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IN VITRO AND *IN VIVO* TOXICITY EVALUATION OF *TRAPA BISPINOSA* ROXB. STARCH

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ABSTRACT

Objective: This study aimed to assess the toxicity of *Trapa bispinosa* Roxb. starch (TBS) through *in vitro* and *in vivo* studies.

Methods: The cytotoxicity of TBS extract (TBSE) was evaluated on RAW 264.7 macrophage and NIH 3T3 fibroblast cell lines and the acute dermal and oral toxicities of TBS were analyzed in rats. To access acute dermal toxicity, the rats received a single application of 200, 1000, and 2000 mg/kg BW of TBS, while for acute oral toxicity, the rats received a single administration of 300 and 2000 mg/kg BW of TBS. All animals were observed for changes in body weight, mortality, and clinical signs of abnormality after application and administration of the TBS.

Results: The *in vitro* results showed that TBSE at concentrations of 6.25–200 µg/ml was non-cytotoxic to macrophages and fibroblasts. From acute toxicity studies, the lethal dose of TBS was considered to be over 2000 mg/kg BW. No mortality, clinical signs of abnormality, or gross pathology were detected at necropsy.

Conclusion: TBS is non-toxic in *in vitro* and *in vivo* studies. Therefore, TBS can be used as pharmaceuticals excipients or cosmetic ingredients.

Keywords: *Trapa bispinosa* Roxb., Macrophage, Fibroblast, Acute toxicity, Pharmaceutical excipient, Cosmetic ingredient.

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INTRODUCTION

Trapa bispinosa Roxb. (family *Trapaceae*) or Krajub in Thai is an annual edible aquatic angiosperm that is found in lakes, tanks, ponds, and shallow water. *T. bispinosa* Roxb. favors locations with a tropical, subtropical, or temperate climate, which include many countries in South Europe, Africa, and Asia [1]. The fruit shape is triangular obovoid with two horns. The color of a fresh *T. bispinosa* Roxb. fruit is green but turns blackish once it is dehydrated. The pulp color varies from whitish to light brown and has a sweet taste. It is a staple ingredient of both human and animal diets in some countries, such as India, China, and those in Southeast Asia. Regarding its medicinal properties, the whole plant is used in Ayurvedic and Unani medicine to treat problems related to the stomach, bladder, liver, kidney, spleen, and reproductive organs. The common conditions include diarrhea, dysentery, spermatorrhea, tuberculosis, dry cough, hemorrhage, strangury, polyuria, sore throat, lumbago, fatigue, and dental caries for instance. In recent years, many studies reported that *T. bispinosa* possesses antioxidant, antimicrobial, antidiabetic, analgesic, anticancer, and enzymatic activities [2,3].

Starches are widely used as drug excipients and cosmetic ingredients. In conventional pharmaceutical applications, they are used as diluents, lubricants, absorbents, binders, disintegrants, and thickeners [4]. However, the pharmaceutical data of excipients (i.e., acceptable physical and chemical stability, safety, and efficacy profile) are required before usage and market launch [5]. *T. bispinosa* Roxb. Starch was previously studied as an alternative pharmaceutical excipient by evaluating its physicochemical and binder properties. The powder characteristics, such as granular shape, particle size, hydration, and swelling capacity, are similar to maize and potato starches [6]. Starch materials used in pharmaceutical applications are non-toxic and are generally considered safe by FDA standards [7]. The toxicological evaluation through safety test methods, however, remains necessary as a measure of reducing the risks associated with new excipients and natural products, and to confirm their safety and effectiveness [8,9]. Since *T. bispinosa* Roxb. is a commonly consumed food, it may be further developed for pharmaceutical and cosmetic applications, though little information on the toxicity of TBS is available. Therefore, the objective of this study was to assess the safety of *T. bispinosa* Roxb. starch (TBS) through both *in vitro* and *in vivo* assays.

