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Evaluation of acute oral toxicity of the Camellia sinensis phytosome formulation in female wistar rats

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***Abstract*---**Acute toxicity study was carried out for evaluation of toxic effect of formulation, safe acute dose for human and potential target organ for toxicity. Tea (*Camellia sinensis*), a cultivated evergreen plant and belonging to *Theaceae* family. The goal of present investigation was to perform acute toxicity study of *Camellia sinensis* phytosome formulation (CSP) for estimation of LD 50 or Median fatal dose and safe acute dose for human. A total of 12 female wistar rats weighing 90 to 100 gm were used in this investigation and divided into four groups, each with three animals, in accordance with 423 OECD guidelines. Group 1, 2, 3 and 4 animals were orally administered with 300 mg/kg bw of CSP, 300 mg/kg bw of CSP, 2000 mg/kg bw of CSP and 2000 mg/kg bw of CSP respectively. All the animals were observed for clinical sign, symptoms, body weight changes and mortality for 14 days. There was no death in any of the animals, and there were no significant variations in clinical signs or body weight in the experimental group. There were no abnormal alterations found during a gross necropsy. Oral administration of *Camellia sinensis* phytosome (CSP) formulation at a dose of 2000 mg/kg body weight was shown to be safe and non-toxic, according to the findings. The LD50 cut-off value was set at 5000 mg/kg body weight.

***Keywords*---**Camellia sinensis, phytosome formulation, 423 OECD guidelines, acute toxicity.

Introduction

There are many different types of toxicity studies carried out for evaluation of toxic effects of therapeutic agents or potential toxicants that could pose threat to the lives of humans and animals. The three categories of toxicological studies based on the length of time that animals are exposed to substances are acute, subacute, and chronic toxicological investigations. The Organization for Economic Cooperation and Development (OECD) defines acute toxicity as an effect that occurs within a short time following oral administration of a single dose of a chemical or multiple doses given within a 24-hour period [1]. Acute toxicity studies are carried out to determine the short time toxicity effect of a toxicant (1 s to 2 weeks). Acute toxicity studies provide information on:

- The potential for acute toxicity in humans
- An estimate of safe acute doses for humans
- The potential target organs of toxicity
- Time course of drug-induced clinical observations
- The appropriate dosage for multiple-dose toxicity studies
- Species differences in toxicity
- Calculates the LD50, or median fatal dose
- Gross behaviour

Subacute testing involves giving animals (typically rats and dogs) daily doses that steadily increase every two to three days until hazardous symptoms develop. Subacute toxicity study is carried out to know the relative long term effect of a toxicant (4 weeks – 6 months). Subacute is sometimes called subchronic, sub means under i.e. considered under acute or below chronic (Saganuwan, 2012). This test is conducted for a period of 90 days (3 months). In this type of study, subchronicity factor gives an indication of the cumulative effects of poisons. It is the ratio of acute LD50 to 90 days LD50. A compound though may have low acute toxicity, but it has the tendency to accumulate in the body tissues and can cause subacute or chronic toxicity. Such toxicants are termed as cumulative poisons, e.g. DDT, lead, fluorides etc. [2]. A satellite group may be included in the study protocol, and this group has both a control group and a high dose group [3]. Tissues are collected for gross pathology and histopathology.

Chronic toxicity is a long term toxicity study that last as long as the life-span of the test animals usually 1–2 years. Chronic toxicity studies are carried out to know the long term effect of a toxicant (1–1½ years). Rodents such as mice and rats are usually used. Chronicity factor gives an indication of the cumulative effects of poisons. It is the ratio of acute LD50 to 1–2 years LD50. This type of test can be conducted on drugs developed for terminal diseases such as cancers, AIDs etc. (Saganuwan, 2012). A satellite group may be included in the study protocol. This group has both a control group and a high-dose group. The animals are observed for normal and abnormal body functions and biochemical parameters should be measured. Tissues are collected for gross and histopathology.

Carcinogenicity testing is under chronic toxicity testing in which both rodent and non-rodent species of animals are used. The test can be terminated after 1½ years in case of mice and hamsters and after 2 years in case of rats. Haematological analysis is performed in healthy animals after 1 and 1½ years in mice and rats respectively and the study is terminated. The animals are sacrificed for gross pathology and histopathology [2].

