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Acute oral toxicity study of "Polyherbal formulation (*Rosmarinus officinals*, Ashwaghandha and Amla) in Wistar rats"

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Abstract
Today in this present globe many part of the continents the use of herbal medicinal products and supplements has increased tremendously over the past three decades with not less than 80% of people worldwide trusting on them for some part of primary healthcare. The study was focused to evaluate the acute oral toxicity of polyherbal formulations (PHFs) and the developed polyherbal formulation includes three herbs *viz.* the roots of *Withania somnifera* (Ashwagandha), leaves of R. *officinalis* (Rosemary) and fruit of *Emblica officinalis* (Amla) detect the effect of this polyherbal formulation on higher mammals. The raw materials were standardized according to the WHO guidelines and the three holistic extracts were mixed in 1:1:1 ratio for further study by bio-guided method and also in combination to control and curing or managing of different diseases. However, to confirm the drug's safety and efficacy in the appropriate dosages we used healthy nulliparous and non-pregnant female Wister Albino rats (Rattus *norvegicus*) with average body weight of 160 g - 180 g and age between 8–12 weeks were used throught the experiments in the present study administered a single dose of 2000mg/kg of body weight by oral gavage in female Wistar rats the observation period of 14 days. The rats were maintained under standard laboratory conditions (room temperature of 22.1 to 24.6°C with optimal air cycle changes 12-15 per hour and relative humidity of 48-61 % with 12 hours light & dark cycle). The animals were fed with laboratory animal feed and UV sterilized drinking water was provided *ad-libitum* throughout the experimental period. Hence the present study is designed to test the acute (limit dose) toxicity of two herbal extracts of rosemary leaf and ashwagandha and amla root in Wister rats following the OECD guidelines 423, which would help in suggesting the minimum safety level of herbal extracts.

Keywords: Acute oral toxicity, polyherbal formulation, bio-guided method, *Ad-libitum* and wistar rats

Introduction
Today in this green planet the Plants are very useful to mankind. Many of them are used exclusively for medicinal purposes. According to the World Health Organization (WHO), "a medicinal plant is a plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are pioneers for chemo-pharmaceutical semi-synthesis." Such plants are in great demand by pharmaceutical companies for their active ingredients. Herbal care or traditional systems of medicine are used from ancient times; herbs have been the original source for most of the drugs. Today approximately 70% of the world population is depending on medicinal herbs. Medicinal plants contain so many chemical compounds which are the major source of therapeutic agents to cure human diseases and Herbal formulations have attained wide recognition in comparison to crude plant materials and extracts, due to reduction in dose levels, convenience and ease of administration. These formulations are popular worldwide as therapeutic agents, in various ailments that impact the quality of life of human beings. Recent research focus has shifted towards the use of herbal medicines due to their diverse biological activities, easy availability, cost effective in nature and safe usage. Polyphenols and flavonoids present in the medicinal plants have been shown to possess significant anticancer properties. The information of extractability of a plant provides an idea regarding the amount of extract present in a definite quantity of drug. The extractability also serves as a tool for quality control of plant-drug by [1] today the Herbal medicines are traditionally given in the form of polyherbal formulations (PHFs) as each ingredient is supposed to have different pharmacological function. Polyherbal formulations are usually prescribed to be taken for a longer period and hence may cause adverse effects in the patients, thereby deserving evaluation of their efficacy and safety profile.

A World Health Organization survey indicated that 70 to 80% of the global population depends on alternative medicine, predominantly herbal in nature, in their primary health care. The uses of medicinal plants as a source of drugs in primary health care have become popular universally, particularly in developing countries as a safe because of natural source [2]. Despite the widespread use of plants for treatment of several ailments there is a little known about their toxicity and safety. However, in contrast to popular view, there are some reports regarding serious adverse effects of herbal therapies, such as renal failure and liver injury caused by some plant species. In this sense, experimental studies to determine the safety of medicinal plants are required. The latest surveys have indicated that many medicinal plants applied in traditional medicine showed adverse effects. Therefore, it should be emphasized that the traditional use of any plant for medicinal purposes, by no means, guarantees the safety of such plant. Therefore, the evaluation of the toxic action of the plant extracts or herbal formulations is important in order to consider them safe before used as medicines.

A key stage in ensuring the safety of drugs is to conduct toxicity tests in appropriate animal models. Toxicity is the fundamental science of poisons. The Organization for Economic Co-operation and development (OECD) mentioned acute toxicity as the advance effect occurring within a short time of oral administration of a simple dose of a substance or a multiple doses given within 24 hours and Acute oral toxicity data is a key component of classification and labeling as well as hazard assessment for industrial substances and is a requirement in diverse regulatory testing regimes. The first acute oral toxicity test was created by the British pharmacologist by [3]. It used to involve up to 150 animals until the OECD Test Guideline (TG) 401 [4] standardization it to 45 animals in the year 1981 by [5]. The acute oral toxicity test aims at establishing the therapeutic index, i.e. defined as the ratio LD_{50}: ED_{50}. However, the term acute oral toxicity is most often used in connection to lethality and lethal dose determinations. It is used to standardize biological products and can serve to establish dosing levels for repeated dose studies. With this keeping in view the impact of stress and the great demand for effective supplement to overcome stress and its consequence, the study was focused to evaluate the acute oral toxicity of polyherbal formulation (PHF).

The developed polyherbal formulation includes three herbs *viz*. the roots of *Withania somnifera* (Ashwagandha), leaves of *R. officinalis* (Rosemary) and fruit of *Emblica officinalis* (Amla) detect the effect of this polyherbal formulation on higher mammals. *Withania somnifera* (*W. sominifera*), commonly known as Ashwagandha and it is also called as Indian ginseng and winter cherry, holds a key place in Ayurvedic herbs being one of the best researched and effective herbs belonging to family Solanaceae and It is an important medicinal plant in Ayurvedic and Unani system of medicine. It is widely used indigenous medicine for more than 3,000 years with high medicinal values. Numerous studies indicated immunomodulatory, anti-depressive, antioxidant, antitumor, anti-stress, anti-aging, anxiolytic, cardio-protective and neuro-protective activity of *W. somnifera.* The plant acts as an anti-inflammatory agent through inhibition of complementary system and lymphocyte proliferation by [6]. The European Food Safety Authority (EFSA) has categorized some of the alkaloids of ashwagandha as toxic and harmful. These include anaferine, anahygrine, withanine, sominiferine, somnine, tropine etc. Apart from alkaloids, withaferine A has also been categorized as cytotoxic lactone by EFSA.