MATERIALS AND METHODS

Materials

Fresh and matured *T. bispinosa* Roxb. (BKF200375) fruit were purchased from Rai Jum Tid Loe, Suphan Buri, Thailand, in January 2018. Murine macrophage (RAW 264.7) and murine fibroblast (NIH 3T3) cell lines were procured from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, penicillin, streptomycin, and trypsin-EDTA were purchased from Gibco, USA. Trypan blue and resazurin were purchased from Sigma-Aldrich, USA.

Preparation of test samples

The outer layer of the fruits was peeled off and the fruit pulp (white parts) was collected. The collected parts were grounded into powder and washed with distilled water until the supernatant became clear. The washed TBS was oven-dried at 50°C for 24 h. Dried TBS was then used as testing material in acute toxicity studies. TBS was soaked in 70% ethanol for 7 days, and the extract was filtered with Whatman filter paper. Excess solvent was removed using a rotary evaporator at 50°C. The product is designated as TBS extract (TBSE), used for *in vitro* studies.

Cell culture

Murine macrophage (RAW 264.7) and murine fibroblast (NIH 3T3) cell lines were sustained in DMEM containing 10% fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO_2. The macrophages were passaged twice a week and dislodged using a cell scraper. Fibroblasts were passaged twice a week and dislodged using 0.25% trypsin-EDTA. Cell viability was determined with 0.4% trypan blue. Cells with more than 85% viability were used in the experiments.

Determination of cytotoxicity in RAW 264.7 macrophage and NIH 3T3 fibroblast cells

RAW 264.7 macrophages or NIH 3T3 cells were seeded at a density of 4×10^5 cells/ml in a 96-well plate and incubated at 37°C for 24 h. The cells were treated with TBSE at various concentrations 6.25, 12.5, 25, 50, 100, and 200 µg/ml for 24, 48, and 72 h. Cell cytotoxicity was determined with resazurin reduction assay, where the treated cells were incubated for 2 h at 37°C in 100 µl DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance was determined at 560 nm against 600 nm. The cell viability of RAW 264.7 macrophages or NIH 3T3 cells was presented as percentage cell viability calculated from the following formula:

$$\%\text{Cell viability} = \left[\frac{(OD_{560} - OD_{600})_{\text{with extract}}}{(OD_{560} - OD_{600})_{\text{without extract}}}\right] \times 100$$

Animals

Female rats (Jcl:SD) were purchased from Nomura Siam International Co., Ltd. (Bangkok, Thailand). All animals were healthy, nulliparous, and non-pregnant. Eight-week-old rats weighing an average of 235.50±8.21 g and 217.92±15.69 g were used for acute dermal and acute oral toxicity test, respectively. Animals were housed in suitable size plastic cages with a filter top cage and kept under standard laboratory conditions at room temperature (22±3°C), 55±10% relative humidity, and a 12 h light-dark cycle. The rats were acclimated for 9 days in controlled conditions. The animals were provided with food pellets and reverse osmosis water. Changes in behavior and any adverse clinical signs were monitored daily. All procedures were conducted in agreement with the Institutional Animal Care and Use Committee of Naresuan University, Thailand (NO. NU-TS620207).

Acute dermal toxicity study

The acute dermal toxicity test of TBS was evaluated in rats according to OECD GLP guidelines No. 402 [10]. Twenty-four hours before the test, the dorsal hair of rats was shaved using a razor blade. Based on procedural guidelines, the test area, which is not less than 10% of the body surface area of rats, was cleared. TBS was wetted with sterile water and applied at designated doses: 200, 1000, and 2000 mg/kg BW. The application site was covered with a porous gauze dressing and non-irritating tape. The rats were observed individually for signs of behavioral changes after 30 min and 2 and 6 h of application. After 24 h, the pad and starch were removed. The test site was wiped with 0.9% normal saline. The response of skin irritation and corrosion was then observed at 24, 48, and 72 h after the test site was cleansed (Table 1). The primary irritation index was calculated as previously reported [11]. The clinical signs were monitored and recorded at least once daily for 14 days. The animals were euthanized by overdosing with thiopental on day 15. Gross pathological abnormalities of internal organs – that is, heart, liver, kidney, lung, spleen, stomach, and sex organs (uterus and ovary), were recorded.

