WALAILAK JOURNAL
http://wjst.wu.ac.th Agriculture Technology and Biological Sciences

In vitro Evaluation of the Cytotoxicity of Starch from *Curcuma comosa* Rhizomes

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Received: 23 June 2013, Revised: 22 December 2013, Accepted: 20 January 2014

Abstract

Objective: The aim of the present study was to evaluate the cytotoxicity of the starch from *Curcuma comosa* rhizomes in HepG2 and Jurkat T cells. **Materials and methods**: The starch powder of *C. comosa* rhizomes was provided from a previous research project on the isolation and characterization of starch from the rhizomes of plants in the Ginger family (Zingiberaceae). Various concentrations of *C. comosa* starch solution were prepared and investigated for cytotoxicity effects in both HepG2 and Jurkat T cell lines after 24, 48 and 72 h incubation by using an 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the trypan blue exclusion test method. **Results:** The results demonstrated that the starch solutions at low concentrations (0.001, 0.01, 0.1 and 5 µg/ml) were not toxic to either type of cells, while at high concentrations (10, 50 and 100 µg/ml) they might be toxic. **Conclusions:** The starch from *C. comosa* rhizomes at lower concentration is safe to HepG2 and Jurkat T cells, and should be further investigated for biocompatibility by using appropriate cell models and methods before developing it for use in pharmaceutical applications.

Keywords: *Curcuma comosa*, starch, cytotoxicity, HepG2 cells, Jurkat T cells

Introduction

Starch is an abundant storage reserving carbohydrate in plants. It is found in many different plant organs, including seeds, fruits, tubers and rhizomes. The composition and structure of starch granules vary considerably among different plants [1]. It has been used for a long time and has many potential uses, including in the pharmaceutical and paper mining and building industries [2-3]. The classical functionalities of native starch in the past were as fillers, disintegrants, and binders in tablets, and as fillers in dermatological powders. Usually, maize starch, potato starch, corn starch and wheat starch are used and monographed in pharmacopoeias [1-2]. Rhizomes of plants in Zingiberaceae also have starch in high content, but at present, there are less pharmaceutical applications for their starch. *Curcuma comosa* Roxb. is an indigenous Thai medicinal plant, the rhizome of which is widely used for treatment of inflammation in postpartum uterine bleeding [4]. Recent research has found anti-inflammatory properties of polar and non-polar extracts of *C. comosa* and its diarylheptanoids, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene by suppressing the release of cytokines TNF-α and IL-1β through attenuating IKK-β expression and NF-κB activation [5]. However, there are no reports of using starch isolated from *C. comosa* rhizome in pharmaceutical applications. If the starch form *C. comosa* is proven to be safe and has appropriate properties, it may be used as an alternative source of cheap starch for pharmaceutical applications, and therefore increase the value of *C. comsa*. Before the starch is prepared to produce any types of pharmaceutical application, the researcher would like to preliminarily screen for *in vitro* cytotoxicity tests, and the outcome will be used in the decision for the appropriate form of pharmaceutical applications.

In general, cell culture systems provide convenient, controllable and repeatable methods for preliminary assessment of biological responses [6-8]. *In vitro* cell culture has been widely used to evaluate cytotoxicity of many biomaterials, such as denture relining [9], root canal filling materials [10], starch-based biomaterials [11], orthodontic composites [12], starch capped water soluble copper nanoparticles [13], denture adhesives [14] and eye drop applications [15]. The human hepatoma cell line (HepG2), and the human leukemic Jurkat T cell line were chosen as the *in vitro* cell culture model in the present study because: 1) they are representative of liver cells [16-20] and immune cells [21-22] in the human body respectively, 2) in human body, there are both adherent cells and suspension cells; HepG2 can also represent adherent cells and Jurkat T cells can also represent suspension cells. While there are many cytotoxicity tests available for measuring a variety of parameters [23-25], this study used an MTT assay measuring mitochondrial activity, and the trypan blue exclusion test method measuring cell membrane integrity, to determine cell viability. Because there are some opportunities that the *C. comosa* starch may be developed for pharmaceutical applications in some products, the objective of this study was to evaluate the cytotoxicity of starch isolated from *C. comosa* rhizome to both human hepatoma cells (HepG2) and human leukemic Jurkat T cells.

