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FULL PAPER

Sub-acute toxicity effects of modified sago starch edible films intake on haematology, histopathology, body and organ weight changes in *Sprague Dawley* rats

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Abstract

The toxicity of edible film from modified sago starch was evaluated through a sub-acute toxicity study on *Sprague Dawley* rats. Rats were given edible film at concentrations of 2 and 5 g/kg bodyweight for 28 consecutive days. Control rats only received commercial pellets. No significant changes were observed in the weight of rats and internal organs in low and high dose groups as well as control groups (p>0.05). The food intake was in a range of 15-19 g/week. The haematology analysis showed significantly higher haemoglobin (HBG), haematocrit (HCT) and red blood cells (RBC) in the high dose group of rats. Elevated haematocrit level might indicate a dehydrated condition in rats as a result of the significant reduction of fluid intake in low and high dose groups as compared to control rats. Besides haematology, observation of histopathology effects of edible film on rats with an experimental diet found insignificant vacuolation in hepatocytes. In conclusion, these results demonstrate that edible film from modified sago starch is safe and non-toxic for consumption at a low dose, 2 g/kg body weight based on the 28 days of sub-acute toxicity study.

1. Introduction

Edible films and coatings are the promising biofilms in the area of food science and technology over the last 30 years. Research on edible films and coatings has continuously evolved as many food issues have been addressed and resolved. For instance, edible films and coatings can improve mechanical properties, sensory perception, convenience and microbial protection. On top of that, edible films and coatings have been successfully extending the shelf life of food products while maintaining their quality attributes (Krochta, 2002; Janjarasskul and Krochta, 2010). Edible films and coatings can be made from organic polymers such as protein, starch, alginate, cellulose derivatives, chitosan, agar and lipid (Min and Krochta, 2005) which are capable of degradation and formation. The usage of edible films in food packaging applications has been partly replacing the consumption of synthetic packaging material from non-renewable sources. According to Wolff *et al.* (1951), starch-based edible films have shown similar physical properties to synthetic polymers, such as being transparent, odourless and tasteless. Besides, the films also are semi-permeable to carbon dioxide and resistant to oxygen passage (Nisperos-Carriedo, 1994). In addition, the starch films have good barrier and mechanical properties (Larotonda *et al.,* 2005). Starch, a biodegradable polymer is commonly used as a material in edible film and coatings production due to its abundance, edible, easy to recycle (renewable), biodegradable and low cost (Mali and Grossmann, 2003; Famá *et al*., 2005). Several researchers have investigated the starch-based edible films from various common sources of starches including high amylose corn, potato, cassava, tapioca, rice, sweet potato, yam, breadfruit, and banana. However, starch from sago palm is not as familiar as common starch because the starch is obtained from an uncommon source (*Metroxylon* sp.) or better known as “rumbia” in South East Asia (Fasihuddin *et al*., 1999). In East Malaysia, sago starch as the main carbohydrate source can compete economically on the higher yield and lower cost of production as compared to cassava and maize starch (Singhal *et al*., 2008). However, the physicochemical properties of sago starch are quite similar to cassava and potato starch (Karim, Nadiah, Chen *et al*., 2008; Karim, Tie, Manan *et al*., 2008; Tie *et al*., 2008). The usage of sago starch has

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widely been used in food packaging applications where sago starch has become a good material for producing biodegradable films and coatings. Even though edible films from starch have desirable properties, plain sago starch films are too rigid and have hydrophilic nature. Therefore, starch is always modified chemically, physically, mechanically and/or added with additives or plasticizers in order to improve its functional properties hence broadening its usage in the industrial application (Robertson, 2008; Ehivet *et al*., 2011). The toxicity of modification of sago starch in edible film formulation had been studied by Hanisa *et al.* (2011), but only on the aspect of the toxicity level of modified starch and the safe dose for oral use. There is not much information has been reported on the side effects of the edible film which may compromise its safety due to sago starch modification. Toxicological studies are necessary to indicate the safety level of edible film in order to ensure the films are safe as food packaging material. Therefore, the objective of the study is to evaluate the effect of the edible film which focuses on haematology, body and organ weight changes and histopathology assessment in rats.