Acute oral toxicity test

The acute oral toxicity test of TBS was evaluated in rats according to OECD GLP guidelines No. 423 [12]. TBS was premixed with distilled water and administered by gavage feeding at a dose of 300 mg/kg BW and 2000 mg/kg BW. First, the 300 mg/kg BW dose was administered. A dose volume of 2 ml/100 g body weight was maintained. The 2000 mg/kg BW dose was further administered to another set of animals once no adverse effects were seen after the 300 mg/kg BW dose. The clinical signs of toxicity, mortality, and behavioral changes were observed and recorded for 30 min, 1, 2, 3, 4, 24, and 48 h post-administration, and once daily for 14 days thereafter. Individual animal body weight was recorded on the 1st, 7th, and 14th days of the study period. At the end of the study, all surviving animals were sacrificed by 100 mg/kg BW thiopental intraperitoneal injection. External and internal gross pathological changes were documented.

Statistical analysis

In vitro results were expressed as mean ± standard error of the mean of triplicate experiments. Cell viability was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's (*post hoc*) using the SPSS 22.0 software and $p<0.05$ was considered statistically significant. Descriptive statistics and mean ± standard deviation (SD) were presented for *in vivo* studies.

RESULTS AND DISCUSSION

Cell viability of RAW 264.7 macrophage and NIH 3T3 fibroblast cells

In vitro cell cultures benefit in the preliminary evaluation of cytotoxicity of many biomaterials [13]. Macrophages are immune cells that play important roles in maintaining immune homeostasis. They are a component of the innate immunity, bearing roles as the first line of defense against pathogens and the initiator of humoral immune response that secretes macromolecules to trigger a systemic immune response [14,15]. Fibroblasts are connective tissue cells, which have a variety of functions including maintenance of the extracellular matrix in homeostasis, and reparation of tissue in wound healing and regeneration [16].

RAW 264.7 macrophage and NIH 3T3 fibroblast cells were treated with variable concentrations of TBSE (6.25, 12.5, 25, 50, 100, and 200 µg/ml) for 24, 48, and 72 h. In Fig. 1, the results showed that the viability of macrophage cells treated with TBSE at concentrations 6.25–200 µg/ml for 24, 48, and 72 h was close to the untreated control. Similarly, fibroblast cells did not show reduced cell viability when exposed to TBSE of the same concentration (Fig. 2). There was no statistical significance between the cell viability of the control and TBSE-treated cells. Therefore, TBSE at concentrations 6.25–200 µg/ml is non-cytotoxic to macrophages and fibroblasts cells after 24, 48, and 72 h of exposure. This corroborates

Table 1: Evaluation of primary irritation/corrosion index

Primary irritation/ corrosion index	Classification of irritation/ corrosion
0	Non-irritant
>0–2	Mild irritant
>2–5	Moderate irritant
>5–8	Severe irritant

