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Citric Acid Cross-Linking of a Polysaccharide-Based Hydrogel of Plant Origin: A Preliminary Study on Toxicity Evaluation using Rat as Model Animal

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ABSTRACT

The regulatory approval of the active pharmaceutical ingredients and their excipient presupposes the development of safety assessment of these substances. The study of acute oral and dermal toxicity of a cross-linked hydrogel made by the esterification of *Artemisia vulgaris* seed mucilage and citric acid was achieved by following the directives and guidelines 420 and 402 of the OECD. For toxicity studies, rat was taken as the model animal and the rats were divided into four different groups. The animals (rats) in group A were not subjected to hydrogel

and used as a control. Whereas the other groups, i.e. B, C and D of were treated with hydrogel dose of 0.05, 0.3 and 2 g/kg of the bodyweight, and were treated as the test groups. The behavior of all animals was observed, food consumption, water intake, allergic reactions, mortality rate, and all other side effects were noted in 14 days. All the observed animals survived without any serious behavior and physiological defects. It was also found that the biochemical analysis and hematological tests were found to be consistent and comparable after 14 days of the treatment in both groups of animals, control and treated. The internal organs of animals (the major ones) were removed in order to establish the weight of the organs. The accumulated results of acute oral experiments showed that the produced hydrogel is harmless and non-toxic when taken orally.

Keywords: Hydrogel; Crosslinking; Toxicity studies; Biochemistry; Hematology

1. Introduction

Toxicology of any new product is essential in determining the suitability of the new product as an excipient, as a carrier of drug delivery systems, and as an active pharmaceutical ingredient [1]. They were believed to be non-toxic and without pharmacological effects; excipients and drug delivery systems safety were not prioritized in the past [2,3]. Recent years have demonstrated the need to perform additional toxicity studies due to the reports of the toxicological reactions, which comprise renal toxicity induced by 8-cyclodextrin, diarrhea induced by mannitol, skin irritation induced by propylene glycol, and digestive system induced by lactose [4-6]. The polymers are known as natural polysaccharides, which can be characterized by a high degree of swell ability, release of drugs based on pH, biodegradability, and biocompatibility. The properties are improved with the help of chemical modification like

copolymerization and crosslinking etc. and the outcome achieved within the work of materials responsive to pH, temperature, salt, and ethanol [7-26].

Artemisia vulgaris is a perennial aromatic plant commonly known as mugwort and belongs to the family Asteraceae**,** which is one of the largest families of flowering plants. *A. vulgaris* species are famous due to their antiseptic, analgesic, soothing, antimicrobial, antioxidant, antifungal, and anti-inflammatory properties. *A. vulgaris* seeds are a source of protein, fats, carbohydrates, dietary fibers, ash contents, minerals, vitamin, and dry matter. Naturally, *A. vulgaris* seeds include antioxidants, fatty acids, fiber (soluble or insoluble), minerals, as well as vitamins. The mucilage of *A.vulgaris* seeds is a polysaccharidal in nature and have hydrophilic functional groups which can be alkylated to make materials with broad biomedical uses. *A. vulgaris* mucilage seeds are natural polysaccharide, which is rated in terms of medicinal applications due to its swelling and de-swelling capacity, pH-sensitive drug release [27-30].

The proposed investigation aims at synthesizing cross-linked hydrogel using *A. vulgaris* seed mucilage and citric acid to assess its acute oral studies to expand its biomedical use. When assessing the acute oral toxicity, guidelines 420 and 402 produced by OECD will be used accordingly, OECD, 402 and 420). The effects of synthetic hydrogel on the biochemical and haemoglobinic traits of rats will be assessed.

2. Material and Methods

2.1. Materials

Retail market sources were used to get *A. vulgaris* seeds. Various chemicals and reagents used in this study were of analytical grade chemical reagents. To prepare solutions, distilled water

(DW) was employed in the whole course of the experiment.

2.2. Isolation Mucilage and Synthesis of Hydrogel

The mucilage of *A. vulgaris* seeds was isolated according to the reported method and it was further esterified with citric acid according to already reported with slight modification [22].

2.3. Acute Oral Toxicity Testing

The rats were the model animals to test the acute toxicity of synthesized hydrogel. The laboratory animal unit provided these model animals. The model animals were maintained in clean and well-organized cages with good environment of 12 h photoperiod, 40 percent humidity and temperature of approximately 25℃. It was an experiment which was carried out in accordance with rules of good laboratory practices (GLP) and Organization for Economic Co-operation and Development (OECD) (OECD, 402 and 420) [31,32]. The model animals (rats) of group B (0.05g/kg), C (0.3g/kg) and D (2g/kg) were administered a dose of co-polymeric hydrogel proportionate to their bodyweight of the model animals (kg), and the model animals (rats) of group A were not administered any dose and they acted as control group of the model animals (Table 1). The model animals were all starved 12 h and then co-polymeric hydrogel was administered. The model animals of each group were supplied with water and food evenly after 1 h of hydrogel administration and monitored periodically after 14 days.

