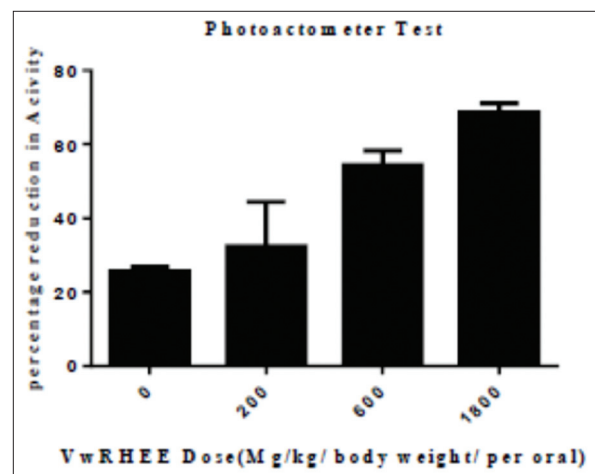


Figure 3: Photoactometer test

Table 1: Chronic toxicity – Haematology and Biochemistry

Parameter	Control	VwRHEE		
		200 mg/kg	600 mg/kg	1800 mg/kg
WBC/cu mm	5273.33±41.1	5290±58.88	5253.33±33.99	5293.33±20.55
RBC (million/cu mm)	3.83±0.13	3.87±0.09	3.9±0.082	3.9±0.16
Lymphocytes (%)	41.67±1.7	39.67±1.7	40±2.16	40±2.21
Platelet count (lakhs per cu mm)	1.87±0.05	1.8±0.08	1.89±0.05	1.84±0.05
Neutrophil (%)	54.67±1.25	54.33±0.47	53.33±0.47	53.67±1.25
Hb (g/dl)	12.5±0.08	12.63±0.13	12.5±0.12	12.7±0.05
Blood sugar (mg/dl)	128.33±1.25	130.33±3.3	131±2.94	129±2.45
SGPT (IU/ml)	24±0.82	23.33±0.47	23.7±0.47	24±1.41
SGOT (IU/ml)	20.33±0.94	20±0.82	19.33±0.47	20.67±0.47

the world for insomnia, stress etc as a nutraceutical or herbal medicine. American and European Pharmacopoeias have published special editions of Valerian. Indian Vedic literature, mainly ayurvedic scripts have mentioned many herbal preparations and are being used, but most of them are not scientifically validated. Many organizations like WHO and ICMR have expressed the urgent need for validation of indigenous medicines as these could be potential alternative to fill in the treatment gap due to lack of sufficient supply and further which may help in the discovery of novel active principles and targets. The pharmacological validation of herbal formulation has to be preceded by preclinical toxicity studies and this study is meant to partly fill that gap. There are several compounds isolated from this herb in the pipeline of drug development like Valerianic acid. These kinds of studies are significant from both health care and economic development of the country. Reports from several preclinical studies and empirical human use suggests pleiotropic effects of the herb, this could be scientifically evaluated with clinical trials if the detailed preclinical toxicity studies are available.

The present study is a preliminary step towards pharmacological and toxicological profiling of the herb to aid the rational clinical application. Acute toxicity and 90 day chronic toxicity study is essential but not enough to meet the requirements prior to commencement of human trial. Considering the long term use of the herb in humans a chronic toxicity study of one year duration is the minimum requirement as per WHO guidelines on evaluation of safety of herbal medicines. Further conventional guidelines also suggest toxicity study in multiple species in order to improve predictive power of the effects in humans. Most of the adverse effects observed in the study were mild and the sedative property in compliance with reports on CNS depressant and GABAergic activities of the extract. *Valerianawallichii* rhizome hydro-ethanolic extract is found to be safe with mild adverse effects in few animals; most of which was not dose dependant indicating that the effects may be related to other variables like artefacts or to handling.

From this work it could be concluded that *Valerianawallichii* rhizome hydroethanolic extract didn't exhibit mortality, morbidity or any other neurologic, hematologic, biochemical adverse effects apart from sedation which is extension of their known pharmacological activity, at 200 mg/kg, 600 mg/kg and 1800 mg/kg oral treatment for 90 days in healthy adult Swiss albino mice.

CONCLUSION

From this work it could be concluded that *Valerianawallichii* rhizome hydroethanolic extract didn't exhibit mortality,

morbidity or any other neurologic, hematologic or biochemical adverse effects apart from sedation which is extension of their known pharmacological activity, after single oral dose of 2000 mg/kg bw (14 days of observation) or after once daily 200 mg/kg, 600 mg/kg 1800 mg/kg oral treatment for 90 days in healthy adult Swiss albino mice.

ACKNOWLEDGEMENTS

We would like to thank Mr. Sudhakar Shetty and Mr. Suresh Kumar. S Bioanalytical Service Department, Sequent Research Ltd, Mangalore, Dr. Vinitha.R.Pai, Professor, Department of Biochemistry, Deputy Director Research Yenepoya University, Dr. Ravi Rao, Professor, Alvas Ayurvedic Medical College, Moodbidri, and Dr. Navneet Sharma, Sr. Lecturer, Rajiv Gandhi Government Ayurvedic College, Paprola, Himachal Pradesh, Mr. S. Purushottam Nayak, Deepak memorial computerized laboratory and Dr. Kalyan Chakravarthi Kosuri.

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Authors Contribution:

LJ - Concept and design of the study, reviewed the literature, conducted experiment, collected and analysed data, manuscript preparation and critical revision of the manuscript; **RE** - Concept and design of the study, reviewed the literature, manuscript preparation and critical revision of the manuscript; **SNR** - Concept and design of the study and critical revision of the manuscript.

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Source of Support: Ethnopharmacology Lab- Department of Pharmacology, Yenepoya Medical College, Mangalore, Karnataka, India. 575018. **Conflict of Interest:** None.