2. Materials and methods

2.1 Preparation of edible film

The edible film was prepared through a casting technique. The film-forming solution was prepared by mixing modified sago starch and glycerol in a beaker with distilled water at ambient temperature. The mixture was then homogenized until starch gelatinized at 95°C for 15 mins. Then, the solution was cast on a Thin Layer Chromatography (TLC) plate (20 × 20 cm) to form thin films. The films were dried in an oven (Memmert, USA) at 50°C for 16 hrs before being peeled off from the TLC plate. The dried edible films were ground into powder form using a grinder and were then mixed with standard commercial rat pellets according to treatments given 2 g/kg body weight (low dose) and 5 g/kg body weight (high dose) which would be used for feeding trials on rats.

2.2 Experimental animals

A total of twenty-four female *Sprague Dawley* rats (age about 2 months) each weighing between 220 -240 g were used in this study. They were fed with a standard pellet diet and water *ad libitum* (free access water for 24 hrs). All rats were acclimatized to the animal room conditions (temperature 21±2°C light 12/12 light/dark cycle) for a week before starting the experiment. The acclimatization period is very important to the rats for adaptation to a new environment or cages (WHO, 1993). According to the guidelines, at least five rats per group for certain sex (male or female) should be used to be statistically validated. In this study, the rats were randomly divided into three groups (I, II and III) which consisted of eight rats per group. Rat identification and treatment groups were identified via coloured-coded identification cards. In group I, rats were given commercial pellets and served as a control. Group II and III rats were given the edible film powder mixed with standard commercial rat feed. All rats were given 250 ml of distilled water every day. This study was approved by the Malaysian Agricultural Research and Development Institute (MARDI) following the Institute's ethical standards with reference number 20200106/R/MAEC00067 dated 29 January 2020.

2.3 Sub-acute toxicity

The sub-acute toxicity test was conducted in accordance with the guidelines published by the Organization for Economic Cooperation and Development (OECD) No. 407, Issue Date 7/27/95 (Derelanko and Hollinger, 2002). Control rats were fed with a commercial standard rat pellet and the two treatment groups of edible film in their feed for 28 days. The rats were then observed daily for mortality, signs of gross toxicity and behavioural changes.

2.4 Body and organ relative weight

Body weights of all rats were recorded once a week which included the initial and final stages of the study. The six main visceral organs which are the liver, the kidney, the spleen, the heart, the lungs and the ovary, were quickly excised of all excess tissues and fats and rinsed in 0.9% NaCl (normal saline) to remove any blood. Then, the organs were weighed and examined macroscopically for any seen abnormalities. The organ relative weight (% body weight) was obtained by dividing the final weight of organs by the final bodyweight of the rat (Abdullah *et al*., 2009). Organ relative weight needs to be determined due to the rat's body weights vary within groups.

2.5 Haematology studies

The whole blood was directly transferred into a 3 mL K_3 EDTA blood collection tube (VACUETTE) for the measurement of haematology profiles. Red blood cells (RBC), white blood cells (WBC), platelet (PLT), haemoglobin (HGB) and haematocrit (HCT) were measured on the day of blood collection using a fully automated haematological analyser (Medonic CA530 VET, Boule Medical, Stockholm, Sweden). Assays were performed twice on a single blood sample.

2.6 Histopathology studies

The liver and kidneys were fixed in 10% formalin

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for histopathological study. Tissues were processed using Leica TP1020 Automatic Tissue Processor. After processing, the tissues were embedded in paraffin wax with Embedding Centre (Leica EG1150H and EG 1159C) and sectioned to a thickness of 5 µm using a Microtome RM2045. Sections were stained with haematoxylin and eosin.