2.4. The Physical Observation and Mortality Rate

The all the animals were monitored on any negative effects or strange symptoms like tremors and salivation or diarrhea and seizures or allergic reaction or change in behavior in the course of 14 days. Also, in case any of the animals died at any point in time during the 14 days-experimental work of the model animals**.**

2.5. Body weight, Food, and Water Intake Evaluation

Any modification in the weights of both the treated and the control groups of the model animals (rats) as well as the food intake by the model animals (rats) served as a measure of any rough effects of the cross-linked hydrogel on the physical condition of the model animals (rats). Therefore, the weight, food and water consumption of the treated and control groups of model animals (rats) were recorded prior to administration of cross-linked hydrogel on the 1^{st}, 2^{nd}, 3^{rd}, 7^{th}, and 14^{th} day.

2.6. Biochemical Analysis and Hematological Analysis

A collection of blood of model animals (rats) in treated and control groups was made after 14 days of study were completed and they were transferred to the already coated test tubes with the help of ethylenediaminetetraacetic acid (EDTA). Blood samples that had been collected on model animals (rats) were tested in terms of white blood cells, red blood cells, hemoglobin content, platelet count and average cell volume. Also, the serum samples were checked in terms of the concentration of various metabolic, kidney, and liver functioning indicators.

2.7. Organ Weight Measurement

The model animals (rats) were euthanized, and major internal organs, including heart, kidneys, stomach, liver, and intestine were excised from model animals of both the groups. The precise weight of the organs was noted, and this weight was evaluated against the model animal (rats) control group's weight.

3. Results and Discussion

3.1. Acute Oral Toxicity Studies Testing

In order to test the toxicity of cross-linked hydrogel, the rats that were used were Swiss albino rats. The animals had a good environment in clean and well arranged enclosures with photoperiod of 12h, 40% humidity with temperature of approximately 25℃. The experiment has been carried out according to the guidelines of good laboratory practices (GLP) to ensure that tests are correct. The rats of treated groups B (0.05g), C (0.3g), and D (2g) were administered a dose of co-polymeric hydrogel per kg of the bodyweights of the animals namely, B (0.05g), C (0.3g), and D (2g), and the rats of the group A were not subjected to any dose and they acted as the control group of model animals. The animals were starved (12 h) and then cross-linked hydrogel was administered to them. Animals of each group were supplied with water and food uniformly after 1 h of co-polymeric hydrogel administration and monitored regularly after 14 days.

3.2. Physical Observation and Mortality Rate

No apparent effect was experienced on the skin, eyes, hair, nails, fur, and in the behavior of animals in the treated group after the administration of the dose of cross-linked hydrogel. Any side effects or strange symptoms e.g. tremors, salivation, diarrhea, seizures, allergic reactions or change in behavior were observed in all the animals and no signs of this abnormal behavior were detected. The 14 day observation proved the animals were all healthy and energetic and there were no mortality cases observed.

3.3. Bodyweight, Food, and Water Intake Assessment

The weight, food, and water consumption of both animals in all groups, i.e. treated and control rats after pre- and post-dose of cross-linked hydrogel were recorded on 1^{st}, 2^{nd}, 3^{rd}, 7^{th}, and 14^{th} day (Tables 1-3). Insignificant reduction of weight among rats was observed in the first 3 days of experimentation because of the starvation of the 1st day and the administration of co-

polymeric hydrogel. The weight of rats was steadily increased during the first week of study, which was the evidence of the normal development and functioning of the animals. The change in weight of animals of the treated and control group was not significantly different at the entire study period. Results were adhering to protocols and regulations 402 and 420 as per OECD and similar to compounds of the control group.

Table 1: Bodyweight (g) of treated and control group of rats (mean ± SD)

Parameters	Group 1	Group 2	Group 3	Group 4
Pretreatment	149.96±2.25	182.69±2.96	161.39±2.11	208.61±2.10
Day 1	149.23±3.57	181.42±2.65	160.80±2.29	207.94±2.93
Day 2	150.71±2.79	182.61±2.97	160.31±2.04	208.67±2.04
Day 3	151.23±2.57	183.07±3.13	161.67±2.90	209.40±2.43
Day 7	152.07±2.40	184.34±2.35	163.56±2.50	210.44±2.85
Day 14	155.28± 2.96	187.86±1.86	167.47±2.56	213.51±2.34

Table 2: Mean values of water consumption (mL) of control and treated groups of rats

Parameters	Group 1	Group 2	Group 3	Group 4
Pretreatment	6.04±0.32	5.84±0.41	5.76±0.39	6.37±0.29
Day 1	5.80±0.40	4.98±0.50	5.66±0.28	5.80±0.34
Day 2	6.32±0.24	5.84±0.12	4.92±0.44	6.10±0.51

Day 3	5.93±0.33	6.29±0.67	5.61±0.57	7.11±0.26
Day 7	6.42±0.21	5.58±0.30	6.54±0.43	6.22±0.37
Day 14	7.05±0.38	6.43±0.40	5.94±0.65	7.32±0.44