Rosmarinus officinalis is commonly called Rosemary and it is an evergreen plant of the Lamiaceae (*Labiatae*) family, in folk medicine, rosemary has been used as a choleretic and a diuretic agent. It has been documented to possess a number of therapeutic applications in medicine for curing or managing of different diseases such as DM, respiratory, gastrointestinal disorders, and inflammatory disease. The active constituents of this plant are phenolic diterpenes and triterpenes. The main active compounds of rosemary include carnosic acid (CA), rosmarinic acid (RA), ursolic acid (UA), caffeic acid and carnosol by [7]. Approximately 90% of the total antioxidant activity of rosemary is attributed to carnosol and CA by [8]. *Emblica officinalis* Geart. (*E. officinalis*) is commonly known as Amla It is also named as *Phyllanthus Emblica or* Indian gooseberry belonging to family Euphorbaceae, is one of the widely used medicinal herb in Ayurveda. *Emblica officinalis* (EO) enjoys a hallowed position in Ayurveda-an Indian indigenous system of medicine. According to believe in ancient Indian mythology, it is the first tree to be created in the universe. It has application as antioxidant, immunomodulatory, antipyretic, analgesic, cytoprotective, antitussive and gastroprotective. Additionally, it is useful in memory enhancing, ophthalmic disorders and lowering cholesterol level, it is useful in memory enhancing, ophthalmic disorders and lowering cholesterol level.

As per the guidelines from Government when any new product is to be put in the market, it is necessary to generate the data regarding its safety. The present investigation is planned with the objective to evaluate the acute oral toxicity of PHF (*W. somnifera*, *R. officinalis* and *E. officinalis*) in female Wister rats and the animals were observed for clinical signs, tremors, Body weight, Behaviour (depression, excitement) and mortality. No mortality was observed in any of the rats. Body weight was recorded at 0,7th and 14th day and all the animals are terminally sacrificed for gross necropsy findings. [9] The method also renders information on the hazardous properties and allows the substance to be ranked and classified according to the Globally Harmonised System (GHS). Detailed toxicological evaluation has to be performed using suitable experimental animals to provide guidelines for selecting a 'safe' dose for human use. In spite of the wide use of "Polyherbal formulation (*Rosmarinus officinals,* Ashwaghandha and Amla) in wistar rats" in traditional medicine, reports of systematic toxicity studies are not available. Hence, the present study is designed to test the acute (limit dose) toxicity of two herbal extracts of rosemary leaf and Ashwagandha root in Wister rats following the OECD guidelines, which would help in suggesting the minimum safety level of herbal extracts. To our best of knowledge, no reports are there on the acute toxicity of polyherbal formulation in Wister rat's studies.

Materials and Methods

a. Distribution and chemical compositions of all three species

Acute oral toxicity study of "Polyherbal formulation (*Rosmarinus officinals,* Ashwaghandha and Amla) in wistar rats" was performed following OECD Guideline 423 in the Liveon Biolabs Private Limited, Yallapura Tumakuru, India. All the 3 plants belonging to the herbal formulation of R. *officinalis* (Rosemary leaves), root powder of *W. somnifera* (Ashwaghandha root) and fruit powder of P. *emblica* (Amla fruit) were collected from the local market, Mandipet, Tumakuru, Karnataka, India. The seeds were dried under shade and powdered before use. Plants used in developing the polyherbal formulations for present study are as follows,

Rosmarinus *officinalis, Withania somnifera & Embalica officinalis.* Rosemary is the native of Mediterranean region and is cultivated in Europe and California in US. It is also grown in Algeria, China, Middle East, Morocco, Russia, Romania, Serbia, Tunisia, Turkey, and to a limited extent in India. Temperate climate is suitable for the cultivation of Rosemary. From fig-1, *Rosmarinus officinalis* L. is a medicinal plant that belongs to the *Lamiaceae* family and is commonly known as rosemary by [10]. Rosemary is a dense bush, branched, evergreen and blue–white flower, reaching a height of about 1 m by [11]. It is characterized by leaves with 1–4 cm long and 2–4 mm wide, sessile, leathery, linear to linear-lanceolate, with curved edges, dark green upper side and granulosa and page bottom tomentous, with prominent midrib, and very characteristic smell by [12, 13]. Rosemary composed of dried leaves and flowers constitutes a particularly interesting source of biologically active phytochemicals as it contains a variety of phenolic compounds including carnosol, carnosic acid, rosmanol, 7-methyl-epirosmanol, isorosmanol, rosmadial and caffeic acid, with substantial *in vitro* antioxidant activity by [14]. Rosemary has been widely used not only in cooking, especially to modify and enhance flavours, but also in traditional medicine, being a highly appreciated medicinal plant to prevent and cure colds, rheumatism, pain of muscles and joints. From fig-2, it is nowadays one of the most popular sources of natural bioactive compounds, and in fact, this plant exerts various pharmacological activities such as anti-bacterial, anti-diabetic, anti-inflammatory, anti-tumor and anti-oxidant, among others.