2.7 Statistical analysis

The significant differences between the control and edible film's treated groups were determined using ANOVA at a 5% level (p<0.05) followed by Duncan Multiple Range Test (DMRT). All values are expressed as group mean ± standard error of mean (SEM).

3. Results and discussion

3.1 Effect of edible film on body weight

All rats subjected to different edible film administrations showed no significant changes in body weight (p>0.05) at low and high dose treatments as compared to control rats (Table 1). Both control and treated rats appeared uniformly healthy throughout the 28 days of the treatment period. The food intake was an average of 15-19 g per week throughout the experimental period, except the in the first week, in which the rats ate a lesser amount of experimental diets compared to standard pellets (control), as depicted in Table 2. One explanation for this fact is the inadequate evolutionary adaption to the rat taste buds with experimental diets in the early week of the study.

3.2 Effect of edible film on organ relative weight

Organ weight measurement is another fundamental judgment to diagnose whether an organ has been exposed to toxicity. Changes in organ weight are an indicator of toxicity since damaged organs will either increase (swell) or decrease based on the organ-to-body weight ratio (Heywood, 1983; Frank, 1996; Rosidah *et al*., 2009). Some exposure to potentially toxic substances will slightly reduce body and internal organ weights (Teo *et al*., 2002). This study showed that there was no significantly different (p>0.05) in organ relative weight in low and high dose treatment groups as compared to control rats (Table 3). This indicates that organ relative weights were not affected by the administration of edible film. Besides, no noticeable changes were observed between groups in terms of gross appearance, weight, size and colour.

3.3 Effect of edible film on haematology values

Red blood cells (RBC), white blood cells (WBT), haematocrit (HCT), platelet (PLT) and haemoglobin (HBG) are normally used for the screening of haematotoxicity (Goldstein, 1988). The highest overall

Table 1. Effect of experimental diets on body weight of rats during 28 days of sub-acute toxicity study

Group	Body Weight (g)			
	Week 1	Week 2	Week 3	Week 4
Control	245.60±12.37[a]	257.38±13.20[a]	266.17±16.23[a]	272.18±17.79[a]
Low Dose	247.56±24.53[a]	250.18±29.84[a]	258.20±27.45[a]	265.46±23.68[a]
High dose	239.24±15.86[a]	244.20±15.86[a]	246.62±18.13[a]	253.56±18.99[a]

Values are presented as mean±SEM, n = 8. Values with different superscript within the same column are significanntly different (p<0.05) between all treatment groups.

Table 2. Effect of the experimental diets on food intake of rats during 28 days of sub-acute toxicity study

Group	Food Intake (g)			
	Week 1	Week 2	Week 3	Week 4
Control	17.75±2.10[a]	17.60±2.55[a]	18.07±1.94[a]	18.18±2.59[a]
Low Dose	15.40±4.22[b]	15.92±4.25[a]	18.43±4.24[a]	17.80±2.84[a]
High dose	16.05±3.20[a,b]	17.93±3.20[a]	18.38±3.57[a]	19.19±3.88[a]

Values are presented as mean±SEM, n = 8. Values with different superscript within the same column are significanntly different (p<0.05) between all treatment groups.

Table 3. Effect of the experimental diets on organ relative weight of rats during 28 days of sub-acute toxicity study

Group	Organ relative weight (%)				
	Liver	Kidney	Spleen	Heart	Lungs
Control	2.88±0.20[a]	0.67±0.06[a,b]	0.17±0.04[a]	0.31±0.02[a]	0.66±0.09[a]
Low Dose	2.94±0.24[a]	0.64±0.05[b]	0.17±0.02[a]	0.32±0.05[a]	0.70±0.11[a]
High dose	3.12±0.34[a]	0.72±0.08[a]	0.19±0.02[a]	0.31±0.03[a]	0.59±0.23[a]

Values are presented as mean±SEM, n = 8. Values with different superscript within the same column are significanntly different (p<0.05) between all treatment groups.