Table 3: Mean values of food (g) of control and treated groups of rats

Parameters	Group 1	Group 2	Group 3	Group 4
Pretreatment	5.75±0.25	5.87±0.36	6.41±0.31	6.59±0.23
Day 1	5.84±0.30	4.91±0.47	5.73±0.90	5.65±0.43
Day 2	6.27±0.18	5.23±0.42	6.05±0.58	6.21±0.16
Day 3	5.72±0.29	6.65±0.35	5.49±0.23	6.35±0.40
Day 7	6.61±0.34	5.09±0.65	6.64±0.31	7.06±0.44
Day 14	5.91±0.47	6.31±0.39	6.80±0.52	7.27±0.32

3.4. Hematology and Biochemical Analysis

The bone marrow produces blood cells and any substance affecting its activity brings about changes in CBC (complete blood count). Serum enzyme biomarkers such as alanine aminotransferase, alkaline phosphatase and total bilirubin are usually used as part of the biochemical tests and any change in their levels results in hepatotoxic liver damage. In the same way, to monitor renal status, the concentration of urea and creatinine in blood is shown.

The hematology tests and serum biochemistry test were conducted to test cross-linked hydrogel toxicity to major internal organs of rats. At the end of 14 days of study, the blood sample of rats in the treatment and the control group was obtained to analyze CBC and serum biochemistry. A blood sample was taken and put in the already coated test tubes with the use of ethylenediaminetetraacetic acid (EDTA). The blood samples obtained in rats were tested in respect to the number of white blood cells, red blood cells, the amount of hemoglobin in the blood, the number of platelets and the mean volume of the cells. The serum samples were also evaluated to have the levels of various indicators of metabolic, kidney, and liver functioning. Combined with hematological findings in both the treated and control groups of rabbits, the cross-linked hydrogel exhibits a comparable result and is within the normal parameter of the hematological findings. Hemoglobin values, serum biochemistry, renal profile, liver profile, and lipid profile values proved that cross-linked hydrogel does not have a significant impact on blood cells, kidney, and liver. Such studies have shown that cross-linked hydrogel is a non-toxic compound; therefore, it is suitable to use as an oral agent (Tables 4 and 5).

Table 4: Hematological parameters of rats

Parameters	Group 1	Group 2	Group 3	Group 4

TLC (μL^{-1})	5.92	7.13	6.32	7.04
RBC (μL^{-1})	7.18	6.27	6.51	6.49
Hb (g/dL)	15.7	16.29	15.41	16.32
HCT (PCV) (%)	46.3	45.5	45.29	46.7
MCV (fL)	68.2	69.10	70.4	72.26
MCH (pg)	23.1	25.41	22.31	23.5
MCHC (g/dL)	35.5	36.2	34.8	36.1
Platelet count (μL^{-1})	842.3	875.1	838.2	859.7
Neutrophils (%)	37.4	36.7	37.1	35.6
Lymphocytes (%)	63.7	65.4	60.8	61.3
Monocytes (%)	2.9	2.3	2.7	2.1
Eosinophils (%)	3.1	2.9	3.0	3.5

Table 5: Clinical biochemistry of rats

Parameters	Group 1	Group 2	Group 3	Group 4

Lipid Profile				
Cholesterol (mg/dL)	131.2	137.3	140.9	136.5
Triglyceride (mg/dL)	114.1	117.5	119.4	120.7
HDL (mg/dL)	46.7	42.6	44.1	45.9
LDL (mg/dL)	73	71.2	76	77.3
VLDL (mg/dL)	24.6	22.5	23.4	22.1
Liver function test				
Bilirubin (mg/dL)	1.0	0.8	0.7	0.9
SGPT (ALT) (U/L)	74.5	72.4	69.8	70.7
SGOT (AST) (U/L)	161.3	153.1	164.6	159.0
ALP (U/L)	89.2	82.7	86	81.5
Total protein (g/dL)	6.1	5.3	5.7	6.4
Albumin (g/dL)	2.2	2.7	2.9	3.0
Globulin (g/dL)	2.0	2.3	2.5	2.6
A/G Ratio	1.1	1.17	1.16	1.15
Renal function test				
Urea (mg/dL)	64.3	72.8	71.6	68.3

Creatinine (mg/dL)	1.2	1.4	1.3	1.2
Hematology				
ESR (mm/h)	3.6	4.1	3.4	3.9
Serum electrolyte				
Potassium (mmol/L)	2.8	3.4	2.9	3.7
Sodium (mmol/L)	125.1	123.2	127.5	129.3
Uric acid (mg/dL)	4.3	4.0	3.9	4.1

4. Conclusion

The toxicological testing of cross-linked hydrogel was performed using the protocol and regulations (420 and 402) of OECD model animals (rats.) showing stability of hematological profile, biochemical assessment and histological parameters. The general outcome of the experiment indicated that the cross-linked hydrogel synthesized is a non-toxic and safe compound and can be used as a carrier in oral drug delivery systems and development of the dressing of wounds. Nevertheless, additional toxicological testing such as chronic toxicity, cytotoxicity and mutagenic tests is required to expand its range of applications.

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