Tea (*Camellia sinensis*), a cultivated evergreen plant, is native to China, later spread to India and Japan, then to Europe and Russia, arriving in the New World in the late 17th century. The chemical component of tea leaves include polyphenols (catechins and flavonoides), alkaloids (caffeine, theobromine, theophylline, etc.), volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), inorganic elements (e.g., aluminium, fluorine and manganese), etc. However, the polyphenols are primarily responsible for the beneficial healthful properties of tea. The flavonoids have antioxidant, anti-inflammatory, antiallergic and anti microbial effects. Green tea contains six primary catechin compounds namely catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate (EGCG), the later being the most active component [4-6]. The polyphenols content of green tea and black tea varies from 30% to 40% and 3% to 10%, respectively. A good number of animal and clinical studies suggest that chemical constituents in tea play an important role in contributing overall human health. Green tea has various medicinal properties and used for the treatment of various types of cancers, diabetes, respiratory diseases, skin disorders, hypercholesterolemia liver diseases, improvement of cognitive function and acts as antioxidants and immunity booster [7-8].

The initial stage in establishing the dangerous nature of bioactive compounds present in plant parts extracts and formulations is acute oral toxicity [9]. In the published paper, a toxicity investigation of *Camellia sinensis* extract was reported. The goal of this study was to use an acute toxicity test to estimate the LD50, or median fatal dose, of a phytosome formulation of *Camellia sinensis* leaf extract in female wistar rats.

Material and Methods

Chemical and Reagents

The standardised hydro alcoholic *Camellia sinensis* leave extract was provided by Arjuna Remedies in Kerala. LECIVA-S70 (Phospholipid) was provided by VAV Life Sciences Pvt. Ltd, Mumbai. The chemicals and reagents that used were of outstanding analytical purity.

Preparation of phytosome formulation

The *Camellia sinensis* leaf extract loaded phytosome (CSP) optimized formulations were prepared by using solvent evaporation techniques. In 250 ml capacity round bottom flask Leciva S70 phospholipid (3 gm), *Camellia sinensis* leaf extract (1 gm) were taken and 50 ml of ethanol was added to this mixture. The round bottom containing reaction mixture was heated at 60^{o}c for 3 hrs in water bath. The reaction mixture was stirred during heating. After specified period of time

resulting solution was evaporated and stored in vacuum desicator overnight to remove any traces of solvent. The dried complex was stored at room temperature in amber colour bottle for further use.

Acute Oral Toxicity Test
Experimental animals

An acute oral toxicity research was used to estimate the median fatal oral dose (LD50) of the *Camellia sinensis* phytosome (CSP) formulation. Healthy, non pregnant and nulliparous young adult female Wistar rats weighing 90 to 100 gm were used in the experiment. The Institutional Animal Ethical Committee of Crystal Biological Solutions, Pune, gave its approval to the experimental procedure, which was given the number CRY/2122/070.

Housing and Feeding condition

Stainless steel grill tops on polypropylene cages with food facilities were used to house the chosen animals in groups. The cage was sufficiently large enough to avoid interference of animals with clear observations of each animal. Each cage was labeled using cage card for recognition [10]. This card was revealed the cage number, study code number, date, group number, mark, sex and dose level. The experimental animal room was kept at 22±3°C with a relative humidity of 55.5 ± 5 percent and a 12 hour light/12 hour dark cycle. The animals were given a diet of commercially available food pellets (supplied by Nutrivet Pvt Ltd) with RO filtered water *ad libitum* using polypropylene water bottle with stainless steel piping [11].

Animal Selection and Assignment

The wistar rats were chosen at random and placed into four groups, each with three female rats. Animals were identified by making mark (Group I: H-Head, B-Back, T-Tail.; Group II: HB- Head Back, BT- Back Tail and HT: Head Tail.; Group III: FLL- Front Left Leg, FRL- Front Right Leg and HLL- Hind Left Leg,; Group IV: RSL- Right Side Legs, LSL- Left Side Legs and W- Without) on each animal using yellow stain of picric acid for ease observation. All the animals were acclimatized to the experimental animal room condition by keeping in their respective cages for 8 days prior to dose administration [12-14].

Preparation and administration of doses

The OECD 423 recommendations were followed for conducting acute oral toxicity testing. The *Camellia sinensis* phytosome (CSP) formulation was dissolved in a 0.5 percent CMC solution and given as a suspension. Before receiving the dose, the animals were fasted overnight, but water was accessible at all times. Following the fasting period, each animal's weight was measured and recorded.