Fig 1: *Rosmarinus officinalis*

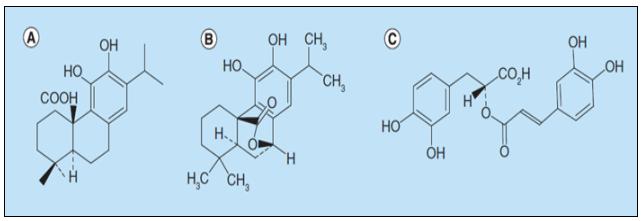

Fig 2: Chemical structure of three major components present in R. officinalis A) Carnosic acid, B) Carnosol, C) Rosmarinic acid

Withania somnifera is a small, woody shrub in the Solanaceae family that grows about two feet in height. It can be found growing in Africa, the Mediterranean, and India. An erect, evergreen, tomentose shrub, 30-150 cm high, found throughout the drier parts of India in waste places and on bunds. Roots are stout fleshy, whitish brown; leaves simple ovate, glabrous, those in the floral region smaller and opposite; flowers inconspicuous, greenish or lubrid-yellow, in axillary, umbellate cymes; berries small, globose, orange-red when mature, enclosed in the persistent calyx; seeds yellow, reniform. The roots are the main portions of the plant used therapeutically. The bright red fruit is harvested in the late fall and seeds are dried for planting in the following spring. It grows in dry parts in sub-tropical regions. Rajasthan, Punjab, Haryana, Uttar Pradesh, Gujarat, Maharashtra and Madhya Pradesh are the major Ashwagandha producing states of the country. From fig-3, Ashwagandha (Withania *somnifera*, fam. Solanaceae) is commonly known as "Indian Winter cherry" or "Indian Ginseng". It is one of the most important herbs of Ayurveda (the traditional system of medicine in India) used for millennia as a Rasayana for its wide-ranging health benefits. Among the ayurvedic Rasayana herbs, Ashwagandha holds the most prominent place. It is known as "Sattvic Kapha Rasayana" Herb by [15]. Most of the Rasayana herbs are adaptogen/anti-stress agents. Ashwagandha is commonly available as a churna, a fine sieved powder that can be mixed with water, ghee (clarified butter) or honey. It enhances the function of the brain and nervous system and improves the memory. Ashwagandha improves the body's defense against disease by improving the cell-mediated immunity. It also possesses potent antioxidant properties that help protect against cellular damage caused by free radicals. Ashwagandha is one of the most valuable herbal drugs used in Indian traditional medicine (Ayurveda) as a *rasayana* drug that is capable of imparting long life, youthful vigor, and good intellectual powers [16]. Ashwagandha is clinically used for the treatment of general debility, consumption, nervous exhaustion, insomnia, loss of memory, and so on [17, 18]. These traditional uses imply that Ashwagandha may possibly be useful at improving neurodegenerative diseases. From fig-4, indeed, this herbal drug has been reported to exert various pharmacological effects such as anti-inflammatory, anti-tumor, anti-oxidant, immunomodulatory, and anti-neuropsychiatric disease effects [19, 20]. The biologically active chemical constituents of *Withania somnifera* (WS) include alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine, etc.), steroidal lactones (withanolides, withaferins) and saponins [20]. Sitoindosides and acylsterylglucosides in Ashwagandha are anti-stress agents. Active principles of Ashwagandha, for instance the sitoindosides VII–X and Withaferin-A, have been shown to have significant anti-stress activity against acute models of experimental stress [21]. Many of its constituents support immunomodulatory actions [22]. The aerial parts of *Withania somnifera* yielded 5-dehydroxy withanolide-R and withasomniferin-A [23].

Emblica officinalis (also known as Amla or Indian Gooseberry) has an important position in Ayurveda- an Indian indigenous system of medicine. According to believe in ancient Indian Mythology, it is the first tree to be created in universe. It also present on the hill slopes up to 2000 meters.

It is found in Pakistan, Uzbekistan, Sri Lanka, South East Asia, China, and Malaysia and commercially cultivated in the state of Uttar Pradesh in India. It is also grown in Tamil Nadu, Rajasthan and Madhya Pradesh by [24]. *Emblica officinalis Gaertn*. (Phyllanthus emblica Linn. Amla. Indian Gooseberry) belongs to the plant family Euphorbiaceae [1].

Fig 3: *Withania somnifera*

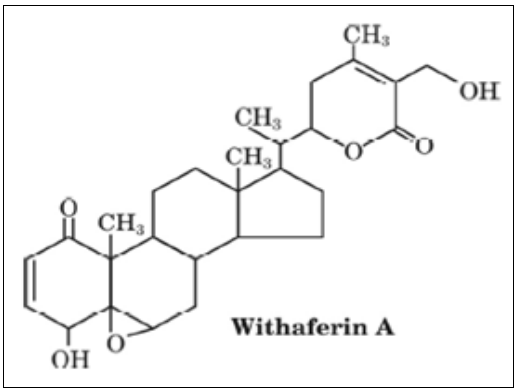

Fig 4: Structure of withaferin A

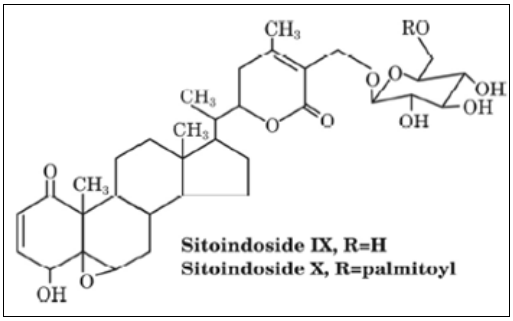

Fig 5: Structure of sitoindosides IX and X

Amla is a small to medium sized deciduous tree. It grows about 8-18m height with thin light grey bark, leaves are simple, light green, sub-sessile, closely set along the branchlets looks like pinnate leaves; flowers are greenish yellow; fruits are globose, fleshy, pale yellow with six obscure vertical furrows enclosing six trigonous seeds in two seeded three crustaceous cocci [25]. The average weight of the fruit is 60-70 g. The fruits of Amla are widely used in the Ayurveda and are believed to increase defense against diseases. Dried fruits, fresh fruit, seed, leaves, root bark and flowers of Amla are mostly used in medicines [24]. From fig-5, *Emblica officinalis* (Amla), a natural, traditional and functional food in Asia, has physiological benefits such as hepato-, cyto- and radio- protection, as well as hypolipidemic effects. In addition, Amla often functions as a potent antioxidant due to the high level of ascorbic acid (ranging from 1,100 to 1,700 mg/100 g of fruit) in its fruit [26].

