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Preclinical Safety Assessment of Starch Humate: Acute and Sub-Acute Oral Toxicity Studies in Rats

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ABSTRACT

Starch humate is a new superdisintegrant prepared from sorghum starch and humic acid. It has been developed for use as excipient in fast-dissolving tablets. Prior to its use in solid dosage form, it is crucial to assess its safety and toxicity profile. This study aimed to evaluate the acute and sub-acute toxicity of starch humate in Swiss mice, and Wistar rats. The acute toxicity study was done by single oral administration of starch humate to Swiss mice at doses of 50, 300, 1000, and 2000 mg/kg body weight. To evaluate the sub-acute toxicity, Wistar rats were administered starch humate at doses of 50, 300, 1000, and 2000 mg/kg body weight over a 28-day period. Throughout the study, the body weight, food and water consumption, hematological and biochemical parameters, and organ weights were monitored. The results showed no sign of toxicity or mortality in the mice up to the highest dose of 2,000 mg/kg in the acute toxicity test. Similarly, for the sub-acute toxicity test, results showed no significant alterations in body weight, hematological and biochemical markers, or organ weights in the groups treated with starch humate compared to the control group. These findings suggest that starch humate is a safe excipient for pharmaceutical use. However, additional research into its long-term safety and effectiveness in drug formulations is necessary.

Keywords: Disintegrant, Starch humate, Toxicity, Safety.

Introduction

The term "toxicity" describes the degree of adversity that can arise from a specific interaction between toxicants and cells. The cell membrane, which controls movement of materials from the extracellular matrix into the cells, and a few chemical characteristics of the toxicants, determine this interaction.[1] Hence, in the search for substances of potential therapeutic value, safety is a concern, therefore, it is important to understand their toxicity levels.[2,3] Acute, sub-acute, chronic, reproductive toxicity, and carcinogenicity are the models used to assess the toxicity profile of medicinal plants, functional food, and nutraceutical preparation.[4,5] Starch is an inexpensive material that can be recycled easily, due to its biodegradability, and biocompatibility. Starch are usually modified by cross-linking through a number of bonding interactions, including covalent bonding, and intermolecular hydrogen bonding.[6,7] This cross-linking contributes to the mechanical properties, making them more flexible, and useful as industrial material. Sorghum millet is a natural source of starch, it is gluten free, and presents an alternative for those with celiac disease or gluten sensitivity, it is high in resistant starch, and is an excellent source of minerals.
Compared to other cereal crops, sorghum has a broader range of phenolic compounds; flavonoids, tannins, and simple phenolic acids are among the most common phenolic compounds. These compounds possess several biological properties, which include anti-inflammatory, antioxidant, antithrombotic, and antidiabetic properties, as well as probiotic activity.[8]

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Humic acid is a naturally occurring organic compound in soil, peat, and certain coal deposits. Modified starch with humic acid is a new excipient that combines the desirable properties of starch such as biocompatibility and hydrophilicity, with the unique characteristics of humic acid, which is known for its antioxidant, and chelating abilities.[9] the use of starch humate in fast dissolving tablets, enhances the dissolution rate and bioavailability, followed by better pharmacological responses. Starch matrix incorporated with humic acid is expected to improve the disintegration and dissolution properties of the resulting superdisintegrant, hence has potential for use as an excipient in drug formulation.[10,11] Accordingly, it is important to conduct toxicity studies before introducing any new excipients into pharmaceutical formulations to ascertain their safety profile. Hence, toxicity assessments are critical in determining any possible adverse effects and defining the tolerable levels of exposure to any excipient in order to ensure the safety of patients who will eventually be using the formulated product containing such excipient. An acute toxicity study is therefore performed to determine any potential adverse effects that a medication could cause if a high dose or amount is administered over a short period. Such study is used to determine or establish a safety margin. Acute toxicity evaluation typically includes studies, such as acute toxicity tests that evaluate immediate or short-term effects resulting from single or multiple exposures within 24 hours and subacute toxicity tests designed to examine the effects of repeated exposure over 28 days or less. Starch humate has been shown to have a superdisintegrant effect, increased solubility, and increased bioavailability of drugs, indicating better pharmacological effects. However, there is no available evidence of its safety or toxic effects. Therefore, the present study was undertaken to determine the potential toxicity of starch humate in an animal model of acute and subacute toxicity. Such studies would provide valuable information about the potential toxicity of excipients on various organ systems, hematological and biochemical parameters.

