Research Article

SAFETY EVALUATION OF *ABELMOSCHUS ESCULENTUS POLYSACCHARIDE*

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ABSTRACT

To evaluate the safety of Distilled water (DW), Hot water (HW) and Hot buffer (HB) pod extract (okra polysaccharides-OKPs) of *Abelmoschus esculentus* using *in vitro* and *in vivo* toxicity studies in mice. MTT Formazan assay was used for the *in vitro* study. The effects of different dilutions of each OKPs on human cancer cell line growth were studied. In acute test, a single dose (50, 300 & 5000mg/kg bw) of OKPs was ingested to mice and were observed for 14 days. In 28 day study, the mice were ingested chronically with 200, 500&1000mg/kg bw of OKPs. The relative organ weights, haematological, biochemical and histopathological values of mice ingested 1000mg/kg bw were estimated**.** The mice of high dose recovery group were observed on 0^{th} (initial) and 14^{th} day (final) after the termination of the treatment. The *in vitro* study showed the percentage viability as 94.11 to 98.71(DW), 94.8 to 99.07 (HW) and 95.12 to 99.72 (HB) on human cancer cell line. In the acute study, the OKPs produced neither mortality nor significant difference ($p < 0.05$) in neurobehaviour and body weight when compared with control group. In sub chronic study, the relative organ weights, hematological, biochemical and histopathological values showed no significant differences ($p < 0.05$) with the control group. It can be concluded that OKPs is a safe Pharmaceutical excipient based on our present preliminary study.

Keywords: *In vitro* toxicity, Acute toxicity, Sub chronic toxicity, Okra polysaccharide.

INTRODUCTION

Natural polysaccharides (excipients) are biodegradable, relatively inexpensive and are generally considered as safe pharmaceuticals. Less attention has been devoted to the safety of excipients obtained from natural source because of their inertia and innocuity. The approved excipients originating from the food industry are generally recognized as safe (GRAS)[1]. The International Pharmaceutical Excipients Council (IPEC), an industry association which published safety evaluation guidance for the excipients[2]. More recently, FDA have issued guidance on nonclinical studies needed to develop excipients with the key message that excipients are potential toxicants and need to be evaluated accordingly. Cytotoxicity tests are more relevant due to the background of current public and expert opinion that leads to a pressure for the reduction of animal experimentation whenever that is possible. The mucilage used for this study was extracted from the plant Okra (*Abelmoschus esculentus*) which differs widely in the molar ratios of sugar moieties depending on the solvent used for the extraction[3]. There are reports about the use of OKPs in the formulation of sustained release tablets[4], bio adhesive tablets[5], and colon delivery systems[6]. This paper examines whether OKPs has the potential to be a safe Pharmaceutical excipient based on our present study with the aim to include the material in future drug formulations. To evaluate the safety of the OKPs, the *in vitro* toxicity studies were carried out using MTT assay method on human cancer cell line and *in vivo* toxicity studies were carried out as per OECD guidelines on mice.

MATERIALS AND METHODS

High quality chemicals were used for the study. The chemicals used for *in vitro* studies were Eagles minimum essential medium, Fetal Bovine serum, Trypsin, EDTA, MTT and Tryphan blue purchased from Sigma. The instruments used were CO_2 Incubator, Lark Chennai; Inverted Phase contrast Microscope, Labomed, Mumbai, India. The instruments used for *in vivo* studies were Rota-rod, Actophotometer and Eddy's hot plate. The instruments used for biochemical study were Semi auto analyzer, Microlab 300, Merck, Mumbai and the kits used were from Merck Specialities Private Limited, Mumbai. Haemometer used was superior Marienfeld laboratory glassware, Germany; Hayem's fluid was purchased from Loba Chemie Pvt.Ltd., Mumbai; Turke's fluid was purchased from Nice Chemicals Pvt. Ltd., Cochin. Leishman's stain was purchased from Qualigen Fine Chemicals, Mumbai.

Collection and authentication of the plant material

The plant *Abelmoschus esculentus (L.) (=Hibiscus esculentus L.)* of family MALVACEAE was authenticated by Dr.G.V.S. Murthy, Scientist 'F' & Head of Office, Botanical Survey of India, Southern Regional centre, Coimbatore, Tamilnadu. The herbarium specimen (specimen voucher, Sep-2010-04) of the plant has been preserved at Karpagam College of Pharmacy, Coimbatore.