The pharmacological research reports on amla reveals its analgesic, anti-tussive, anti-atherogenic, adaptogenic; cardio, gastro, nephro, neuro protective and anticancer properties. From fig-6, Amla is also reported to possess chemopreventive, radio, chemo and immunomodulatory, free radical scavenging, antioxidant, anti-inflammatory, anti-mutagenic activities. These properties are efficacious in the prevention and treatment of various diseases like cancer, atherosclerosis, diabetes, peptic ulcer, anemia, liver, heart diseases and various other disorders [25]. *Emblica officinalis* is one of the most extensively studied plants, primarily it contains tannins, alkaloids, phenolic compounds, amino acids, carbohydrates and other compounds (like Vitamin C, Flavanoids, Ellagic acid, Chebulinic acid, Quercetin, Chebulagic acid, Emblicanin-A, Gallic acid, Emblicanin-B, Punigluconin, Pedunculagin, Citric acid, Ellagotannin, Trigallayl glucose, Pectin). Its fruit juice contains the highest concentration of vitamin C (478.56 mg/100 mL).. Fruits have 28% of the total tannins distributed in the whole plant. The fruit contains two hydrolysable tannins Emblicanin A and B, which have antioxidant properties; one on hydrolysis gives gallic acid, ellagic acid and glucose wherein the other gives ellagic acid and glucose respectively. The fruit also contains Phyllemblin.

Fig 6: *Emblica officinalis*

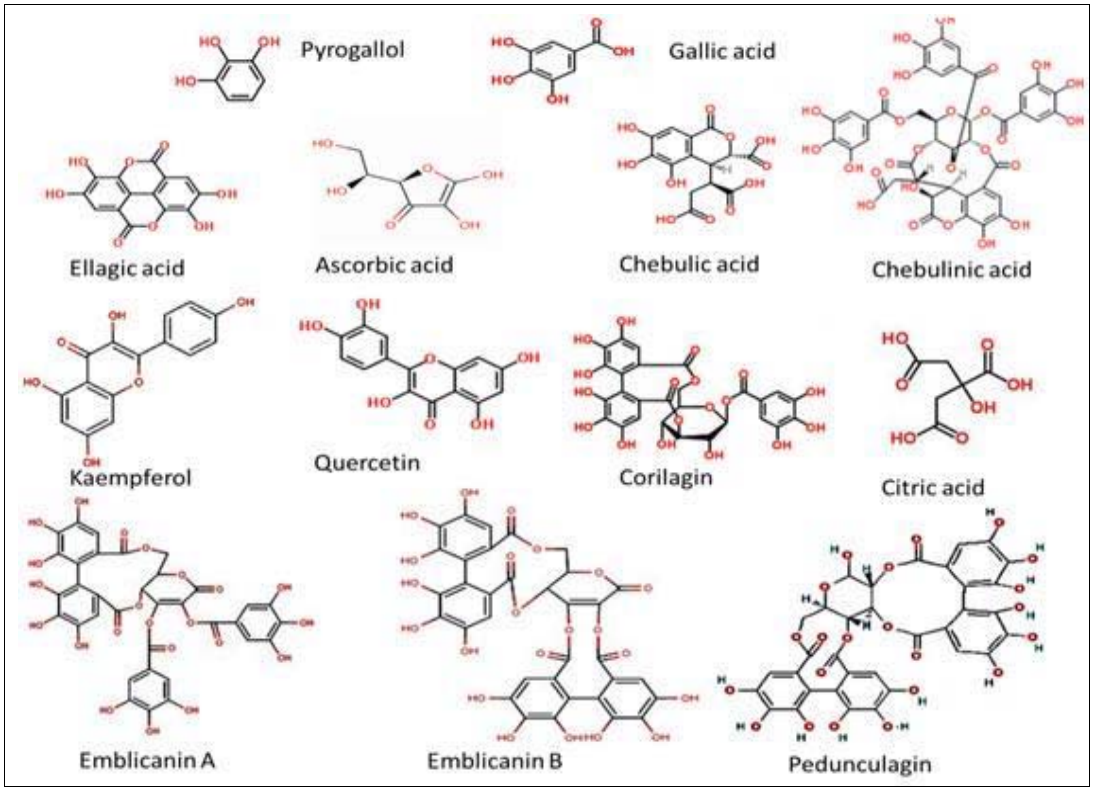

Fig 7: Chemical structure

b. Extraction and preparation of polyherbal formulation

Extraction of rosemary leaves

The plant leaves were dried under shade, coarsely powdered and stored in air tight container. This shade dried leaves powder of *Rosemarinus officinalis* was first defatted with petroleum ether (at 60-80°C) and then extracted 95% v/v ethanol in a sox let apparatus. The alcohol extract filtered, the solvent was evaporated and accurate weight of the extract was taken. The color and constituency of the extract were noted down (light brown and powderly mass).

Extraction of ashwaghandha root and amla

Ready dried powder of extracted ashwaghandha root and amla fruit powder were used.

Preparation of polyherbal formulation

The Polyherbal formulation was prepared by mixing all the 3 holistic extracts taking equal Quantity (666mg) of each powdered extract of Rosemary leaves, Ashwagandha root powder and Amla fruit powder were mixed in 10 ml of distilled water and formulated into 1:1:1 ratio for further study.

c. Experimental animal and their maintenance

A laboratory rat or lab rat is a rat of the species *Rattus norvegicus domestica* which is bred and kept for scientific research. While less commonly used for research than mice, rats have served as an important animal model for research in psychology and biomedical science [27]. The rat is the commonly used species for toxicity studies and recommend by the international guidelines (i.e. OECD 423) and it meets the regulatory requirements for conducting preclinical toxicological studies among rodents.

