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Assessment of Acute and 28-Day Sub-Acute Oral Toxicity of a Polyherbal Formulation in Rats

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Research Article

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Abstract

Purpose: Most of these studies are conducted to assess the degree to which substances are toxic (poisonous) for humans, animals or the environment, to investigate the mechanism of toxic chemicals, or to develop new or improved tests for specific types of chemically induced effects. The present study was designed to evaluate the acute and 28 days repeated oral toxicity study of Polyherbal formulation according to OECD guidelines. Materials and Methods: In acute oral toxicity study, Herbal mixture was administered at 2000mg/kg orally and animals were observed for toxic signs at 30 min, 1, 2 and 4 hrs and thereafter once a day for the next 14 days. In repeated dose-28-day oral toxicity study, the animals were divided into four groups of 6 animals each. Group-1 animals served as a control. Group II animals received low dose of herbal mixture 50 mg/kg, Group III animals received middle dose 100 mg/kg and Group IV animals received high dose of 200 mg/kg (orally) once daily for 28 days respectively. Results: The assessment study results showed that neither the acute toxicity study of herbal mixture at the dose level of 2000mg/kg nor the 28 days repeated oral toxicity study produced any toxic signs or mortality during study. In 28 days repeated oral toxicity study, no significant changes were observed in the haematological and biochemical parameters, relative organ weight, gross necropsy and histopathological examination with herbal mixture treatment. Conclusion: The results of the present study suggest that LD50 of newly developed Polyherbal formulation was >2000 mg /kg and the mixture is completely safe and non-toxic for therapy.

Keywords: Acute Oral Toxicity; Sub Acute Toxicity; Herbal Mixture; Haematology; Liver Function Test; Histology

Introduction

Medicinal plants have surged in recent times due to increased effectiveness of drugs from plant sources, increased use of natural products and raising apprehension about the ill effects of available medicine [1]. Herbal mixtures have shown that they achieved better efficiency with limited side-effects in relative to single herbal drugs.

The World Health Organization estimated that about 80% people around the world believes on these "alternative" plant-derived drugs as their primary medical need especially in the developing and developed countries where modern medicines are prevalent practice [2]. In the recent years, the use of herbal medicines for various illnesses has been very fruitful and its past usage has been beneficial in new drug discovery. Herbal prescriptions and natural medicines are routinely prescribed in developing nations for the treatment of various illnesses [3,4]. Toxicology can be defined as the study of harmful/poisonous effects of drugs and other chemicals with emphasis on detection, prevention and treatment of poisonings. After obtaining relevant information on the harmful effects of a compound, the levels for its safe usage or the degree of its safety is established, this is known as its (compound) Biosafety level [5]. Acute toxicity testing in animals is typically the initial step in the assessment and evaluation of the health effect characteristics of a test substance and its primary aim is to provide information on potential health hazards that may result from a short-term exposure.

Traditional and alternative medicine is largely practiced in the prevention, diagnosis and treatment of various illnesses. It has drawn public attention over the past 2-3 decades as these medicines are easily accessible in some regions [6]. Medicinal plants contributed greatly in daily life by offering wide variety of nutrients, vitamins and other compounds which widen their therapeutic applications. In general, natural products play a major role in the development of novel drug leads for the prophylaxis and therapy of diseases [7]. Assessment of efficacy and safety of herbal medicines is necessary because many people using these agents as self-medication. Since, there is limited data available about the safety of the commonly used herbal mixtures, therefore, efforts to explain health benefits and risks of herbal medicines should be intensified. It is the need of the hour to test acute and chronic toxicities of herbal drugs [8].

Plant based formulations available with a range of indications such as liver protective, appetite and growth promoters, gastrointestinal and hepatic regulator; despite of extensive use, there is a lack of scientific evidence on their quality, safety and efficacy of many herbal preparations. Although many herbal preparations are non-toxic, many plants currently used for medicines have been shown to be highly toxic when given either acutely or sub-chronically [9,10]. The increasing practice of plant based medication all over the world and lack of experimental reports on their safety make it basic to direct toxicological evaluation on natural herbal products [11,12].