Materials and Methods

Chemicals

The chemicals used in this study included starch humate (prepared in the laboratory), mercuric chloride (Merck, Mumbai), sodium chloride (Merck, Mumbai), crystalline sodium sulfate (Qualigens Fine Chemicals, Mumbai), ethanol (Changhu Yanguan Chemicals, China), glacial acetic acid (Merck, Mumbai), gentian violet (Qualigens Fine Chemicals, Mumbai), ammonium oxalate (Merck, Mumbai), Wright's stain (Qualigens Fine Chemicals, Mumbai), methanol (Qualigens Fine Chemicals, Mumbai), sodium hydrogen phosphate (Merck, Mumbai), potassium dihydrogen phosphate (Merck, Mumbai), disodium hydrogen phosphate (Himedia Laboratories Pvt Ltd, Mumbai), and Merck analytical kits for serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, blood urea nitrogen, and serum creatinine.

Collection of sorghum millets

Sorghum millets were collected from an agricultural research station, Vizianagaram, India.

Extraction of starch

Unwanted materials and debris are removed from the sorghum millets. Starch was extracted from the millets by wet milling method. Firstly, the millets were steeped in water for 2 – 4 days to remove the kernels, followed by the addition of 0.25% w/v sodium hydroxide, and allowed to stand for 18 – 24 h to soften the grains. The mixture was filtered through a muslin cloth, and centrifuged (REMI P24, Mumbai) at 5000-7000 rpm for 10 min to remove proteins. The above procedure was repeated several times to increase starch yield. The final starch residue was dried in a hot air oven at 40ºC for 24 h. The percentage yield, chemical composition, and other properties of isolated starch highly depend on the method adopted for isolation.[11-17]

Preparation of starch humate

Ten parts of starch was dissolved in 25 parts of distilled water in a beaker, the mixture was stirred for at least 1 h with a magnetic stirrer. Ten parts of humic acid was dissolved in another breaker containing 10 mL of water. The mixture was stirred for 1 h using a magnetic stirrer. The starch-humic acid dispersion was adjusted to a pH of 5-6 by the addition 0.1 N NaOH, then allowed to stand at room temperature for 16 h. the clear liquid was separated from the suspension to eliminate unreacted humic acid. The suspension was washed with distilled water, then transferred to a stainless-steel tray and dried at 60°C. The resulting solid mass was sieved through a #120 sieve, and the dried product was stored in a desiccator.

Toxicity screening

Experimental animals

For acute and subacute toxicity studies, colony-bred Wistar rats and Swiss mice were used. animals were housed in polycarbonate cages lined with heat-treated hardwood chips and covered with polyester filter sheets. The cages were stored on stainless steel racks with an automatic watering system. The animals were maintained under controlled environmental conditions, with a temperature range of 68–76 °F (20–24°C) and a relative humidity between 42% and 72%. The incoming air was filtered to remove particulates, and the air exchange rate ensured complete air replacement at least ten times per hour. A controlled light cycle of 12 hours of daylight and 12 hours of darkness was maintained. The Wistar rats used in the study were of either sex, weighing 130–150 g for males and 120–150 gr for females. Swiss mice were of either sex, with body weights ranging from 20 to 25 g. All experimental procedures involving animals were approved by the Institutional Animal Ethics Committee (IAEC), with the approval number CPCSEA Number SVCP/IAE/2023/018. The experiment was conducted following the guidelines for the care and use of laboratory animals.