Fig 8: Wistar rat (female)

Healthy nulliparous and non-pregnant female Wister Albino rats (*Rattus norvegicus*) from fig-7**,** with average weight of 160-180 g and age between 8–12 weeks were used for all the experiments in the present study. Before placing animals, the experimental room was decontaminated by fumigation and microbial load was checked by settle plate's method. The copies of results were kept in the raw data. The experimental room floor was mopped with disinfectant solutions daily once. The maximum of 3 animals were housed in a standard polycarbonate cages Size: L43cm X B27cm X H15cm) with stainless steel mesh top grill having facilities for holding pelleted feed and drinking water. The water was provided in polycarbonate water bottles fitted with rubber cork and a stainless-steel sipping tube. Clean sterilized corn cob was provided as bedding material. The corn cob was analysed periodically for any fungal and microbial contaminations. The animals were fed *ad libitum* with laboratory animal feed and UV sterilized drinking water was provided *ad libitum* throughout the experimental period. The rats were maintained

under standard laboratory conditions (room temperature of 22. 1 to 24.6 °C with optimal air changes 12-15 per hour and relative humidity of 48-61 %, with 12 hours light & dark cycle).

Animal care and the experimental protocol followed the principles and guidelines suggested by the "Association for Assessment and Accreditation of Laboratory Animal Care" (AAALAC-2012) [28] International accredited facility and registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA-2004) [29] guidelines, Ministry of Environment, Forests and Climate change, Government of India. Also Liveon Biolabs Private Limited ensures that animals experiments are performed in accordance with the recommendation of the guidelines for laboratory animal facility published in the gazette of India, 2018. The care and use of all experimental animals complied with relevant animal welfare laws. Before the experiment animals were allowed to acclimatize for Step I five days and Step II eight days before the experiment (each step 3 rats were used). From fig-8, during acclimatization period, each animal was identified by tail marking with permanent marker pen and cage cards and Clinical sign of individual animals were observed daily during the acclimatization period.

Fig 9: Acclimatization of rat

d. Experimental design and procedures

i) Acute oral toxicity study

This study was performed in accordance with the OECD guideline for the testing of chemicals, "acute oral toxicity study (acute toxic class method)", guideline no. 423, adopted on december 17, 2001 [30]. From fig-9, it is a single dose study for a period of 14 days. Three female wister rats were used for each step. The study was conducted in 2 steps. in step-i three female wistar rats were kept fasting for 16 hrs period of time by providing only water was given *ad libitum* prior to dosing and the dose volume calculated based on the fasted body weight of each animal and administrated in a single dose of 2000mg/kg of body weight by gavage using a 18g intubation canula. The animal administrated the maximum dose volume were administrated 10 ml/kg body weight. Test item was administrated in constant volume over the ranges of doses to be tested by varying the concentration of dose preparation. The 2000 mg of the test item polyherbal formulation was taken into the mortar and triturate using pestle. 3 ml of distilled water was slowly added in to mortar and mixed. Transfer the formulation to the measuring cylinder and make the volume up to 10 ml. the dosing solutions was freshly prepared prior to dose administration. after test item administration feed was provided at 3rd hour observation and animal were observed periodically for clinical sign of toxicity and body weight for every 30 min of the administration for first 24hrs and then daily for 14 days. No mortality was observed in all the treated animals. Hence step-ii was conducted with three naïve female wistar rats were treated with same dose of 2000 mg/kg body weight, no mortality was observed in throughout the observation period. Hence further dosing was not performed as per dosing criteria. During treatment period, each animal was identified by body marking with turmeric solution and cage cards and terminally all the animal were subjected to grass necroscopy findings.

ii) Clinical observation and body weight

For acute toxicity study all the animals were observed twice daily for mortality and morbidity throughout the experimental period. Clinical examination included any abnormal physical and behavioural changes and Animals were observed individually for toxic signs and mortality after dosing at least once during the first 30 min, 1, 2 and 4 (± 10 minutes at each point) hours and daily thereafter for a total of 15 days. Observations were include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern tremors, convulsions, salivation, diarrhoea, lethargy and others and Bodyweight of all animals was measured on day 0,8 and 15. All the animals were normal in behavior throughout the study.

iii) Pathology

At the completion of 14 days of observation period, all the animals were subjected to gross necropsy examination. Microscopic examination was performed.