The dose administration was performed in stepwise manner and given below

Step 1: Group I containing animals was treated with the CSP formulation in the form of suspension made in 0.5% CMC at the dose of 300 mg/kg body weight.
Step 2: After the animals were found to be safe in step 1, again it was confirmed

by administration of CSP formulation to Group II at the same dose i.e. 300 mg/kg body weight.
Step 3: After establishing safety at 300 mg/kg body weight in step 2, group III animals were administered the CSP formulation at a higher dose of 2000 mg/kg body weight.
Step 4: After the animals were found to be safe in step 3, it was again confirmed by administration of same dose i.e. 2000 mg/ kg body weight to group IV animals. CSP formulation was administered orally using oral feeding needle of size 16. Animals were treated as per the above steps. The all group animals were withheld for food for a further 3 hours after respective treatment. Throughout the trial, water was available at all times. For the first four hours and thereafter for a total of fourteen days, the animals were continuously monitored. [13, 15-16].

Observations
Clinical Signs and Symptoms

For the first 30, 60, 120, 180, and 240 minutes after dosing, all animals were observed individually for mortality and clinical signs, which included changes in respiratory, circulatory, autonomic and central nervous systems, skin, fur, eyes and mucous membranes, somatomotor activity, and behavioural pattern, then once daily for the next 14 days. Salivation, lethargy, tremors, convulsions, diarrhoea, sleep, and coma were all observed with great interest. Food and water consumption were also tracked [13, 17].

Body Weight

On test day zero (before to the administration of the CSP formulation, i.e. during the fasting period), as well as days 7 and 14, (post treatment or at death) each animal's body weight was recorded [18].

Necropsy and Pathology

Gross necropsy was performed on all of the animals. In gross necropsy the animals were observed at all the body openings, opened up and observed it with naked eye for any alterations in normal body organs. External orifices, cerebral cavity, thoracic cavity, abdominal cavity, and external surfaces of each animal were examined. At this point major organs like liver, lungs, ovaries, kidneys, adrenal gland, spleen, pancreas, heart, brain etc. were observed [19].

Result and Discussion

Mortality

The CSP formulation was tested for toxicity using twelve female rats at dose levels of 300 mg/kg and 2000 mg/kg body weight. There was no mortality in any of the treatment groups. Table 1 shows a summary of the mortality results. Experimental animals from each group were observed after dosing with special attentions upto 4 hours and daily thereafter for a total of 14 days.

Table 1. Summary of Mortality Result

Step	Group	Dose	Number of Treated rat	Terminally sacrificed	Found dead
1	I	300 mg/ kg bw (CSP formulation)	3	3	0
2	II	300 mg/ kg bw (CSP formulation)	3	3	0
3	III	2000 mg/ kg bw (CSP formulation)	3	3	0
4	IV	2000 mg/ kg bw (CSP formulation)	3	3	0
Total			12	12	0

Clinical sign and symptoms

Table 2 shows observations of clinical signs and symptoms in wistar rat females treated with doses of 300 mg/kg and 2000 mg/kg body weight (bw). The animal from each group were not revealed any clinical signs of toxicity immediately after 30 minutes of dosing then in first 4 hours observations as well as upto 14 days in daily observations at dose of 300 mg/kg and 2000 mg/kg body weight. Parameters such as alertness, eyes, touch response, lacrimation, tremor, convulsion, salivation, diarrhoea, lethargy, sedation, skin & fur colour, mucous membrane, etching, comma, gripping, urination, hematuria, moribund state, respiration, heart rate and grooming were observed and appeared normal without clinical sign of intoxication in each animal from four experimental groups at two different doses. There was no hazardous or deadly effect in any of the animal groupings [20].

Table 2. Observations of clinical sign and symptoms of wistar rat treated at dose 300 mg/kg & 2000 mg/kg bw

Observation parameters	30 minutes				4 hours				14 days			
	Group				Group				Group			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Alertness	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Touch response	N	N	N	N	N	N	N	N	N	N	N	N
Lacrimation	N	N	N	N	N	N	N	N	N	N	N	N
Tremor	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Convulsion	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhoea	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Lethargy	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Sedation	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Skin & Fur colour	N	N	N	N	N	N	N	N	N	N	N	N
Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N

Itching	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Comma	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Gripping	N	N	N	N	N	N	N	N	N	N	N	N
Urination colour	N	N	N	N	N	N	N	N	N	N	N	N
Haematuria	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Moribund state	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Respiration	N	N	N	N	N	N	N	N	N	N	N	N
Heart Rate	N	N	N	N	N	N	N	N	N	N	N	N
Grooming	N	N	N	N	N	N	N	N	N	N	N	N