Acute toxicity study

Acute toxicity study was performed according to the Organization for Economic Co-operation and Development (OECD) guideline 423 with minor modifications. Swiss mice of either sex, weighing 20–25 g, were randomly distributed into four groups, each consisting of six animals. The animals were fasted overnight before administration of the test substance (starch humate). The superdisintegrant was administered orally and in distilled water at four doses: 50, 300, 1000, and 2000 mg/kg body weight.[18,19]

After dosing, the animals were closely observed for the first 12 h for signs of toxicity or adverse effects, such as increased motor activity, anesthesia, tremors, convulsions, ptosis, lacrimation, salivation, muscle spasms, depression, ataxia, sedation, cyanosis, and algesia. Mortality rates were monitored during this period and for the subsequent 12 h.[20,21]

Subacute toxicity study

Sub-acute toxicity study was conducted according to OECD Guideline 407. (Test No. 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents, 2008). Wistar rats of either sex, weighing 130–150 g, were assigned to five groups with six animals per group.[22,23]

Group I: The control group received distilled water for 28 days. Group II: Received 50 mg/kg of starch humate for 28 consecutive days. Group III: Received 300 mg/kg of starch humate for 28 consecutive days. Group IV: Received 1000 mg/kg of starch humate for 28 consecutive days. Group V: Received 2000 mg/kg of starch humate for 28 consecutive days. The body weight of the animal, food intake, and water intake were recorded for the entire period of study. On the 29th day, the animals were sacrificed. Blood samples were collected for hematological analysis, the parameters estimated were hemoglobin, red blood cell count, white blood cell count, erythrocyte sedimentation rate, and differential count for neutrophils, lymphocytes, eosinophils, monocytes, and basophils. Moreover, the levels of various biochemical parameters such as serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, bilirubin, blood urea nitrogen, and serum creatinine were estimated in a Merck semi-autoanalyzer using Merck analytical kits. Different organs like the liver, kidneys, and heart were also isolated and weighed to evaluate the organ-specific toxicity.[24]

The sub-acute toxicity study was carried out to investigate any adverse effects of repeated 28-day exposure to starch humate by monitoring alterations in body weight, food and water intake, hematological and biochemical parameters, and organ weights.

Histopathological analysis

At the end of the 28-day treatment period, animals from each group were sacrificed, and vital organs (liver, kidneys, and heart) were excised and preserved in 10% neutral buffered formalin. The preserved tissues were dehydrated with a graded series of alcohol solutions (70, 90, and 100% ethanol). Dehydrated tissues were cleared with xylene and then embedded in paraffin wax. Thin sections of 4-5 µm thickness were prepared by rotary microtome from the paraffin blocks. The tissue sections were mounted on glass slides and dried. Thereafter, the tissue sections were deparaffinized, and rehydrated with xylene and a sequence of alcohol solutions in a decreasing order. The tissue was stained with haematoxylin and eosin for general histological examination.

Statistical analysis

Data obtained from the acute and subacute toxicity studies were presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the GraphPad PRISM 5 software. The results were statistically analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. The minimum level of significance was set at $P \leq 0.05$.

Results and Discussion

Acute toxicity of starch humate

In the acute toxicity study, starch humate was administered to Swiss mice at doses up to 2000 mg/kg body weight. No lethality or toxic symptoms were observed in any of the treated animals during the observation period of 12 h after dosing and the subsequent 12 h. The rats exhibited normal behavior with no signs of increased motor

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activity, anesthesia, tremors, convulsions, ptosis, lacrimation, salivation, muscle spasms, depression, ataxia, sedation, cyanosis, or algesia. Furthermore, no abnormalities in body weight, food or water consumption, or respiratory patterns were observed. The absence of any kind of toxic effect and mortality at the highest dose of 2000 mg/kg body weight indicates that the LD_{50} value for starch humate would be higher than 2000 mg/kg. Thus, starch humate was classified as non-toxic in acute single dose administration. The findings from the acute toxicity study suggest that even at high doses, starch humate is relatively safe. This becomes very important for its potential use as a superdisintegrant in pharmaceutical formulations where accidental overdose or misuse may take place.[25-28]

Subacute toxicity study

In the *in vivo* subacute toxicity study, Wistar rats were treated with starch humate at 50, 300, 1000, and 2000 mg/kg body weight for 28 consecutive days. Throughout this 28-day treatment period, there were no remarkable changes in the body weight of starch humate-treated groups compared with the control group. (Tables 1 and 2). This indicate that starch humate does not harmfully affect the general growth and development of animals.