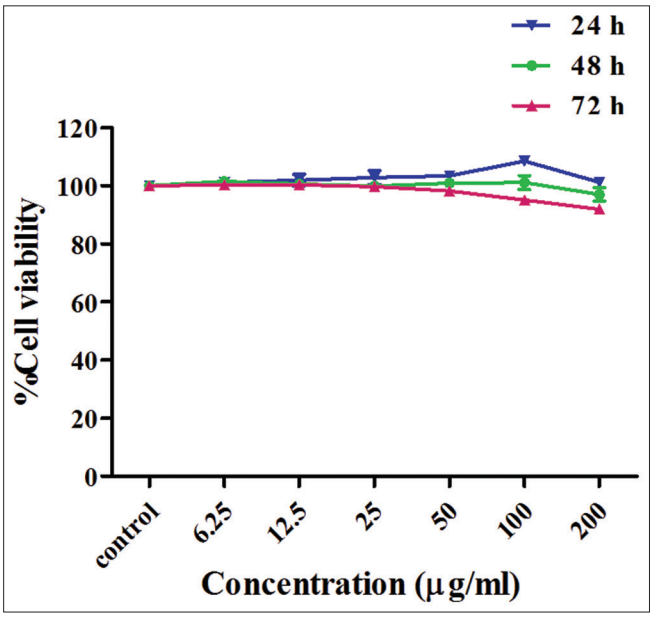

Fig. 1: Effects of *Trapa bispinosa* Roxb. starch extract at variable concentrations on macrophage cell viability after 24, 48, and 72 h of treatment

Table 2: The primary irritation/corrosion index of *Trapa bispinosa* Roxb. starch in rats

Skin characteristics	Time (h)	Primary irritation/corrosion index					Total
		200 mg/kg BW	1000 mg/kg BW	2000 mg/kg BW	2000 mg/kg BW	2000 mg/kg BW	
Irritation	1	0	0	0	0	0	0
	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
	72	0	0	0	0	0	0
Corrosion	1	0	0	0	0	0	0
	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
	72	0	0	0	0	0	0

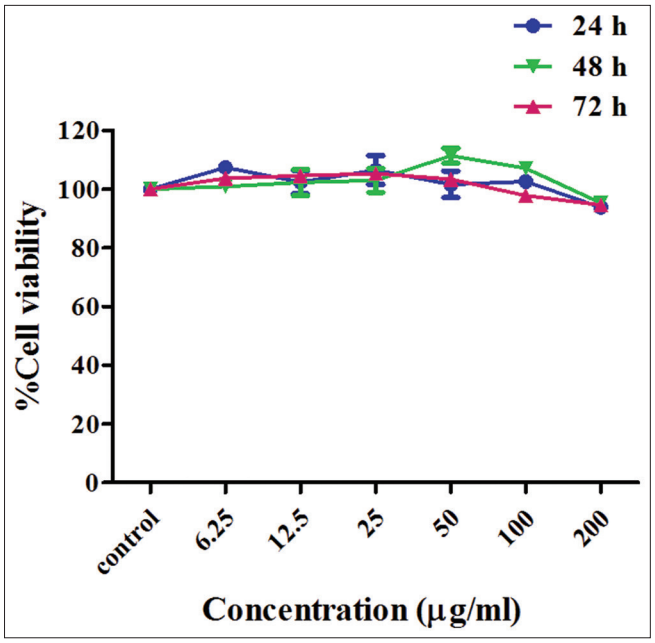

Fig. 2: Effects of *Trapa bispinosa* Roxb. starch extract at variable concentrations on fibroblast cell viability after 24, 48, and 72 h of treatment

with the results from a previous study that demonstrated the non-cytotoxic effects of methanolic and ethanolic *T. bispinosa* Roxb. fruit and peels extracts on RAW 264.7 macrophage cells [17].

The properties of TBS as a stabilizer in yogurt [18] and binder in diclofenac sodium tablets [6] have been studied. *T. bispinosa* Roxb. was also investigated for its potential development into starch-based biomaterials for medical applications such as wound dressing [19] and other applications in pharmaceutics and cosmetics [20]. The results from this study demonstrate the non-cytotoxic effects of TBS, which confirms its safety for further development in medical and pharmaceutical applications, or as ingredients of cosmetics products.