Herbal medicines have attained the widespread acceptability as natural therapeutic agents for various diseases like diabetes, arthritis, renal and liver diseases, obesity and cardiovascular disorders. It has proved that herbal combination of different herbs produce better therapeutic effectiveness than the individual herbs. These combinations are employed for the cure of various chronic disorders. Currently worldwide there is need to find out the safe, less toxic, cost effective polyherbal remedies that can be effective against various chronic diseases like diabetes, obesity, liver dysfunction. Here we developed polyherbal formulation which is made up of three Indian medicinal plants with minimum quantity and maximum therapeutic potential. We hope the newly developed herbal medicine may be very effective to treat the various chronic diseases. Hence it has become necessary to standardize the preclinical safety and efficacy study on animal model for further therapeutic study to establish the formulation as a drug. The polyherbal formulation contained proven antidiabetic, antioxidant, antihyperlipidemic and cardio tonic herbs that alone or in combination control diabetes and diabetic complications.

The selected plants for preparation of polyherbal formulation includes *Momordica charantia* (bitter melon), commonly referred as bitter gourd, karela and balsam pear. Its fruit are widely used for the treatment of diabetes and related conditions amongst the indigenous populations of Asia, South America, India and East Africa. Abundant pre-clinical studies have documented its anti-diabetic and hypoglycaemic effects through various postulated mechanisms [13].

Syzygium cumini (Myrtaceae) commonly known as Malabar plum, Java plum, or black plum, is traditionally used against cardio metabolic disorders such as antihyperglycemic, antihyperlipidaemic, anti-inflammatory, cardio protective and antioxidant activities. These properties have been attributed to the presence of bioactive compounds such as phenols, flavonoids, and tannins in different parts of the plant [14].

Aegle marmelos (L.) Correa, (Rutaceae) commonly known as Bael, has been widely used in indigenous systems of medicine due to its various medicinal properties. Bael is having great potential to cure the diseases like diabetes, hyperlipidaemia, ulcer, inflammation, diarrhoea, cancer, constipation and shows antibacterial, antifungal, radio protective, antipyretic, analgesic, antioxidant, hepatoprotective, wound healing and many more activities [15].

The safety of these individual herbs is well documented, but the effects of these herbs in combination are unclear. Thus, it becomes necessary to evaluate the safety and toxicity of the combination of herbs (PHF), before their use in human. Preclinical toxicity studies are necessary for determining

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a safe dose for human trials. Hence, the aim of the present study, to assess the safety of polyherbal formulation by acute oral toxicity (single dose, 14 days) and sub-acute oral toxicity (repeated doses for 28 days) in albino Wistar rats. The toxicity study was performed as per guidelines of organization for economic cooperation and development (OECD) 423 and 407 respectively.

Materials and Methods

Plant Materials

Fruits of *Momordica charantia* (Cucurbitaceae), *Syzygium cumini* (Myrtaceae), *Aegle Marmelos* (Rutaceae) were collected from the aromatic and medicinal plant garden, MPKV, Rahuri and were authenticated by Dr. J. Jayanti, Botanist, Botanical Survey of India, Pune. (BSI/WRC/Tech./2011/581)

Preparation of Polyherbal Formulation

The fruits of *Momordica charantia, Syzygium cumini, Aegle Marmelos* were collected, dried under shade and then coarsely powdered. The dried powder sieved through sieve No.40 and stored for the extraction purpose. The 500 gm of powdered plant materials were packed in the soxhlet extraction assembly and extracted by using solvents of increasing polarity by continuous hot extraction, for 48 Hrs. The aqueous extraction was carried out by cold-maceration method.