The liver, kidneys, and heart weights were checked at the end of the treatment period. There were no statistically significant differences in the organ weights of starch humate-treated groups compared to the control group, meaning that starch humate does not evoke animal organ-specific toxicity or anomalies. Hematological parameters, including hemoglobin (Hb), red blood cell (RBC) count, white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), and differential count (neutrophils, lymphocytes, eosinophils, monocytes, and basophils), did not show any significant changes in the starch humate-treated groups compared to the control group (Table 3). These results suggest that starch humate does not adversely affect hematopoiesis or cause alterations in the hematological profile.

Table 1: Effect of 28-day oral administration of starch humate on the body weight of rats

Group	Weight before administration (g)	Weight after 1st week (g)	Weight after 2nd week (g)	Weight after 3rd week (g)	Weight after 4th week (g)
I	132 ± 0.12	139 ± 0.13	146 ± 0.15	150 ± 0.11	157 ± 0.12
II	131 ± 0.1[a]	139 ± 0.12[a]	144 ± 0.2[a]	151 ± 0.3[a]	158 ± 0.3[a]
III	132 ± 0.11[a]	141 ± 0.3[a]	147 ± 0.3[a]	154 ± 0.3[a]	160 ± 0.4 [a]
IV	133 ± 1.1[a]	145 ± 0.2[a]	149 ± 0.1[a]	153 ± 0.2[a]	159 ± 0.1[a]
V	134 ± 0.1[a]	140 ± 0.3[a]	149 ± 0.2[a]	154 ± 0.1[a]	159 ± 0.2[a]

Values are the mean ± S.E.M, (n = 6). Values with the same superscript letters in a column are not significantly different.

Table 2: Effects of 28-day oral administration of starch humate on organ weights in rats

Group	Liver (g/100 g body weight)	Kidney (g/100 g body weight)	Heart (g/100 g body weight)
I	2.12 ± 0.11	0.58 ± 0.1	0.21 ± 0.2
II	2.23 ± 0.12[a]	0.59 ± 0.1 [a]	0.23 ± 0.3 [a]
III	2.42 ± 0.19[a]	0.61 ± 0.2 [a]	0.24 ± 0.1 [a]
IV	2.49 ± 0.18 [a]	0.62 ± 0.1 [a]	0.27 ± 0.3 [a]
V	2.52 ± 0.14 [a]	0.68 ± 0.32 [a]	0.29 ± 0.1 [a]

Values are the mean ± S.E.M, (n = 6). Values with the same superscript letters in a column are not significantly different.

Table 3: Effects of 28-day oral administration of starch humate on hematological parameters in rats

Group	ESR (mm/h)	Hb (%)	RBC (× 10^6 mm^-3)	WBC (× 10^3 mm^-3)	N (%)	L (%)	E (%)	M (%)	B (%)
I	6.11 ± 0.12	10.35 ± 0.1	6.12 ± 1.2	7.21 ± 1.1	66.58 ± 1.1	28.74 ± 1.2	1.2 ± 1.1	2.48 ± 1.2	-
II	6.41 ± 0.23[a]	10.21 ± 0.1[a]	6.21 ± 1.1[a]	7.27 ± 1.2[a]	67.12 ± 1.2[a]	28.41 ± 1.1[a]	1.3 ± 0.1[a]	2.31 ± 1.3[a]	-
III	6.33 ± 0.41[a]	10.32 ± 0.1[a]	6.47 ± 1.2[a]	7.28 ± 1.3[a]	66.14 ± 1.1[a]	28.33 ± 1.2[a]	1.1 ± 0.2[a]	2.12 ± 1.1[a]	-
IV	6.12 ± 0.21[a]	10.14 ± 0.1[a]	6.35 ± 1.1[a]	7.38 ± 1.3[a]	66.28 ± 1.3[a]	28.75 ± 1.1[a]	1.2 ± 0.1[a]	2.12 ± 1.1[a]	-
V	6.21 ± 0.33[a]	10.12 ± 0.3[a]	6.49 ± 1.3[a]	7.88 ± 1.2[a]	67.12 ± 1.2[a]	28.28 ± 1.3[a]	1.2 ± 0.2[a]	2.54 ± 1.2[a]	-

Values are the mean ± S.E.M, (n = 6). ESR: Erythrocyte sedimentation rate, Hb: Hemoglobin, RBC: Red blood cells, WBC: White blood cells, N: Neutrophils, L: Lymphocytes, E: Eosinophils, M: Monocytes, B: Basophils. Values with the same superscript letters in a column are not significantly different.