Collection and extraction of polysaccharide from okra ponds

Fresh immature pods were purchased from local market. The pods were sliced, homogenized and extracted with

cold distilled water (DW) or hot distilled water (HW) or hot buffer (HB) after removal of the seeds. The crude mucilage was filtered through muslin cloth and centrifuged at 5000rpm for 30 min. The obtained mucilage was precipitated with three volumes of acetone. The precipitated gum was washed several times with acetone to get a white to cream colored product. The product was dried in microwave oven at 800 micro powers. The dried gum was pulverized using mortar and pestle. The powder was passed through sieve no.80 and stored in a desiccator until used for further studies.

Assessment of *in vitro* toxicity study

The cell viability in presence of OKPs was assessed by a MTT assay[7]. The human cancer cell line was obtained from National Centre for Cell Science (NCCS), Pune. The cells were grown in Eagles minimum essential medium containing 10% fetal bovine serum (FBS). The various dilutions (0.5mg, 0.25 mg, 0.31mg, 0.62mg, 0.125mg and 1.0mg) of OKPs (DW, HW & HB) were used for the study. For screening experiment, the cells were seeded into 96-well plates in 100 µl of respective medium containing 5% FBS, at plating density of 10,000 cells/well and incubated at 37^{0}C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of compounds. The compounds were solubilized in hot water and filtered through 0.45µm syringe filter. The compound was then diluted in serum free medium at various concentrations and 100 µl was added to respective wells and incubated at 37^{0}C, 5% CO2, 95% air and 100% relative humidity for 48h. Triplicate was maintained and the medium containing without extracts were served as control.

After 48h, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{0}C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to positive control as follows

% cell viability = [A] Test / [A]control x 100

Assessment of *in vivo* toxicity study

Animals and its ethical approval

Swiss albino mice were used for this study. The welfare of the mice were maintained in accordance with the general principles governing the use of animals in experiments of OECD 423[8] & 407[9] guidelines. This study was approved by The Institutional Animal Ethics Committee (IAEC) of Karpagam University, Coimbatore, Tamilnadu, India (Approval No. Ku/IAEC/Pharm/074 & 076).

Acute toxicity study

The total number of female mice used for the studies were 30. They were divided in to 09 groups of 03 each and a control group. A single oral dose (50, 300 & 5000mg/kg bw) of OKPs (DW, HW& HB) were administered. The muscle relaxant property by Rota-rod method, the analgesic response by Eddy's hot plate method, locomotor activity by actophotometer, the body weight of the mice were measured at 0th (initial) and 14th (final) days. The mice were observed daily for abnormal signs, diarrhea, food and water intake.

Sub chronic toxicity study

The numbers of mice used for the sub chronic toxicity study were 140. The study included 09 groups of five mice per sex which were ingested a dose (200, 500 & 1000mg/kg bw) of OKPs (DW, HW& HB) from the start of the study (day 0) until scheduled sacrifice day. Another 05 groups of 05 mice per sex were used as a control, high dose recovery groups (DW, HW&HB) & control recovery group. On 29th day, after overnight fast, the mice (administered with 1000mg/kg bw) were anaesthetized with ether and blood samples for haematological and biochemical analysis (serum obtained after centrifugation of total blood, without anticoagulant) were collected in tubes with and without EDTA respectively. Necroscopy of all animals was performed and the relative organ weights (heart, liver, kidney, spleen, lungs, small intestine, large intestine and colon) were recorded. The mice in a high dose recovery groups and control recovery group were kept for follow-up observations for a further 14 days without treatment. The mice were observed daily for abnormal signs, diarrhea, food and water intake. Their body weights were evaluated on treatment termination day (initial) and 14 days after treatment termination (final).

Biochemical parameters and haematological parameters

Blood Urea Nitrogen (BUN), total proteins, cholesterol and plasma sugar values were estimated using standard diagnostic kits on semi-auto analyzer. Total R.B.C. counts were carried out as per the instruction supplied along with the Hayem's fluid using Haemocytometer. W.B.C. counts were carried out using WBC diluting fluid (Turk's fluid). The differential counts were carried out by spreading a drop of blood on a glass slide with the staining fluid and the cells were counted using oil immersion microscope.