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concordance of toxicity in animals with humans is seen for haematological, gastrointestinal and cardiovascular adverse effects, whereas reactions of idiosyncratic and hypersensitivity are poorly correlated with observed toxicity in animals (Olson *et al*., 2000). Besides, adverse effects such as headache, dizziness, abdominal pain and visual disturbances are difficult to ascertain in animals. For an instance, this analysis is one of the series of tests that are normally carried out in pathology laboratories to determine a disease or toxic effect in humans (Halimah, 2001). This study revealed statistically significant polycythemias in the high dose treated rats. This feature is caused by the significant increases in RBC count, HGB and HCT (p<0.05). The situation will result in thickened blood, retarded flow and increased danger of clot formation within the circulatory system. Polycythemias can be occurred due to restricted food intake by rats (Wohl and Merskey, 1964) however, there were no significant differences in the food intake of rats (control and low dose groups) compared to high dose group. The polycythemias in high dose rats are yet to be known and could be investigated in the future. The measurement of HCT and HBG is related to the concentration of RBC. Haematocrit is a measure of the relative volume of plasma, the RBC mass and the concentration of RBC (as a percentage of the total volume of blood). Results showed that the HCT and HBG values were increased significantly (p<0.05) in high dose treatment rats as compared to control rats (Table 4). Dehydration is the most common cause of high haematocrit. Our study has shown to be consistent with this finding as the fluid intake in low and high dose treatment of rats was reduced significantly (Table 5). One of the possible causes of dehydration is adrenal fatigue due to stress-treated rats. Laboratory routine handling was reported as a major cause of animal stress. Animal responses to stress such as elevated heart rate, body temperature, blood pressure, plasma glucose and serum concentrations of hormones in proportion to handling time when cardiac puncture or decapitation (Jonathan *et al*., 2004). This also suggested the RBCs per volume rise as the volume in the blood drops. Besides, the platelet level shows significantly higher (p<0.05) in low and high dose treatment diet rats, compared to the control, yet in the normal range of rat's platelet count (893 - 1340×10^9/l) (Barnhart, 2011). However, no significant difference in WBC value was observed in all treated and control rats, indicating that the administrated doses of edible film from modified sago starch were not toxic and did not interfere with the production of circulating RBC, WBC and platelet.

3.4 Histopathology observations

The modified sago starch edible film at 5 g/kg body weight showed no significant effects on the kidney tissues as described in Figures 1 and 2. The intact structure of the glomerulus, the medullary rays, the collecting ducts and the interlobular artery were similar to control rats. It can be seen clearly that the modified sago starch did not disrupt kidney function. The histopathology findings were supported with results obtained from the kidney function test as reported by Hanisa *et al.* (2011), in which urea and uric acid concentrations in all treated rats were not significantly different (p>0.05). Urea is synthesized in the liver as the primary by-product of the deamination of amino acids (Vaughn, 1999). An elevated level of urea concentration or plasma creatinine would indicate renal damage. The plasma creatinine showed significantly lower (p<0.05) in the high dose group and this was a good effect of modified sago starch in increasing the excretion of creatinine in urine. The classic lobule of the liver showed a normal appearance in rat standard pallets. The only prominent effect of experimental diets on the liver was

Table 4. Effect of the experimental diets on blood profile of rats during 28 days of sub-acute toxicity study

Group	Haematology				
	RBC (10*12/L)	HCT (%)	PLT (10*9/L)	WBC (10*9/L)	HBG (g/L)
Control	9.05±0.74^{b}	50.48±4.60^{b}	1045.31±211.16^{b}	6.72±1.75^{a}	178.19±17.39^{b}
Low Dose	9.65±0.83^{a,b}	52.31±5.17^{a,b}	1226.94±155.88^{a}	6.93±1.68^{a}	185.44±16.56^{b}
High dose	10.20±1.19^{a}	55.26±6.06^{a}	1276.56±199.85^{a}	6.54±1.96^{a}	198.61±20.21^{a}

Values are presented as mean±SEM, n = 8. Values with different superscript within the same column are significanntly different (p<0.05) between all treatment groups.