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Assessment of biochemical parameters, including serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, blood urea nitrogen (BUN), and serum creatinine, did not show any statistically significant differences between the treated and control groups (Tables 4 and 5). These findings indicated that starch humate did not induce hepatic or renal toxicity, as evidenced by the expected levels of liver and kidney function markers.

Histopathological findings

Liver sections from the starch humate-treated groups at all dose levels of 50, 300, 1000, and 2000 mg/kg body weight did not show any significant pathological changes when compared to the control group (Figure 1). The hepatocytes were normal with intact cell membranes and well-defined nuclei. There was no evidence of inflammation, necrosis, or fatty acid changes. Similarly, kidney sections from starch humate-treated groups did not manifest significant pathological changes compared with the control group (Figure 2). Glomeruli appeared intact, without any evidence of glomerular hypertrophy or hypercellularity. There was no evidence of necrosis or degeneration in the tubular epithelial cells, and the interstitial spaces were unremarkable with no inflammation or fibrosis.

Heart sections from the starch humate-treated groups did not demonstrate any significant pathological changes compared to the control group. The myocardial fibers were intact; neither necrosis nor inflammation, with fibrotic alterations, was observed. The vasculature appeared normal, without any evidence of vascular abnormalities.

Histopathological examination of the liver, kidney, and heart tissues did not show any significant pathological changes in starch humate-treated groups compared with the control group, even at the highest dose of 2000 mg/kg body weight. These histopathological findings further substantiate the safety and non-toxicity of starch humate.

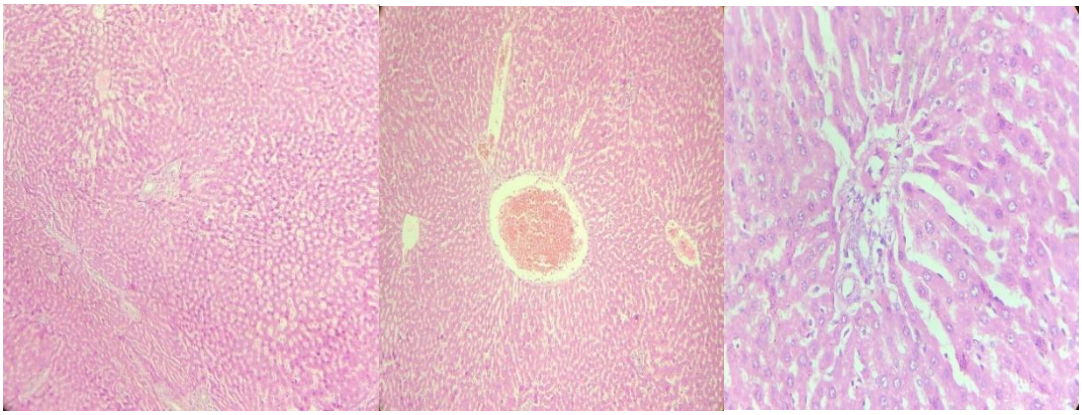

Figure 1: Photomicrograph of the histopathological features of liver of the rats after treatment with starch humate

