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Acute and Sub-Chronic Oral Toxicity Studies of *Hibiscus esculentus* Mucilage on Swiss Albino Mice

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Abstract:

Objective: The objective of present study was carried out to evaluate the safety of novel biomaterial mucilage obtained from the hibiscus species plants of the Malvaceae family in Swiss albino mice.

Materials and Methods: The pod of *Hibiscus Esculentus* powder was subjected to cold/ hot maceration by water followed by precipitation with acetone to get mucilage. *In-vivo* acute toxicity effect of the mucilage was assessed by monitoring the survival, behavioral changes, effect on hematological parameters and cell count. The studies included the gross observation such as changes in behavior, body weight and food intake.

Results: The mice treated with *Hibiscus Esculentus* mucilage with dose of 5000 mg/kg body weight were safe. The dose of *Hibiscus Esculentus* was reduced to 500 mg/kg and observed for consecutive 30 days. The changes in body weight and haematological parameters were statistically insignificant compared to that of control group of mice.

Conclusion:From the result it can be found that isolated mucoadhesive *Hibiscus Esculentus* mucilage showed insignificant ($p<0.001$) in-vivo toxic effect when compared to the control group. It was proved to be safe regarded as (GRAS) category natural polymers and free from toxic or adverse effects for use in pharmaceutical products.

Keywords: Acute toxicity, *Hibiscus Esculentus* mucilage, swiss albino female mice

INTRODUCTION

The traditional use of excipients in drug formulations was to act as inert vehicles to provided necessary weight, consistency and volume for the correct administration of the active ingredient, but in modern pharmaceutical dosage forms they often fulfil multi-functional roles such as modifying release, improvement of the stability and bioavailability of the active ingredient, enhancement of patient acceptability and ensure ease of manufacture. In India, natural gums and mucilage are well known for their medicinal use. Natural gums can also be modified to meet the requirements of drug delivery systems and thus can compete with the synthetic excipients available in the market [1]. But the substance from plant origin also pose several potential challenges such as being synthesized in small quantities and in mixtures that are structurally complex, which may differ according to the location of the plants as well as other variables such as the season. This may result in a slow and expensive isolation and purification process [2]. Hibiscus plants of the malvaceae family are available in India in large quantities, and the plants contain mucilage. *Hibiscus Esculentus* is a genus of about 15 species of flowering plants and an annual or perennial climber, growing up to 2 m tall. The fruit is a capsule up to 18 cm long. It was formerly included within the hibiscus but now classified as a distinct genus. The stem leaves and pods of this plant have mucilage.

In order to establish the safety and efficiency of a new drug or excipient, toxicological studies are very essential in animals like mice, rat, guinea pig, dog, rabbit, monkey etc under various conditions of substance. Toxicological studies help to make decision whether a new drug should be adopted for clinical use or not. Depending on the duration of drug exposure to animal toxicological studies may be three types such acute, sub-acute and chronic toxicological studies. In acute toxicity studies, single dose of drug given in large quantity to determine immediate toxic effect. Acute toxicity studies are commonly used to determine LD_{50} of drug or chemicals and natural products. In sub-acute toxicity studies, repeated doses of drug are given in sub-lethal quantity for a period of 15 to 20 days. Sub acute toxicity studies are used to determine effect of drug on biochemical parameters of tissues [3]. In chronic toxicity studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug. The aim of this study is an investigate toxicity of *Hibiscus Esculentus* (HEC) mucilage to design to bioadhesive system.

MATERIALS AND METHODS

Collection and authentication of plant material

Hibiscus Esculantus unripe fruits were purchased from the local market, Amravati district Maharashtra, in the month of September 2012. Authentication of plant was carried out by Dr. N. M. Dongarwar, Assistant Professor, Department of Botany; RTM Nagpur University Campus, Amravati Road, Nagpur. All excipients were of USP grades and all other chemicals used were of analytical grades.