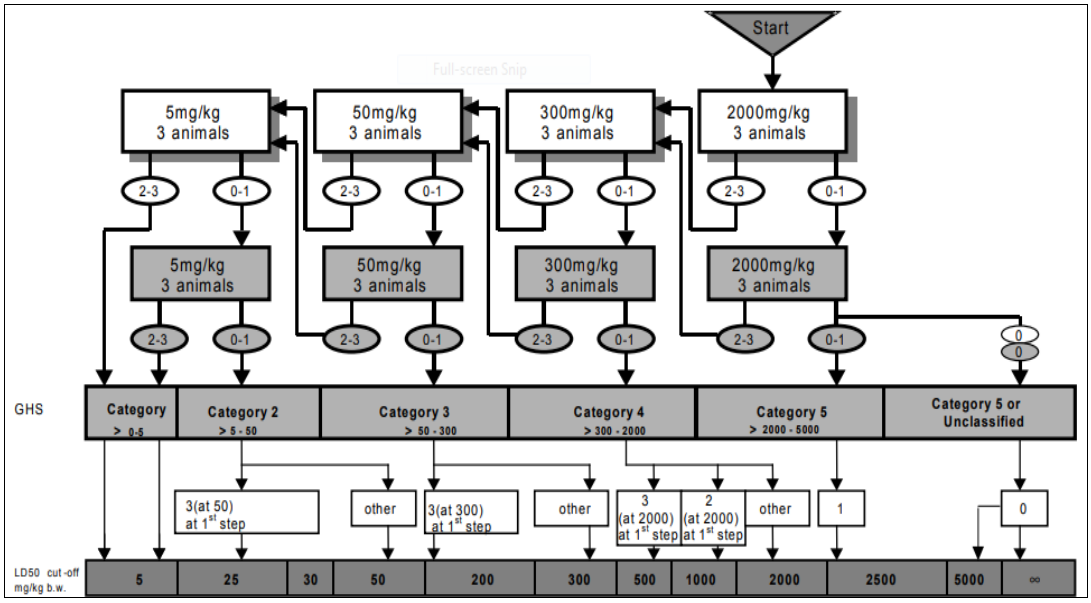

Fig 10: Test procedure with a starting dose of 2000 mg/kg body weight

Result

a. Acute oral toxicity

The Acute toxicity study of poly herbal formulation (*Rosmarinus officinals*, Ashwagandha and Amla) in female Wistar rats were dosed with 2000mg/Kg had no adverse effect on the behavioral responses of the tested rats up to 14 days of observation. Physical observations indicated no signs of changes in the skin, fur, eyes mucous membrane, behaviour patterns, tremors, salivation, and diarrhoea of the rats. There was no mortality was observed and No body weight changes or anatomical abnormalities were noted at necropsy. Therefore, poly herbal extracts have a low risk of acute toxicity, and the oral lethal dosage (LD_{50}) for female rats is higher than 2,000 mg/kg of body weight. It is very crucial to evaluate acute toxicity signs in attempt to establish a lethal dose causing of 50% of animals death (LD_{50}). According to these findings, it was assumed that poly herbal formulation LD_{50} dose is above 2000 mg/kg.

b. Body weight

There were no treatment related changes in body weight in any of the treated animals and showed gain on day 8 and 15, as compared to day 1 body weight. The Individual animal body weights (g) are presented in Table-1 showed the change observed before and after the administration of the poly herbal extracts.and food and water consumption thus were unaffected by administration of poly herbal extracts as a single oral dose of 2,000 mg/kg of body weight.

c. Clinical signs

In Step I (2000 mg/kg body weight), no clinical signs of toxicity was observed in any of the treated animals during Day 1 at 30 min, 1st hour, 2nd hour, 3rd hour and 4th hour after treatment and throughout experimental period of 15 days. In Step I (2000 mg/kg body weight), no clinical signs of toxicity was observed in any of the treated animals during Day 1 at 30 min, 1st hour, 2nd hour, 3rd hour and 4th hour after treatment and throughout experimental period of 15 days. The detail of clinical signs were observed.

d. Gross pathology

All animals were sacrificed at the end of the study and they did not reveal any abnormality of pathological significance. External examination of terminally sacrificed rats also did not reveal any abnormality of pathological significance. On the day of necropsy (Day 15), no external and internal macroscopical lesions were observed in gross pathological examination. The detail of individual gross pathological findings were observed. (Based on these findings of the acute oral toxicity (Acute Toxic Class Method) of the poly herbal formulation in Wistar rats, the LD_{50} of the extract may be classified as GHS (Globally Harmonized System) category 5 (LD_{50} >2000 mg/kg bw) as per OECD Guideline No. 423, December 2001) [30].

Table 1: Individual animal body weight (g)

Step	Dose (mg/kg body weight)	Sex	Animal No.	Volume Administered (ml)	Time of dosing	Body weight (gram), Days			Body weight gain (%)	
						Day 1	Day 8	Day 15	Day 1-8	Day 1-15
I	2000	F	1	1.7	10:20 to 10:25	171.39	187.32	203.05	9.29	18.47
			2	1.8		189.73	200.65	208.87	5.76	10.09
			3	1.9		185.99	202.18	218.7	8.70	17.59
			Mean			179.06	195.38	182.37	196.72	210.21
			SD			7.37	9.06	9.69	8.17	7.91
II	2000	F	4	1.8	10:18 to 10:23	179.9	197.58	213.91	9.83	18.90
			5	1.8		183.06	200.11	215.32	9.31	17.62
			6	1.9		187.89	204.15	222.44	8.65	18.39
			Mean			183.62	200.61	183.62	200.61	217.22
			SD			4.02	3.31	4.02	3.31	4.57

Key: F: Female, SD – Standard deviation

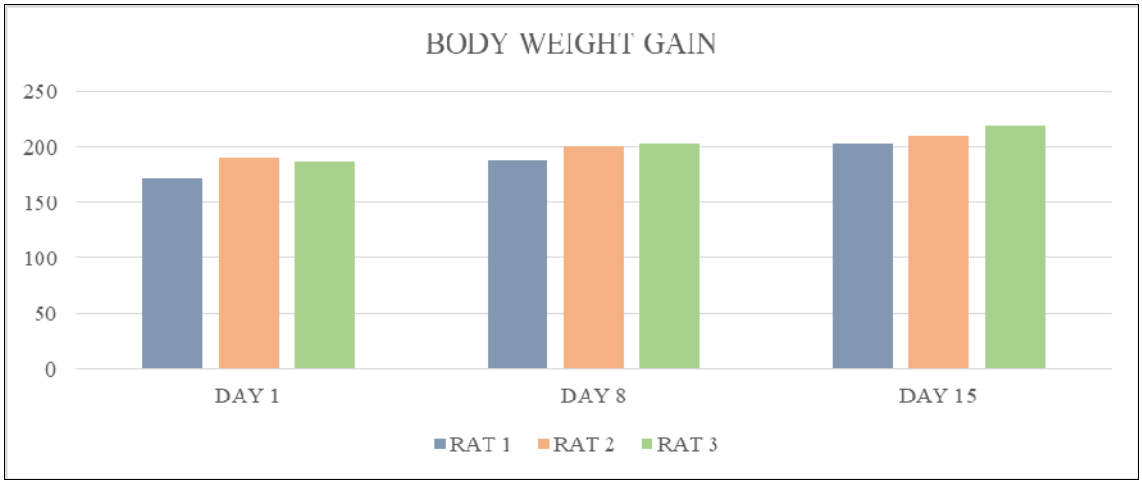

Fig 11: Step-I: The graph showing body weight in grams v/s experimental period in days