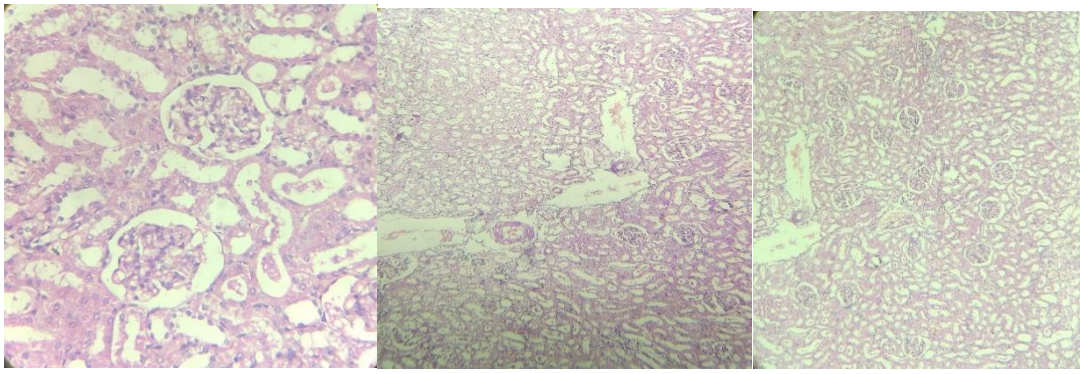

Figure 2: Photomicrograph of the histopathological features of the kidneys of the rats after treatment with starch humate

Table 4: Effects of 28-day oral administration of starch humate on hepatic function in rats

Group	Liver Glycogen (mg%)	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	Bilirubin (mg/dL)
I	125.8 ± 1.1	41.44 ± 1.1	116.41 ± 1.2	207.44 ± 1.1	0.67 ± 0.12
II	126.1 ± 1.4[a]	41.74 ± 1.2[a]	116.84 ± 1.2[a]	207.58 ± 1.2[a]	0.68 ± 0.11[a]
III	128.5 ± 1.32[a]	41.94 ± 1.1[a]	116.53 ± 1.1[a]	208.59 ± 1.3[a]	0.69 ± 0.12[a]
IV	129.4 ± 1.2[a]	42.22 ± 1.2[a]	117.22 ± 1.2[a]	209.94 ± 1.2[a]	0.70 ± 0.12[a]
V	130.5 ± 1.3[a]	42.74 ± 1.3[a]	117.54 ± 1.1[a]	209.71 ± 1.1[a]	0.71 ± 0.13[a]

Values are the mean ± S.E.M, (n = 6). SGPT: Serum glutamate pyruvate transaminase, SGOT: Serum glutamate oxaloacetate transaminase, ALP: Alkaline phosphatase. Values with the same superscript letters in a column are not significantly different.

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Table 5: Effects of 28-day administration of starch humate on blood urea and serum creatinine in rats

Group	Blood Urea (mg%)	Serum Creatinine (mg/dL)
I	37.42 ± 0.2	0.68 ± 0.1
II	37.38 ± 0.1[a]	0.69 ± 0.3[a]
III	37.49 ± 0.3[a]	0.71 ± 0.2[a]
IV	37.48 ± 0.1[a]	0.73 ± 0.1[a]
V	37.55 ± 0.1[a]	0.72 ± 0.2[a]

Values are the mean ± S.E.M, (n = 6). Values with the same superscript letters in a column are not significantly different.

Conclusion

The toxicity of starch humate was evaluated in acute and sub-acute toxicity tests where essential information about the safety profile was obtained. In an acute toxicity study, starch humate was found to be non-lethal and non-toxic as well. There were no clinical signs of toxicity in Swiss mice even up to a dose of 2000 mg/kg body weight. This finding suggests that the LD_{50} of starch humate is more than 2000 mg/kg, which indicates its non-toxic nature in acute oral consumption. In the sub-acute toxicity study, Wistar rats were given starch humate up to 2000 mg/kg body weight for 28 days, and they did not develop any of the major changes like body weight gain, organ weights, haematological and biochemical parameters when compared to the control group. These findings showed that continuous exposure to starch humate did not affect the general health status of the animals. From the above studies, Starch humate which is a new superdisintegrant prepared from sorghum starch and humic acid, has relatively low toxicity, good safety margins and is reported to be well tolerated in acute and sub-acute studies. The findings of the current study therefore substantiates the prospect for the use of starch humate as a safe and efficient excipient in pharmaceutical formulations, particularly in the field of fast-dissolving oral dosage forms.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original, and any liability for claims related to the content of this article will be borne by them.

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