Experimental Animals

Healthy adult, Wistar rats (both male and female) weighing 150-175 g, were obtained from the animal house of the M.E.S's College of Pharmacy, Sonai. All the animals were housed in clean polypropylene cages placed in well-ventilated conditions of temperature and humidity with a 12 h light/dark cycles and are allowed free access to food (standard pellet diet) and drinking tap water *ad libitum* except when fasting was required during the study. The experimental procedures were carried out in accordance with IAEC rules and CPCSEA regulation (MES/COP/IAEC/ 02/16-17).

Assessment of Acute Toxicity Study

The acute toxicity study was performed as per organization for economic cooperation and development (OECD) revised fixed dose procedure for acute toxicity testing (OECD guideline 420, 2001). Two groups of five healthy albino wistar rats of either sex (3-month old, 150–200 g b.wt.) were administered a limit dose of 2000 and 5000 mg/kg of the PHF and animals were observed for mortality and clinical signs for the first hour, then hourly for next 3 hrs and finally periodically until 48hrs for changes in skin and fur, eyes and mucus membranes, behavior pattern, tremors, salivation, diarrhea, sleep, coma, mortality, moribund, ill health or any visible reaction to treatment.. All of the experimental animals were maintained under close observation up to 14 days, and the number of rats dead within the study period was recorded. The LD_{50} was predicted to be above 2000 or 5000 mg/kg if three or more rats survived [16].

Sub-Acute Toxicity Study

The sub-acute oral toxicity study was carried out as per OECD guidelines OECD-407 2. Rats were divided into four groups of 10 animals each (5 males and 5 females) and their body weights were recorded. Control rats (group I) received distilled water, while groups II–IV received PHF at the dose of 50,100 and 200 mg/kg respectively. All treatments were given by oral route once a day for 28 days. Animals were observed for signs of abnormalities during the treatment period. Besides, the body weights of animals were recorded at the end of treatment. On 28th day of treatment, animals were placed in individual metabolic cages for 24hr. Excreted urine was collected and kept at −20°C for ion and biochemical analysis. At the end of the treatment, animals were fasted overnight, but allowed free access to water. They were then anaesthetized with ether and blood samples were collected with and without anticoagulant (ethylene diamine tetra acetate) by retro-orbital puncture, using capillary tubes for hematological and biochemical analysis respectively.

Haematological Study

On the 28th day of drug treatment all animals were kept for overnight fasting (water *ad libitum*). On next day fasted animals were anaesthetized using isoflourane and blood samples were collected using heparinised microhematocrit tubes by ROP into a potassium EDTA containing tubes (for haematological) and 11% w/v Tri-sodium Citrate (TSC) containing tubes (for biochemical analysis). Blood smear was prepared from the EDTA containing blood sample, air dried and stained (Hemacolor rapid staining of blood smear, E.Merck, Mumbai, India) for Differential Leukocyte Count (DLC). Haematological analysis were performed using automated haematology analyser (Model PE 6000 Rapid Diagnostics Pvt Ltd, New Delhi, India), which includes analysis of haemoglobin (HGB), Red Blood Cell count (RBC), White Blood Cell count (WBC), platelet count and Hematocrit (HCT).

Liver Function Test

The blood samples were further analysed for glucose, triglyceride, cholesterol, Alkaline Phosphatase (ALP), Aspartate Transaminase (AST), Alanine Transaminase (ALT), Lactate Dehydrogenase (LDH), total bilirubin, creatinine,

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urea, protein and albumin by using different biochemical kits (Accurex Biomedical Pvt. Ltd, India) in semi-automated biochemical analyser (Model: Benespera C-61, Lablife India Pvt Ltd, New Delhi).

Histopathology

After blood sample collection, rats were sacrificed by decapitation and organs such as heart, liver, kidney, lung and spleen were collected, blotted dry and the relative organ weight was calculated and compared with that of control. The liver and kidneys were fixed in NaCl buffer containing 10% formaldehyde for histopathology examinations.