Table 5. Effect of the experimental diets on fluid intake of rats during 28 days of sub-acute toxicity study

Group	Fluid Intake (ml)			
	Week 1	Week 2	Week 3	Week 4
Control	67±16^{a}	76±14^{a}	76±17^{a}	79±14^{a}
Low Dose	56±19^{b}	53±12^{b}	52±7^{b}	53±8^{b}
High dose	52±15^{b}	57±12^{b}	52±9^{b}	56±8^{b}

Values are presented as mean±SEM, n = 8. Values with different superscript within the same column are significanntly different (p<0.05) between all treatment groups.

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the insignificant presence of fat vacuoles (Figure 3). There were no significant differences between the initial and the final body weight both for the control and the treated rats. In addition, the final rats' body weight was slightly lower (p>0.05) in low and high dose treatment, compared to the control which was 265.46 g and 253.56 g, vs. 272.18 g respectively. However, food intake was similar in all group treatments (p>0.05) (Table 2). Due to that, other parameters which were serum enzymes including aspartate transaminase (AST), alanine transaminase (AST) and alkaline phosphatase (ALP) showed no significant differences (p>0.05) in all these experimental rats (Hanisa *et al*., 2011). These three serum enzymes are mainly used in the evaluation of hepatic damage. In addition, the portal tract also showed a normal appearance in all treated rats, indicating there were no liver and renal toxicity (Figure 4).

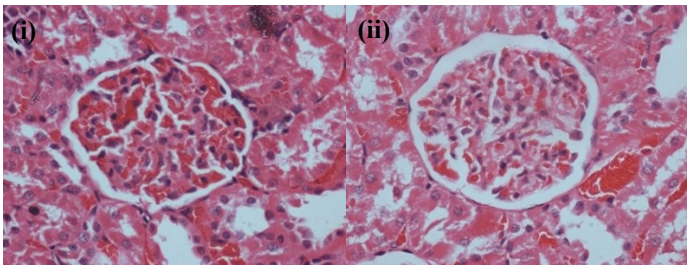

Figure 1. Photomicrograph of rat kidney's cross section from different treatment groups; (i) control rat group (ii) high dose treatment (5 g/kg body weight) rat group during 28 days of sub-acute toxicity study.

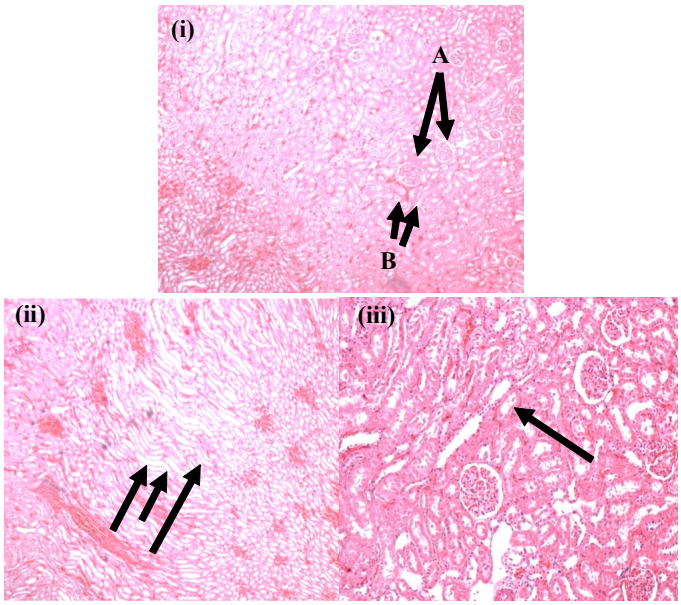

Figure 2. Photomicrograph of rat kidney's cross section from high dose treatment group (5 g/kg body weight); (i) a normal structure of glomerulus of a kidney (indicated by arrow A) and the medullary rays, served the adjacent of glomerulus (indicated by arrow B); (ii) a normal appearance of collecting ducts of a kidney (indicated by arrows) (iii) the interlobular artery of kidney (indicated by arrow) during 28 days of sub-acute toxicity study