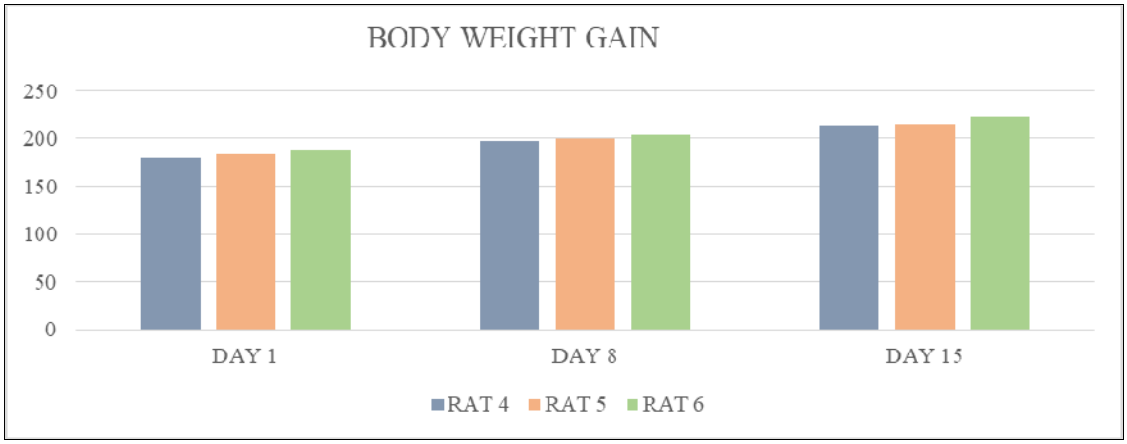

Fig 12: Step-II: The graph showing body weight in grams v/s experimental period in days

Discussion

In order to assess the acute toxicity of polyhebal formulation (rosemerry, ashwaghandha and amla) in wistar rat, we applied OECD Guideline 423, which comprises a single dose and a 14-day observation period (OECD Guideline 423, 2001) [30]. Our findings indicate that polyhebal formulation was not acutely toxic to female wistar rats according to the Globally Harmonized System (GHS) for the classification of chemicals (OECD Guideline 423), with an LD_{50} cut-off value of 2000 mg/kg b.w. Medicinal plants are been used since centuries to treat different ailments. Hence, this project was designed to check the toxic effect of the plant by oral route using 425 toxicity guidelines. Toxicological assessments in experimental animals usually were categorized into four classes: acute, subacute, subchronic and chronic. Acute toxicity test is defined as a single exposure for less than 24 h, subacute toxicity refers to repeated exposures for 1 month or less, subchronic toxicity refers to repeated exposures for 1 to 3 months and chronic toxicity assay refers to repeated exposures for more than 3 months. 24 The oral route of drug administration is the most convenient and commonly used method for toxicity evaluations in pre-clinical animal models. Acute oral toxicity test is conducted at a limit dose (2000mg/kg) primarily in situations where the experimenter has information indicating that the test material is likely to be non-toxic (OECD, 1998) [31]. The acute study was hence, conducted at the limit dose of 2000mg/kg and was found to be well tolerated. This maximum tolerated dose was taken as the highest dose for the sub-acute toxicity study. Two fold decreases were made from the highest dose to arrive the medium and low doses of the study, i.e. 1000 and 500 mg/kg body weight, respectively (OECD, 1995) [32]. Many official guidelines have been developed by various regulatory agencies for toxicity testing. These guidelines relate to the use of botanical products as medicinal preparations and provide standard methods for toxicological studies to assess the safety of medicinal products. Not all tests are necessarily performed for each herbal product and the need for each toxicity test should be evaluated depending on the availability of published literature related to safety, efficacy, established and traditional claims and intended uses [33]. 5 status (LD50> 5000mg/kg), which was the lowest toxicity class. According to the study by Kennedy *et al*. Substances with LD_{50} values higher than 5000mg/kg by oral route are regarded as being safe or practically nontoxic. However, many medicinal plants have also been reported to be toxic to both humans and animals. Therefore, it should be emphasized that the traditional use of any plant for medicinal purposes, by no means, guarantees the safety of such plant.

Toxicity studies are considered necessary, especially on drugs that are to be used in chronic conditions. About 80% of the world's people depend largely on traditional plant-derived drugs for their primary health care. Plant-derived drugs have an important place in both traditional and modern medicine. For this reason, a special effort to maintain the great diversity of plant species would undoubtedly help to alleviate human

suffering in the long term. Medicinal plants constitute the base of health care systems in many societies. The recovery of the knowledge and practices associated with these plant resources are part of an important strategy linked to the conservation of biodiversity, discovery of new medicines, and the bettering of the quality of life of poor rural communities [34]. Plant origin drugs are known to play a vital role in the management various chronic diseases and have received a great preference by the researcher as an alternative source for allopathic pharmaceutical drugs in recent times. Botanical medicines have become popular as alternative remedies as they are believed to be efficacious and have over a thousand years' experience in treating patients. Medicinal plants behave as authentic medicines because the chemical substances of which they are formed can have a biological activity in humans. Determination of efficacy and safety of herbal remedies is necessary because many people using these agents as self-medication by [35]. Although there is a limited data available about the pharmacology and toxicology for the most commonly used herbal remedies by [36]. Therefore, efforts to elucidate health benefits and risks of herbal medicines should be intensified. Current study was design to assess safety and efficacy of herbal medicine. The result of assess toxicity after oral administration reveals the LD_{50} value greater than 2000mg/kg. These findings suggest that an herbal combination is comparatively safe and does not possess acute untoward effects.

The drug under investigation herbal combination has been used for several years of its bio-stimulating, revitalizing and fertility enhancing effects by [37]. There is, in recent times, a growing and increasing interest in herbal medicines. Consequently herbal medicines have received greater attention as an alternative to clinical therapy leading to increasing demand by [38]. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease. Even when efficient, the toxicity of the used preparations is usually unknown, and the population does not care, believing that if the preparation has been used so far, it should be devoid of toxicity by [39]. However, animal toxicity testing have shown many plants currently used, to name a few Momordica charantia by [40-44], as highly toxic when given either acutely or sub-chronically. The concept of polyherbalism has been highlighted in Sharangdhar *Samhita*, an Ayurvedic literature dating back to 1300 AD. From the long-term traditional folk use and clinical applications, it is fairly understood that most herbal plants or products derived from them have an excellent safety record [45-47].