Statistical Analysis

All results were expressed as mean ± SEM. Data obtained from the toxicity studies were analysed by Student's t-test using Graph Pad prism 5.0 to determine significant difference between the means of control and test groups. P value < 0.05 was considered statistically significant.

Results and Discussion

Traditional medicine has maintained greater popularity all over the world and the use is rapidly increasing. Despite this, the safety of herbal medicine use has recently been questioned due to reports of illness and fatalities, hepatotoxicity and nephrotoxicity. Although there are many traditional herbal medicines available, only a few have been verified by clinical trials, their safety and efficacy are still questioned by patients.[13-14]

Acute toxicity study monitors changes in skin, fur, eyes and mucus membrane. Toxic symptoms related to central nervous system, Cardiovascular system and Autonomic nervous system such as occurrence of tremors, convulsions, sedation, stereotypic behaviour, respiratory distress, cardiovascular collapse, response to sensory stimuli, salivation, diarrhea, pilo erection, muscular co-ordination, muscular grip, posture, gait, limb paralysis, lethargy, sleep, coma and mortality were observed and recorded with special attention (Table 1). The observed results indicate that no any death or signs of toxicity in the treated animals (Table 2). Changes in body weight of both male and female rat were recorded and are compared with animals in control group. Further, there were no any gross pathological abnormalities, which prove that the LD_{50} was found to be greater than 2000 mg/ kg b.wt.

Signs and symptoms	Clinical observations at Day		
	1	7	14
Behavior	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal
Eyes And mucous membranes	Normal	Normal	Normal
Salivation	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent
Death	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil

Table 1: Clinical observations of rat at 2,000 mg/kg dose of Polyherbal Formulation (PHF).

Groups	Dose	D/T	Mortality
Control	D/W 10 ml/kg p.o.	None	None
PHF treated	2000 mg/kg p.o.	None	None

D/T: Dead/Treated rats;
None: No toxic symptoms were seen during the observation period.
Table 2: Acute toxicity study Polyherbal formulation.

The sub-acute toxicity study showed that repeated administration of PHF up to 28 days didn't produce any clinical signs of toxicity or death. Changes in body weight, Food and water intake of treated groups were found to be insignificant when compared to the control groups. There was a significant rise (P < 0.05) in the body weight of rats treated with PHF 200mg/kg, while no significant difference was observed in other groups (Tables 3 & 4). This suggests that, there was no any hazardous effect on sub-acute administration of poly herbal formulation.

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Parameters Body wt. (gm)	Control	PHF mg/kg		
		50	100	200
Initial	147.17± 1.35	147.83± 1.01	148.67 ±0.91	149.33±0.80
Final	155.00± 1.39	164.67 ±1.04**	170.50± 0.99**	175.83±0.94**

Table 3: Effect of PHF on relative body weight of rats.

Parameters Organ wt.(gm)	Control	PHF mg/kg		
		50	100	200
Heart	0.32± 0.006	0.35± 0.007**	0.38 ±0.01*	0.35± 0.006**
Liver	3.51± 0.004	3.44± 0.05^{ns}	3.90±0.01**	3.72± 0.007**
Lung	0.74 ±0.01	0.71± 0.005*	0.66± 0.005**	0.61± 0.006**
Spleen	0.29± 0.006	0.31± 0.005^{ns}	0.33±0.04**	0.33± 0.007**
Kidney	0.59 ±0.005	0.64 ±0.006**	0.63± 0.006**	0.62 ±0.004**

Table 4: Effect PHF on relative organ weight of rats.

Haematological values such as Packed Cell Volume (PCV), Red Blood Cells (RBC), White Blood Cell (WBC), Platelets, Haemoglobin (Hb), Mean Cell Haemoglobin Concentration (MCHC), Mean Red Cell Volume (MCV), Neutrophils, Eosinophil's, Basophils, Lymphocytes and Monocytes were found to be within the normal physiological limits for rodents and no significant changes were observed when compared with the control groups (Table 5). Hence, there was no any serious toxicological implication.