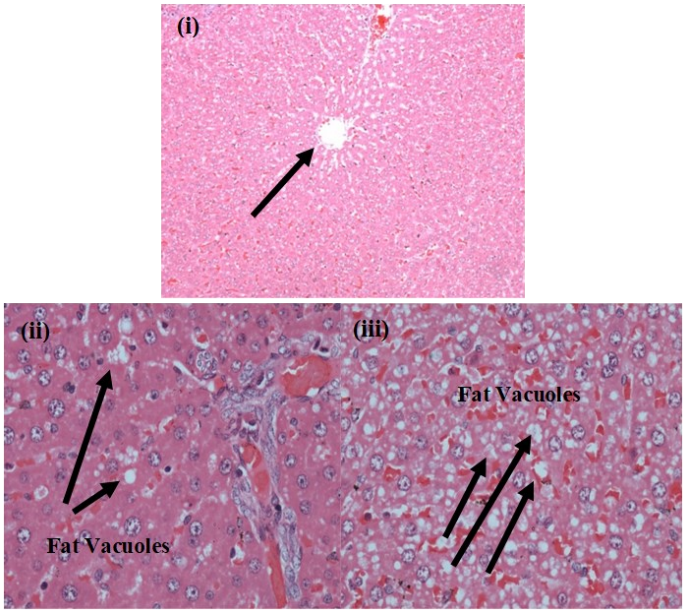

Figure 3. Photomicrograph of rat liver's cross section showed normal appearance of classic lobule in control rats and mild fatty changes in low (2 g/kg body weight) and high dose treatments (5 g/kg body weight). (i) a classic lobule of liver in control rats (indicated by arrow); (ii) mild fatty changes in low dose treatment (indicated by arrow); (iii) mild fatty changes in high dose treatment (indicated by arrow) during 28 days of sub-acute toxicity study.

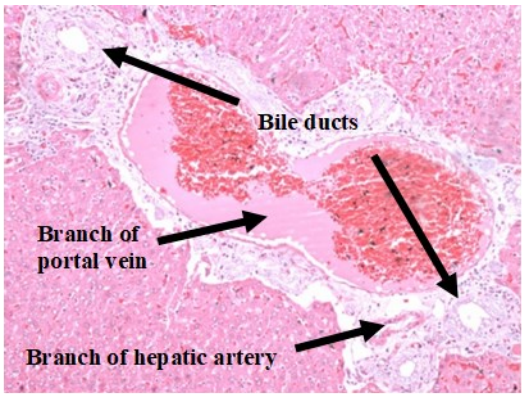

Figure 4. Photomicrograph of rat liver's cross section showed normal appearance of portal tract in high dose treatments rats (5 g/kg bw) during 28 days of sub-acute toxicity study.

4. Conclusion

The administration of repeated different doses of modified sago starch edible films for sub-acute toxicity study did not produce any toxicological significance in body weight and organ relative weight. However, it is possible significant polycythemia due to the significant increase in RBC, HCT and HMG in high dose treatment rats, but needs to be investigated and could be an area for future research. In addition to that, platelet level also elevated significantly and was thought to be toxicologically irrelevant because the platelet was physiologically within the normal range. Besides haematological observations, histopathology studies found that the edible sago film did not significantly affect kidney and liver tissues. In conclusion,

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administration of the edible film from modified sago starch at a low dose (2 g/kg body weight) of Sprague-Dawley rats in the feed did not induce any significant toxicological effects during 28 days of sub-acute toxicity study.

Conflict of interest

The authors declare that is no conflict of interest

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