Extraction and purification of biomaterial mucilage

The pieces of fruits were dried in oven dryer maintained between 40°C to 50°C for 72 h to obtain dry mass which were milled to a coarse powder using multi mill motor driven milling machine. The mucilage was obtained by the optimum extraction methods described by shende et.al 2015 [4]. For the extraction of the mucilage, an immature pods powder, homogenized with water in the ratio 1:7 and allow soaking in water for 6 hrs at 40 ± 2^0C, boiled for 30 min, and kept aside for 1 h for complete release of the mucilage into water. The material was squeezed from an eight-fold muslin cloth bag to remove the marc from the solution. The acetone in 4:1 ratio of that mucilaginous mixture was added to the filtrate to precipitate the

mucilage. The crude mucilages were homogenized with cold dilute trichloro acetic acid solution (5 % v/v). The solutions were centrifuged (2000 rpm for 20 min), neutralized with sodium hydroxide by drop wise addition and then dialyzed for 30 hrs against distilled water. The mucilage was precipitated with acetone, filtered and dried in an oven at a temperature < 50°C, collected, dried-powdered, passed through a sieve #80 and stored for further use in desiccators.

Experimental Animals

The experiment was carried out on healthy, young, nulliporous, non-pregnant albino female mice weighing between 18 to 36 gm (5 weeks old). The experimental protocol has been approved by institutional animal ethics committee. The acute toxicity of dried mucilage of hibiscus Linn was determined in overnight-fasted swiss albino mice by following fixed dose method as per OECD guideline no. 425. The study was undertaken with due approval of the study protocol by the Institutional Animal Ethical Committee (IAEC) and the approval number was 1370/ac/10/CPCSEA/IAEC/04 dated 17/01/2015.

Acute toxicity study [5]

The animals were acclimatized to laboratory conditions for one week prior to experiment. Swiss albino female mice were divided into four different groups comprising of three animals each. Animals were kept fasting for the overnight (>15hrs) before starting the experiment. The control group received normal saline 20 ml/kg (i.p) and the other groups received 500, 1000 and 5000 mg/kg of mucilage suspension in normal saline orally with consideration of body weight. The calculated dose was administered with the help of oro-gastric tube. Animals were observed individually for acute toxicity signs and behavioural changes 4 h post-dosing, and at least once daily for 14 days. The sign of toxicity and mortality were observed over two weeks. The toxicological effect were assessed on the basis of mortality for 14 days, which is expressed by the median lethal dose value (LD_{50}) estimated from the regression of log-probit mortality rate.

Sub-chronic toxicity study [6]

For sub-chronic toxicity test, twelve animals were used and divided into two groups 4 as control and 8 as test animals. Repeat-dose oral toxicity study was carried out according to OECD guideline. The first control group receives normal saline (5 ml/kg body weight) and the test group a dose of 500 mg/kg was administered orally to every 24 h for four weeks. The toxic effect such as body weight, mortality and food and water intake was observed. Twenty four hours after the last dose (i.e., at the 29^{th} day), blood was collected from overnight fasted mice of each group by cardiac puncture for estimation of hematological and serum biochemical parameters. Haemoglobin, haematocrit, red blood cell count, white blood cell count, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and platelet count were determined using an automatic counter. Biochemical analysis was performed on serum obtained after centrifugation of total blood (without anticoagulant) at 2500 rpm for 15 min. After blood collection animals were humanely killed and removed internal organs, then weighed to determine the relative organ weights and observed for gross lesions. Removed internal organs were preserved in 10% buffered formaldehyde solution for histological examination.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey test to evaluate significant differences between groups. Values of $p < 0.05$ were considered significant. All statistical analyses were carried out using Graph Pad Software, Inc., USA.

RESULTS AND DISCUSSION

The isolation of mucoadhesive substances from the natural edible sources was carried out by cold and hot aqueous extraction process followed by the organic solvent precipitation. The selection of the process was based on previous literature giving utmost importance to preserve the components against thermal, enzymatic and hydrolytic degradation. The optimized condition 1:4 leaves or pad/water ratio for time 6 hrs at 40^{o}C reported as earlier could be used for better extraction yield. The process used was found to be effective in selective isolation and purification of the interested constituents and the yielded components possessed good handling properties [4].

Acute toxicity

The acute toxicity of dried mucilage of *hibiscus linn* was carried out in overnight-fasted swiss albino mice by following fixed dose method as per OECD guideline no. 425. All the experimental procedures were conducted in accordance with the CPCSEA guidelines as per principles of laboratory animal care. Assessments of the behavior of animals were carried out by general observations of each animal on a daily basis from the stage of dosing to the end of the study. Cage-side observations included daily recording of condition for damaged areas of skin; subcutaneous swellings or lumps, areas of tenderness, abdominal distension, eyes-for dullness, discharges, opacities, pupil diameter, posies, the colour and consistency of the faces, wetness or soiling of the perineum, condition of teeth, breathing abnormalities, gait, etc. The results of the acute toxicity study indicate that the LD50 of the extract of *Hibiscus Esculentus* mucilage is more than 5000 mg/kg. The test animals at all dose levels showed no significant changes in behaviour after the administration of an oral dose of hibiscus mucilage slurry (table 1). Mortality is the main criteria in assessing the acute toxicity (LD_{50}) of any drug or excipients. The limit dose of 5 g/kg did not cause mortality or any sign of acute toxicity in the three rats dosed for a short period (48 h) and long period (14 days). The limit test is primarily used in situations where the investigator has information indicating that the test material is likely to be non-toxic or of low toxicity [7]. This finding, therefore, suggests that the extract at the limit dose tested is essentially non-toxic and safe in oral formulation.