Parameters	Control	PHF mg/kg		
		50	100	200
WBC ($\times 10^3$ uL^{-1})	9.50±0.42	5.50±0.42**	17.33 ±0.66**	15.83 ±0.60**
RBC ($\times 10^6$ uL^{-1})	6..01±0.06	6.38 ±0.04**	6.98 ±0.06**	7.08 ±0.06**
Haemoglobin(gm/dl)	11.27±0.0.6	11.45±0.09^{ns}	12.23 ±0.09**	12.41± 0.09**
Haematocrit (PCV) (%)	36.66±0.33	40.50±0.42**	42.33± 0.42**	41.33 ±0.42**
Platelets ($\times 10^3$ uL^{-1})	439.00±1.99	634.00 ±3.76**	738.67 ±5.04**	805.67± 1.62**
Monocytes (%)	5.40±0.10	6.03 ±0.06**	6.38± 0.04**	6.33 ±0.06**
Lymphocytes (%)	67.16±0.60	64.00±0.57**	63.50 ±0.42**	63.83± 0.47**
Neutrophils (%)	21.66±0.42	23.00±0.36^{ns}	21.83± 0.47^{ns}	23.50± 0.42*
Eosinophils (%)	2.08±0.06	2.95±0.07**	3.33± 0.06**	4.00± 0.05**
Basophils (%)	0.47±0.005	0.42±0.005*	0.41± 0.004**	0.40 ±0.007**

Table 5: Effect of PHF on haematological values of rats.

Lipid parameters such as HDL, LDL, VLDL, TGL and Total Cholesterol didn't show any significant changes. The main product of protein metabolism is urea and an increased level of urea in the blood is an indicative of renal impairment (Table 6). The present study showed no significant changes pertaining to renal parameters. Serum marker enzymes are biochemical parameters associated with health indices and are of diagnostic significance in routine clinical evaluation of the state of health. Alanine amino Transaminase (ALT) and Aspartate amino transaminase (AST) are largely used in the assessment of liver damage by drugs or any other hepatotoxin (Table 6). So, to elucidate the toxicity produced during liver metabolism of drug, transaminase markers play a vital role. Aspartate Transaminase (AST), Alanine amino Transaminase (ALT) which is the indicators of hepatocellular injury didn't show any significant alterations in the polyherbal formulation treated groups.

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Parameters	Control	PHF mg/kg		
		50	100	200
Glucose (mg/dl)	90.16± 0.30	90.33. ± 0.42^{ns}	91.33± 0.42^{ns}	91.00± 0.57^{ns}
BUN (mg/dl)	9.00± 0.36	11.00 ±0.36**	12.33± 0.33**	16.00± 0.57**
Creatinine (mg/dl)	0.30± 0.004	0.29± 0.003^{ns}	0.31 ±0.004^{ns}	0.32± 0.003*
Total Protein (g/dl)	5.45 ±0.04	6.15 ±0.04**	6.34± 0.04**	6.53± 0.04**
Albumin (g/dl)	3.15± 0.044	3.38 ±0.04**	3.28± 0.03**	4.26± 0.05**
Total bilirubin (mg/dl)	0.43± 0.006	0.53± 0.005**	0.55 ±0.006**	0.67 ±0.007**
AST (U/L)	83.83± 0.47	82.83± 0.60^{ns}	87.50± 0.42^{ns}	89.00± 0.57**
ALT (U/L)	26.66± 0.42	27.16 ±0.60^{ns}	29.66 ±0.33^{ns}	25.33 ±3.28^{ns}
ALP (U/L)	333.17±0.79	315.50± 0.76**	324.67 ±0.61**	295.83± 0.60**
Total Cholesterol (g/L)	0.65± 0.004	0.68± 0.004**	0.55± 0.004**	0.58± 0.004**
LDL (g/L)	0.16 ±0.004	0.19± 0.004**	0.20 ±0.004**	0.21± 0.003**
HDL (g/L)	0.48± 0.004	0.47 ±0.004^{ns}	0.51±0.003**	0.43 ±0.004**
TG (g/L)	1.13± 0.006	1.15± 0.004^{ns}	0.97± 0.01**	0.92± 0.007**

Table 6: Effect of PHF on biochemical values of rats.