Statistical analysis[10]

Values obtained were expressed as mean-standard deviation. The quantitative data were analysed using Dunnett's multiple comparison test and the qualitative data were analysed using Mann-Whitney's U test. For both the tests, one-sided test with $p<0.05$ was applied. In case of high dose recovery group Dunnett's test was performed (Graph Pad Prism 4).

RESULTS AND DISCUSSION

***In vitro* toxicity study on human cancer cell line**

The cell viability on exposure of cells to the OKPs were same in various concentrations (0.5mg, 0.25mg, 0.31mg, 0.062mg, 0.125mg and 1.0mg). The results obtained demonstrated that all the test dilutions of OKPs (DW, HW&HB) compared with the control showed the cell viability percentage as 94.11 to 98.71, 94.8 to 99.07 and

95.12 to 99.72 respectively on human cancer cell line growth (fig.1).

In vivo toxicity studies on mice

In the oral acute toxicity study, LD_{50} cut-off value may exceed 5000 mg/kg bw. The number of scores (the locomotor's activity) obtained in 10 minutes, of mice administered with OKPs obtained at 0^{th} day and 14^{th} day were 339.33 ± 1.15 & 341.33±0.57 (DW), 341 ± 1.00 & 340.66±0.57 (HW) and 340 ± 1.00 & 340.66±0.57 (HB) respectively. The time took by the treated and control group mice to fall off from the rotating rod were 300 seconds in all the test conducted days. The reaction time (the latency) for the mice administered with OKPs on the study conducted days (0^{th} & 14^{th}) were 7.33 ± 1.15 & 6.66 ± 0.57 (DW), 7 ± 1.00 & 7 ± 1.00 (HW) and 7 ± 1.00 & 7 ± 1.00 (HB) respectively. The initial and final body weight of mice ingested with 5000mg/kg bw showed 21.96 ± 1.2 & 24.01 ± 0.96 (DW), 22.46 ± 1.3 & 24.26 ± 1.2 (HW) and 23.22 ± 1.2 & 25.12 ± 1.1 (HB) respectively. Their food and water intake per day were 4.16 ± 0.13 (DW), 4.04 ± 0.16 (HW) & 4.18 ± 0.11 (HB) and 9.98 ± 0.61 (DW), 10.01 ± 0.41 (HW) & 9.89 ± 0.62(HB) respectively.

The 28 days daily dose of 1000mg/kg bw showed initial and final body weight as 22.16 ± 0.84 & 25.94 ± 0.98(DW), 22.02 ± 1.1 & 25.86 ± 1.2 (HW) and 23.26 ± 0.94 & 27.02 ± 0.92 (HB) respectively. Their food and water intake per day were 4.18 ± 0.13 (DW), 4.14 ± 0.14 (HW) & 4.22 ± 0.12 (HB) and 10.04 ± 0.62 (DW), 9.98 ± 0.54 (HW) & 9.86 ± 0.66 (HB) respectively. In sub chronic toxicity study the biochemical parameters and the hematological values of mice administered with high dose (1000mg/kg bw) were showed in the Table No.1 and Table No.2 respectively. The relative organ weights of mice ingested with high dose of OKPs were shown in Table No.3.

The histopathological scores (not shown) and some of the images of the mice ingested with high dose of OKPs are presented (fig. 2).

After the termination of the treatment, the high dose recovery group showed the initial and final body weights as 26.18 ± 0.98 & 28.04 ± 1.1 (DW), 26.28 ± 1.2 & 28.02 ±1.4 (HW) and 25.56 ± 1.2 & 27.88 ±1.4 (HB) respectively.

In vitro toxicity study

The human cancer cell line had no morphological changes and the cell viability was nearly 100%. Reduction of MTT by cells indicates mitochondrial activity, which may be interpreted as proof of cell viability. None of the OKPs induced cytotoxic effects at the used concentrations.