Table 1: Behavioral observations for all animals

Sr. No.	Parameters	Cage side observations
1	Condition of the fur	Normal
2	Condition of damaged areas of skin;, areas of tenderness	Normal
3	Subcutaneous swellings or lumps	Nil
4	Abdominal distension	Nil
5	Eyes - for dullness, discharges, opacities	Nil
6	Eyes – opacities	Nil
7	Pupil diameter	Normal
8	Posies (drooping of upper eyelid)	Nil
9	Colour & consistency of the faces	Normal
10	Wetness or soiling of the perineum	Nil
11	Condition of teeth	Normal
12	Breathing abnormalities	Nil
13	Gait	Normal

Changes in food intake and body weight

The suitability of mucilage for oral drug delivery was access by monitor body weight profile. Loss in body weight is frequently the first indicator of the onset of an adverse effect. A dose, which causes 10 % or more reduction in the body weight, is considered to be a toxic dose. During 14 days of observation the body weight and food intake of mice were weighed. It is considered to be the dose, which produces minimum toxic effect, irrespective of whether or not it is accompanied by any other changes. All the animals from treated groups did not show any significant decrease in body weights for all the 14 days as compared with the 0 day values, indicating no signs of toxicity. The daily body weight is shown in figure 1. Measured quantities of water and feed were supplied daily in each cage. The consumption of water and food was estimated from the amount of water remaining in feeding bottles and from the amount of feed remaining in the feed hopper. The data for food and water consumption is given in table 2. There was no significant change in food and water intake of the test animals at all dose levels.

Sub-chronic toxicity

No behavioural changes and death were observed at the end of the treatment. Similarly, no significant differences in body weight were observed between control and treated groups during this period. After 28 days of treatment, there were no treatment-related changes in haematological parameters between control and treated groups, indicating that the extract was not toxic to circulating red cells, nor interfered with their production and that of platelets. The haematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in man and animals [8]. In addition; most of the biochemical parameters were not also altered by the extract.

Table 2: Daily food and water consumption record

Period	Food intake (gm)				Water intake (ml)			
	Group I	Group II	Group III	Group IV	Group I	Group II	Group III	Group IV
Day 0	16	14	6	10	12	11	20	14
Day 1	15	14	18	11	20	11	20	14
Day 2	15	15	20	12	25	12	25	13
Day 3	5	10	14	9	22	12	15	13
Day 4	5	10	5	7	25	12	20	15
Day 5	5	7	6	10	22	13	25	15
Day 6	5	8	9	11	20	12	22	14
Day 7	10	12	12	10	18	12	23	14
Day 8	12	10	10	11	17	12	16	14
Day 9	13	11	11	10	17	11	15	14
Day 10	10	12	10	8	17	12	16	13
Day 11	10	11	12	7	18	11	16	13
Day 12	13	14	10	9	18	12	17	14
Day 13	15	14	8	10	19	11	17	13
Day 14	14	13	7	10	19	11	18	14

(All the values expressed as mean of three animals in each group)