The histopathological examinations shows no significant weight changes and normal architectural changes in the vital organs such as heart, brain, kidneys, liver, lungs and spleen suggesting that the PHF is free from risk of serious organ degenerative potential at all dose levels (Figure 1).

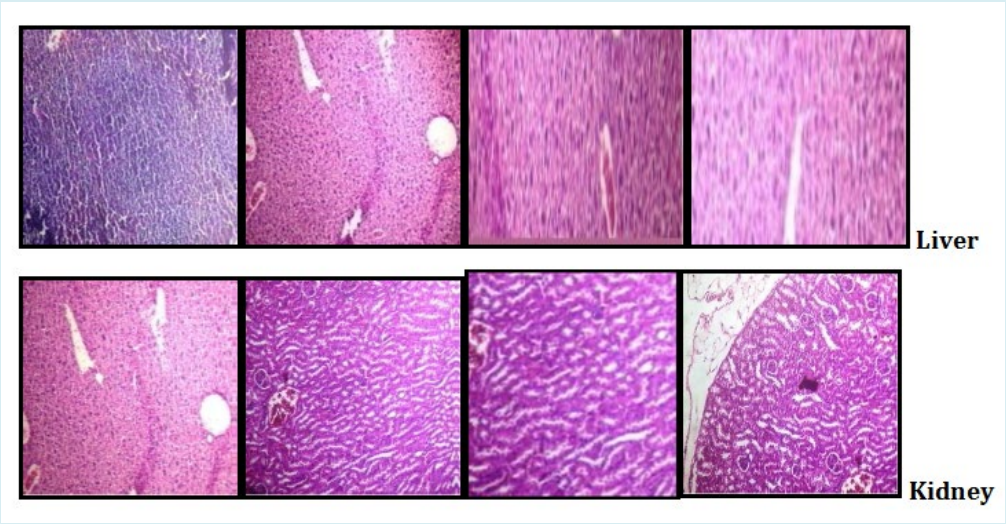

Figure 1: Histopathology (H&E stain10x) of treated groups rats after 28th days Control 50mg/kg 100mg/kg 200mg/kg.

World Health Organization estimated that 80% of the world's population still depend mainly on traditional medicines for their health care. The subcontinent of India is well-known for major biodiversity centres with almost 45,000 plant species. In India, about 15,000 medicinal plants have been identified, in which the communities used 7,000-7,500 plants for cure of different diseases and ailments. In *Ayurveda*, single or multiple herbs (polyherbal) are used for the treatment. The '*Ayurvedic Literature Sarangdhar Samhita*' highlighted the concept of polyherbalism to achieve greater therapeutic efficacy. The active phytochemical constituents of individual plants are insufficient to achieve the desirable therapeutic effects. When multiple herbs are combined in a particular ratio, it will achieve better therapeutic efficacy and safety. We have developed a herbal formulation which is prepared by mixing three well known Indian medicinal plants

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in a fixed ratio. The above study results clearly indicated that the newly developed poly herbal formulation was free from any risk of serious organ degenerative potential at all dose levels

Conclusion

The present Acute and sub-acute toxicity results suggest that LD_{50} of developed formulation was >2000mg/kg. Further studies on long term toxicity and clinical trials may be rational to substantiate the study results.

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Conflict of Interest

Authors disclose no conflicts of interest for publication of the manuscript.

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