In vivo toxicity study

The mice in this study were administered a range of doses from 50, 300 and 5000 mg/kg/bw of OKPs chosen, in order to assess the possible toxic parameters. The mice did not show any statistically significant difference ($p < 0.05$) in the muscle relaxant property, the analgesic response, locomotor activity, the body weight, food and water intake when compared with the control group. The mice did not show any abnormal signs and diarrhea. The acute toxicity study of orally administered OKPs at the doses used was indeterminable due to the nonoccurrence of deaths. The absence of signs of morbidity and mortality in the oral dose study is a probable indication of the relative safety of the short-term administration of OKPs.

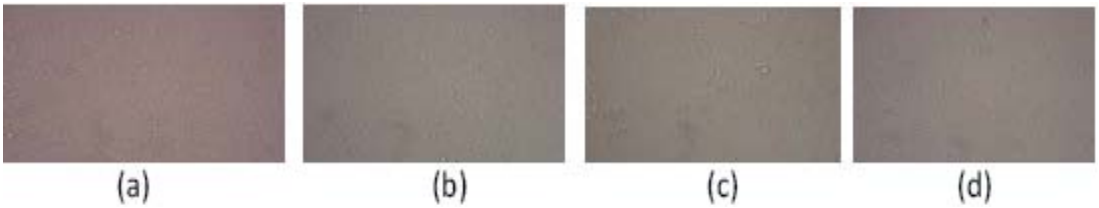

Figure 1: Human cancer cell line grown in a 96-well plate containing 1mg of OKP extracted with DW (b), HW(c) and HB (d) and the control (a) immediately before the addition of the dye MTT.

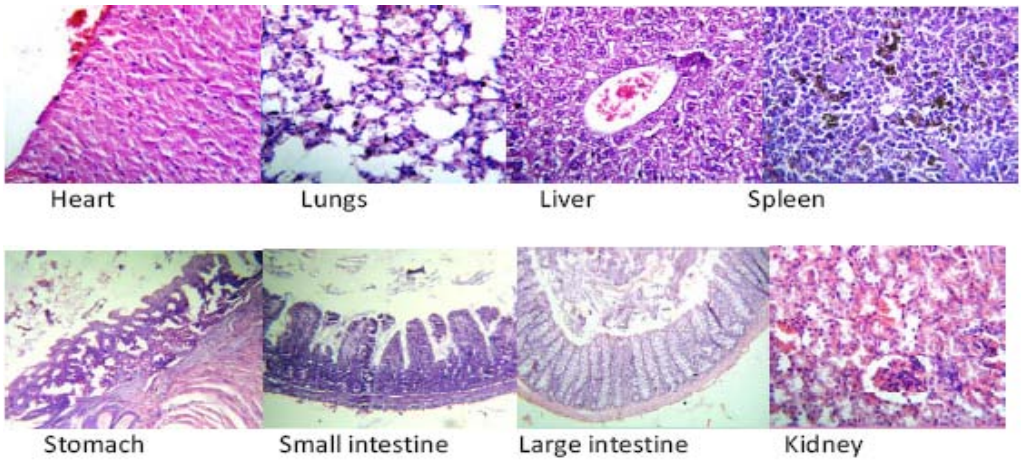

Figure 2: Photomicrographs of mice organs in sub chronic toxicity study.

Table 1: Biochemical parameters of mice ingested with high dose of OKPs in sub chronic toxicity study.

OKPs	SEX	GLU (mg/dl)	CHO (mg/dl)	TPRO (g/dl)	UN (mg/dl)
DW	Male	129 ±12	107 ± 10	4.8±0.3	34 ± 4
	Female	133 ± 10	102 ± 12	5.0 ± 0.9	30 ± 6
HW	Male	128 ±14	106 ± 11	5.2 ± 0.7	32 ± 3
	Female	131 ±13	98 ± 13	5.3 ± 0.5	28 ± 7
HB	Male	130 ±10	104 ± 11	4.9 ± 0.8	33 ± 3
	Female	132 ±12	100 ± 12	5.1 ± 0.6	29 ± 6

Values are mean ± SD; n=5; A significant difference from control, $p < 0.05$.
GLU, glucose; CHO, cholesterol; TPRO, total protein; UN, urea nitrogen.

Table 2: Haematological parameters of mice ingested with high dose of OKPs in sub chronic toxicity study.