Acute dermal toxicity test

There was no evidence of skin irritation and corrosion after 1, 24, 48, and 72 h contact periods. The primary irritation and corrosion index were 0.0 (Table 2). TBS at 200, 1000, and 2000 mg/kg BW did not exhibit clinical signs of toxicity or mortality after 30 min, 2, 6, and 48 h of exposure or at the end of the observation period (14 days). Adverse effects were evaluated through changes in body weight and at necropsy. The treatment did not have any adverse effect on body weight, which increased progressively throughout the study period (Table 3). The external and internal gross pathological lesions in rats did not show the difference between the control and treated groups. These results indicated the

Table 3: Acute dermal toxicity tests of *Trapa bispinosa* Roxb. starch in rats

Parameters	200 mg/kg BW	1000 mg/kg BW	2000 mg/kg BW
Body weight, g (day 0)	231.89	240.08	234.94 ± 10.67[a]
Body weight, g (day 7)	246.78	242.18	244.56 ± 7.10[a]
Body weight, g (day 14)	259.06	268.82	256.86 ± 6.32[a]
Clinical signs (day 1–14)[b]	NAD	NAD	NAD
Mortality (day 1–14)[b]	NAD	NAD	NAD
Necropsy (day 15)[b,c]	ND	ND	NAD

[a] n=3; Values are expressed as mean ± standard deviation. [b]NAD: No abnormalities detected. [c]ND: No detected

Table 4: Acute oral toxicity tests of *Trapa bispinosa* Roxb. starch in rats

Parameters	300 mg/kg BW	2000 mg/kg BW
Body weight, g (day 0)[a]	205.98±10.90	229.86±8.33
Body weight, g (day 7)[a]	232.19±14.58	236.89±9.66
Body weight, g (day 14)[a]	242.89±13.71	251.91±9.69
Clinical signs (day 1–14)[b]	NAD	NAD
Mortality (day 1–14)[b]	NAD	NAD
Necropsy (day 15)[b]	NAD	NAD

[a] n=3; Values are expressed as mean±standard deviation. [b]NAD: No abnormalities detected

safety of the dermal TBS application. Moreover, according to the criteria for acute toxicity hazard categories ranked by the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), TBS is classified as category 5, which is considered to be relatively low acute dermal toxicity in rat [21]. Likewise, the primary dermal irritation study of modified gum acacia hydrocolloid, which is used as an emulsifier in cosmetics, showed non-irritating to slightly irritating [22].

Acute oral toxicity test

The results for acute oral toxicity of TBS are presented in Table 4. No deaths occurred in any animals that received 300 and 2000 mg/kg BW of the test item. The treatments did not have an effect on body weight during the study period. There were no adverse signs or behavioral changes in any treatment group. At necropsy, the test item revealed abnormalities in neither external nor internal gross pathological observation. Therefore, the oral lethal dose of TBS in rats was greater than 2000 mg/kg BW. Based on the GHS criteria for acute toxicity hazards, TBS demonstrated low acute oral toxicity [21]. There are many toxicity researches on starch that is used as pharmaceutical

excipients. Corn starch, a commonly used bulking agent, showed no-observed-adverse-effect level after 90 days of oral ingestion at 10,000 mg/kg BW/d in Sprague Dawley rats [23]. Another example, glutamate derived from potato starch is used as a superdisintegrant for drug tablets and capsules. Its acute and subacute toxicity study results showed no adverse effects on the behavior and gross pathology of rats up to the dose of 2000 mg/kg BW [24]. Banana starch, used as a viscosity modifier and thickening agent in the pharmaceutical industry, incurred neither death nor abnormal behaviors in mice with at the dose of 2000 mg/kg BW in an acute oral toxicity study [25].

CONCLUSION

According to the results of this study, TBS showed no cytotoxic effects on macrophage and fibroblast cells at concentrations 6.25–200 µg/ml. The acute dermal and oral toxicity studies demonstrated that TBS is safe and non-toxic up to the dose of 2000 mg/kg BW in rats. Therefore, TBS could be used as a pharmaceutical excipient or as an ingredient in cosmetic products. However, further subchronic and chronic toxicity studies should be conducted to ensure the long-term safety of perpetual TBS consumption.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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