The diverse therapeutic properties of *R. officinalis* has incremented its use by the population by [48, 49], however the presence of chemical compounds, such as fl avonoids, that have been reported to have toxic by [50-52] and antifertility activities by [53, 54] as well as the anti-implantation effect reported on female rats by [55] suggests a potential reproductive toxic role of this plant. Although rosemary extract did not produce acute toxicity, however, the European Commission has approved the internal use of rosemary leaf for dyspeptic complaints and the external use as supportive therapy for rheumatic diseases and circulatory problems. Because of its anti-inflammatory and antioxidant properties, rosemary is used to treat localized injuries. *Withania somnifera* is a vital medicinal plant traditionally utilized in the treatment of numerous diseases. Owing to the presence of some toxic alkaloids in the root powder and crude extracts, safety of the commercially available extracts has been raised. In few cases, it has been reported that ingestion of ashwagandha might cause stomach upset, diarrhea, and vomiting. The alkaloid rich part of commercial ashwagandha extract was found to be toxic in a zebra fish embryo acute toxicity test as per OECD guideline 236 [56] whereas detoxified ashwagandha extract (rich in glycowithanolides and containing very less alkaloids) was found to be non-toxic under the conditions of the study (under publication). *Emblica officinalis* prevents the retrain stress-induced oxidative stress and balancing the anti-oxidant system, since *Emblica officinalis* is reported as a rich source of Vitamin C [57]. In an acute oral toxicity study, it was observed that the lethal oral toxicity of the PHF was estimated to be higher than 2000 mg/kg, classified as category 5 according to OECD Guide 423, indicating a certain safety margin associated with the use of PHF as therapeutic agents [2]. The median acute toxicity value (LD_{50}) of the extract must be above 10ml /Kg body weight. According to [58, 59] the extract can be classified as non-toxic, since the LD_{50} was found to be more than 15.0 g/Kg.

It suggested that the normal processing of all the nutrients like carbohydrate, proteins and fats are been metabolised appropriately within the body as these are the nutrients that play a major role in physiological function. Postmortem toxicology of treated animals is customarily recommended in the adopted guidelines for acute toxicity testing by [60]. The gross pathological finding for each animal is genuinely considered as potential source of information on the target organ/system and the toxic nature of the chosen test substance. Necropsy examination conducted at the termination of 14 days observation study on individual animal did not show any significant treatment related macroscopic changes of organs or other structures. Brain, Liver, Kidney and heart are the major vital organs of the body that are been not affected by the toxic substance. When animals were sacrificed at the end of study, there were no lesions found on histological examination of Brain, heart, kidney and liver in comparison with vehicle control group. According to [61], substances with LD_{50} of 2000 mg/kg body weight (oral route) are regarded as being safe or of low toxicity.

The results of acute toxicity assay showed that "Polyherbal Formulation (*Rosmarinus officinals*, Ashwagandha and Amla)" at dose of 2000 mg/kg did not cause death and behavioural changes in the animals. Therefore, it can be concluded that according to OECD guidance, the poly herbal extract may be assigned to be the lowest toxicity class 5 (LD_{50}> 2000 mg/kg). In conclusion, acute oral toxicity testing of screened poly herbal products did not produce mortality, toxicity signs or any significant pathological changes on the dose level of 2000 mg/kg body weight and an overall normal body weight gain was observed in all the treated female rats and hence resulted in tested products being labelled unclassified in the hazard category according to Globally Harmonized System.

Conclusion

Polyherbal formulation treating for various disease bring attention to the human society. The toxicity and its safe dosage should be studied for dose optimization and formulation. The aim of this study was to evaluate the oral toxic effect of the polyherbal formulation of Ashwagandha, Rosemary and Amla on female Wistar albino rats. The acute oral toxicity was evaluated as per OECD (Organization for Economic Co-operation and Development) guidelines 423.

The animals will be acclimatized for a minimum period of five days to laboratory conditions. Veterinary examination will be performed before selecting the animals and healthy and active animals will be used in the study. Acute oral toxicity was tested by single dose oral administration of the drug at dose of 2000mg/kg body weight. The animals were identified by tail marking and using cage cards. The animals were observed daily for clinical signs of abnormality/ mortality for 14 days. After 14 days, the animals were weighed before necropsy. Body weight changes will be calculated and recorded. All the animals will be sacrificed by using carbon dioxide asphyxiation and subjected to gross necropsy examination. Microscopic examinations, haematological, biological parameters of treated rats were compared with control animals. Histopathology of all the major organs was also studied. In the present oral toxicity study of polyherbal formulation, the result shows no mortality, no significant clinical signs of toxicity were observed in any of the animals at maximum recommended dose level of 2000 mg/kg body weight. No treatment related gross/histopathological lesions were observed. The haematology and biochemistry profile of treated rats was similar to control animals and difference was non-significant. The histopathology of major organs of all control animals was normal. In this study the NOAEL (No Observed Adverse Effect Level) was calculated as 1000 mg/kg b.w. for rats.

The present study clearly indicates that polyherbal formulation of Ashwagandha, Rosemary and Amla does not have any significant toxic effects in animals at the dose evaluated as oral acute toxicity studies in female Wistar rats. The present study concludes that the oral toxicity results of polyherbal formulation of Ashwagandha, Rosemary and Amla and their individual components emphasized to consider it and no toxic effect to the rats. This study also validated the traditional use of natural remedies as indigenous plants origin for the treatment of diseases. The active constituents present in the Ashwagandha, Rosemary and Amla improves their antioxidant capacity and anti-inflammatory property. So, the present study demonstrated that the polyherbal formulation of Ashwagandha, Rosemary and Amla to be safe without significant toxicity, as it neither caused mortality nor produced any significant haematological, biochemical, gross and histopathological changes, in female Wistar rats. Since, the oral dose of 2000mg/kg bw of polyherbal formulation was the highest dose (limit dose) in an acute oral studies and that it did not cause any adverse effects, it is concluded as the no observed-adverse-effect level for female Wistar rats under the experimental conditions used. Further, studies have to be undertaken to assess the other aspects of toxicities like long term toxicity (90 days/1 year), reproductive toxicity, teratogenicity and carcinogenicity.

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