OKP	Sex	WBCx10^3/ml	RBCx10^6/ml	HGBg/100ml	PCV%	NEU%	ESO%	LYM%	MON%
DW	M	7.42±1.03	8.42±1.04	12.2±0.66	44.26±4.14	14.0±6.4	0.4±0.1	84.4±8.6	2.4±1.2
	F	6.46±1.01	7.24±1.02	12.3±0.88	42.02±3.16	11.0±6.8	0.6±0.1	86.4±8.7	2.3±1.6
HW	M	7.63±1.24	8.14±0.98	11.9±0.72	40.22±4.26	13.8±7.1	0.5±0.2	83.4±7.3	2.2±1.7
	F	7.38±1.12	7.42±0.94	12.1±0.82	43.16±3.24	10.4±6.1	0.7±0.2	86.2±7.2	2.1±1.6
HB	M	7.56±1.06	8.62±0.88	12.5±0.64	41.86±3.28	12.9±6.6	0.3±0.1	82.6±9.4	2.0±1.8
	F	6.72±1.12	7.84±0.96	12.1±0.76	45.12±2.98	10.8±6.4	0.5±0.2	85.4±9.2	1.9±1.5

Values are mean ± SD; n=5. A significant difference from control, $p < 0.05$; WBC, white blood cells ; RBC, red blood cells; HGB, hemoglobin ; PCV, packed cell volume ; N, Neutrophils ; ESO, eosinophils ; LYM, lymphocytes ; MON , monocytes

Table 3: Mean relative organ weight of mice ingested with high dose of OKPs in sub chronic toxicity

OKPs	Sex	Heart	Lungs	Liver	Spleen	Kidneys	Stomach	Small intestine
DW	M	0.46±0.04	0.16±0.09	6.52±0.34	0.29±0.04	1.66±0.18	0.90±0.05	4.80±0.35
	F	0.43±0.02	0.13±0.06	4.69±0.27	0.38±0.04	1.46±0.16	1.22±0.11	5.30±0.38
HW	M	0.44±0.05	0.15±0.03	5.34±0.29	0.28±0.03	1.61±0.19	0.95±0.08	4.96±0.29
	F	0.47±0.04	0.12±0.04	4.88±0.36	0.33±0.06	1.38±0.17	1.25±0.12	5.34±0.28
HB	M	0.49±0.03	0.17±0.07	5.92±0.36	0.25±0.05	1.64±0.22	0.98±0.04	5.03±0.34
	F	0.44±0.05	0.11±0.05	5.12±0.22	0.35±0.07	1.42±0.13	1.29±0.12	4.99±0.36

Values are mean ± SD; n =5. A significant difference from control, $p < 0.05$

In sub chronic toxicity study, the mice ingested with 1000mg/kg bw OKPs did not show any significant difference ($p<0.05$) in body weight, water and food intake when compared with control group and also diarrhea was not observed in all the mice throughout the study. All animals survived until the scheduled euthanasia. OKPs did not show statistically significant difference ($p < 0.05$) in haematological values and biochemical parameters when compared with the values of the control group. At autopsy, macroscopic observation of the organs did not show any change due to the consumption of OKPs. The histopathological scores were statistically insignificant ($p<0.05$) in treated groups when compared with control group. The histopathological sections study of all the mice:

i) Heart shows cardiac muscle fibers which do not show branching. The epicardium shows adipose tissue and cross sections of coronary arteries.

ii) Lung shows no evidence of inflammation / edema / granuloma formation.

iii) Spleen shows no evidence of pathological changes.

iv) Liver shows no evidence of inflammation or significant congestion.

v) The stomach shows no evidence of erosion or ulceration, gastritis or dysplasia.

vi) The small intestine shows no evidence of villous inflammation or atrophy or collections of macrophages in lamina propria or dysplasia.

vii) The large intestine shows no evidence of cryptitis or crypt abscess or ulceration.

viii) The kidney shows the glomeruli, proximal, distal convoluted tubules, collecting ducts, blood vessels and interstitium appear normal.

After the termination of the treatment, the high dose recovery groups did not show any statistically significant difference ($p < 0.05$) in neurobehaviour and body weight compared with the control recovery group and their daily food and water intake also.

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