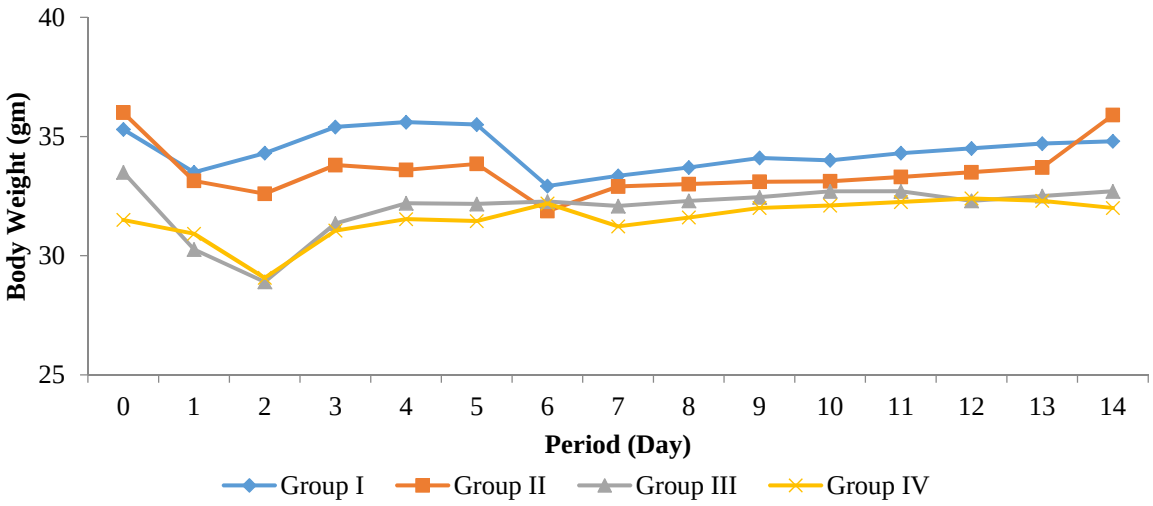

Figure 1: Mean body weight of mice after 28 days treatment with *Hibiscus Esculentus* mucilage

Changes in the biochemical parameters of different tissues

Tables 3 showed the haematological and biochemical parameters for the rats after subchronic toxicity dosing. The results shows that no significant change in blood liver tissues parameters in the animals from all dose groups (500 mg/kg body weight) which proves that the hibiscus mucilage has no significant toxic effect in mice. The histological section of vital organs viz; brain, heart, kidney and liver after treatment of HEC for 28 day for any sign of pathological changes in both control and in treatment group results that there was no sign of pathological changes in both control and in treatment group. The acute and subacute toxicity studies of extracting mucilage showed did not cause any toxic effects on animals. Hematological parameters showed no changes in the normal blood counts. Table 4 shows that the weight of all organs in the group treated with 500 mg/kg insignificantly that of the control group.

Table 3: Haematological parameters for mice after 28 days treatment with *Hibiscus Esculentus* mucilage

Sr. No.	Parameters	Control*	Test**
1	Hematocrit (%)	42±1.34	41±3.41
2	RBC ($\times 10^6$ Cells/mm^3)	2.3±0.21	2.3±0.32
3	Hemoglobin (g/dl)	12.6±0.26	12.4±0.98
4	MCV (μm^3/red cell)	66.0±1.21	64.3±1.21
5	MCH (pg/red cell)	10.3±0.21	10.1±0.48
6	MCHC (g/dl RBC)	34±0.27	34±0.24
7	WBC ($\times 10^3$ cells/mm^3)	8.9±0.26	8.9±0.25
8	Platelet ($\times 10^3$ cells/mm^3)	700±26	668±29
9	Neutrophil	35±2.81	34.2±3.54
10	Eosinophil (%)	4±0.46	3.7±0.56
11	Lymphocyte (%)	77±6.21	76±4.54
12	Monocyte (%)	3±0.35	2.8±0.72
13	Basophile (%)	0.8±0.07	0.7±0.05

Data represent as the mean ±SD of 4 animals; ** Data represents as the mean ±SD of 8 animals, *No. of rats/group indicated in parenthesis

Table 4: Mean organ weight after 28 days treatment with *Hibiscus Esculentus* mucilage

Organ	Mean weight (gm, ± SEM)	
	Control	*Hibiscus Esculentus* mucilage dose (500 mg/kg)
Heart	0.32±0.01	0.33±0.01
Liver	2.60±0.12	2.50±0.13
Spleen	0.17±0.01	0.18±0.013
Kidney	0.56±0.07	0.55±0.03

No. of mice/group indicated in parenthesis; * p <0.05, control group vs test

CONCLUSIONS

From the above findings it could be concluded that the isolated mucoadhesive mucilage exhibited safe due to no mortality and clinical signs of toxicity observed at the doses of 5000 mg/kg. Moreover, no lethality, no significant change in body weights and consumption of food and water by the animals was recorded for dose of 500 mg/kg during the 30 days of treatment. There was no single mortality recorded even at the highest dose level i.e. 2 gm / kg body weight, which proves that hibiscus mucilage has no significant toxic effect and safe regarded as category natural polymers in mice.

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