N: Normal and NO: Not observed

Body weight

Table 3 shows the effects of CSP formulation on female wistar rat body weight in an acute toxicity investigation. The body weight of experimental animals in the treatment groups was measured during the fasting period, i.e. Day 0, as well as on Days 7 and 14 following formulation administration. No significant variations in weight gain were found in the experimental animals' body weight. The body weights of experimental animals were increased normally and progressively during the 14 days [19-20]

Table 3. Effect of CSP formulation on female wistar rat body weight

Group and Dose	Animal Identification Marking	Body weight in gram		
		Day 0	Day 7	Day 14
Group I- 300 mg/kg bw (CSP formulation)	H	92.7	98.6	104.6
	B	94.5	100.5	105.5
	T	96.4	101.9	107.9
	Mean	94.53	100.3	106.0
	SD	1.850	1.656	1.706
Group II- 300 mg/kg bw (CSP formulation)	HB	95.6	102.6	107.6
	BT	97.2	105.8	108.8
	HT	99.5	103.2	111.2
	Mean	97.43	103.9	109.2
	SD	1.960	1.701	1.833
Group III- 2000 mg/kg bw (CSP formulation)	FLL	94.5	101.5	107.5
	FRL	97.9	100.9	106.9
	HLL	96.5	103.5	109.8
	Mean	96.30	102.00	108.1
	SD	1.709	1.361	1.531
Group IV- 2000 mg/kg bw (CSP formulation)	RSL	93.5	100.5	106.9
	LSL	95.5	100.2	105.7
	W	96.5	103.1	108.9
	Mean	95.17	101.3	107.2
	SD	1.528	1.595	1.617

Gross necropsy and pathology

Experimental animals were subjected to gross necropsy and checked for animal organs. There was not observed any gross pathological alternations in any of the experimental female rat organs [21,22]. Result of gross necropsy and list of observation during necropsy are shown in table 4 and 5.

Table 4. Gross Necropsy

Group	Animal Mark	Dose mg/kg	Fate TS/FD	Gross observation
I	H	Group I- 300 mg/kg bw (CSP formulation)	TS	NAD
	B		TS	NAD
	T		TS	NAD
II	HB	Group II- 300 mg/kg bw (CSP formulation)	TS	NAD
	BT		TS	NAD
	HT		TS	NAD
III	FLL	Group III- 2000 mg/kg bw (CSP formulation)	TS	NAD
	FRL		TS	NAD
	HLL		TS	NAD
IV	RSL	Group IV- 2000 mg/kg bw (CSP formulation)	TS	NAD
	LSL		TS	NAD
	W		TS	NAD

TS: Terminally sacrificed and NAD: No abnormalities detected

Table 5. List of Observations during Necropsy

Particulars of region	Organs observed
External Orifices	Nose, Mouth, Ears, Eyes, Urethra, Vaginal opening and Anus.
Cranial Cavity	Brain, Eyes
Thoracic Cavity	Oesophagus, Trachea, Pharynx, Salivary glands, Lungs, Heart and Thymus
Abdominal Cavity	Pancreas, Liver, Spleen, Stomach, Duodenum, Ileum, Caecum, Colon, Rectum, Peritoneum, Kidneys, Adrenals, Ovaries, Uterus, Urinary bladder.
External surfaces	Fur, Tail and Paws

GHS classification based upon LD50

According to the "Globally Harmonized System (GHS)" for classification of chemicals which cause acute toxicity, following categories for chemicals under test.

LD50	**GHS Category**
>0-5	Category 1
>5-50	Category 2
>50-300	Category 3

>300-2000	Category 4
>2000-5000	Category 5

Conclusion

OECD 423 guidelines were used to test the acute toxicity of the optimized *Camellia sinensis* phytosome formulation. The animals were evaluated for mortality during the acute toxicity research, and no deaths were observed. No significant variations in body weight or clinical signs were found in the experimental animals during observation. There were no abnormal alterations in the studied organs in the necropsy investigation. The formulation's LD50 value was determined to be in the GHS category under laboratory settings, ranging from 2000 to 5000 mg/kg body weight, with an LD50 cut off value of 5000 mg/kg, and it was declared to be harmless for use. As a result, it may be stated that at a greater dose of 2000 mg/kg body weight, the prepared *Camellia sinensis* phytosome formulation was proven to be safe and nontoxic. A more complete examination of this phytosome formulation's subacute and chronic toxicity is still needed to back up these findings.

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Potential for conflict of interest

The authors claim that there are no conflicts of interest in this work.

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