Materials and methods

Chemical

All chemicals and reagents were cell culture grade. Dulbecco's Modified Eagle medium powder (DMEM), Roswell Park Memorial Institute medium 1640 powder (RPMI 1640), fetal bovine serum (FBS), sodium pyruvate, non-essential amino acids, 0.25 % trypsin solution, penicillin, streptomycin, and amphotericin B were purchased from Gibco BRL,USA. 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 0.4 % trypan blue in Hanks' balanced salt solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *C. comosa* starch powder was provided from a previous research project on the isolation and characterization of starch from the rhizomes of plants in the Ginger family (Zingiberaceae) [26].

Cell culture

Cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The human hepatoma cells (HepG2) were routinely grown in a monolayer culture in the presence of DMEM supplemented with 10 % heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) at 37 °C in a humidified atmosphere of 5 % CO_2. The medium was replaced twice a week and cells were trypsinised and diluted at a ratio of 1:3 every 7 days. The cells were trypsinised and transferred into 96 well-plates and 60 mm cultured dishes for a cell viability assay.

The human leukemic Jurkat T-cells were maintained at 0.5 - 1.5×10^6 cells/ml in RPMI-1640 supplemented with 10 % heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) at 37 °C in a humidified atmosphere of 5 % CO_2. Cell suspension was transferred to 24 and 96 well-plates for a cell viability assay.

Sample preparation

The selection of the experimental concentrations was based on the fact that the substance could be absorbed by or make contact with individual cells. The concentrations were usually in the level of µM or µg/ml or mg/ml, and the starch was dissolved in a proper vehicle. The starch powder of *C. comosa* rhizomes was provided from a previous research project on the isolation and characterization of starch from the rhizomes of plants in the Ginger family (Zingiberaceae). Twenty mg/ml of the starch was taken and dissolved in 90 % dimethyl sulfoxide (DMSO) for 24 h at room temperature with constant shaking (60 rpm) [27], which was further sterilely filtered and diluted in a DMEM or RPMI 1640 medium to make a substock solution of 1,000 µg/ml, and then serially diluted to the final concentrations of 0.001, 0.01, 0.1, 1, 5, 10, 50 and 100 µg/ml. The maximum of DMSO concentration was 0.45 % (v/v), which was not toxic to cultured cells.

Cytotoxicity studies

HepG2 cells (adherent cells)

The MTT assay assesses cell viability by determining mitochondrial activity (succinate dehydrogenase), which reduces tetrazolium salt, MTT, to form a purple formazan product [22]. HepG2 cells were seeded at a density of 2×10^4 cells/well (200 µl/well) in 96 well-plates. Culture medium was replaced by the starch solution at 0.001, 0.01, 0.1, 1, 5, 10, 50 or 100 µg/ml (100 µl/well) 24 h after seeding, and incubation was continued at 37 °C for 24, 48 or 72 h. After incubation, the medium was removed. Each well was treated with 20 µl/well of MTT (5 mg/ml) in DMEM medium without phenol red, and were incubated for a further 4 h at 37 °C in a humidified atmosphere of 5 % CO_2. At this stage, the MTT was removed, and 200 µl/well of DMSO was added in order to dissolve the formazan crystals. The absorbance was measured at 540 nm in a multiwell microplate reader (Anthos Zenyth 200 microplate spectrophotometer). Cell viability percentage was calculated as 100 × (OD value derived from test wells - OD value derived from blank wells) / (OD value derived from control wells - OD value derived from blank wells).

The trypan blue exclusion test, determines the number of viable cells presented in a cell suspension, based on the principle that live cells possess intact cell membranes that exclude trypan blue dyes. Cells were seeded at 1×10^6 cells/dish in 60 mm culture dishes and cultured for 24 h. Then, cultures were treated with starch solution at 0.001, 0.01, 0.1, 1, 5, 10, 50 or 100 µg/ml, and incubated at 37 °C for 24, 48 or 72 h. At the end of incubation with starch solution, culture medium was aspirated and reserved. The HepG2 cells were detached from culture dishes by treatment with 0.25 % trypsin. After trypsinization, cells were suspended in DMEM medium and the culture medium was returned. The mixture was centrifuged at 800 × g for 5 min to concentrate the cells. Cell suspension and 0.4 % trypan blue in Hanks' balanced salt solutions were mixed, and the number of viable cells was counted using a hemocytometer. The percentage of viable cells (cell viability) was calculated as 100 × (unstained cells) / (stained + unstained cells).

Jurkat T cells (suspension cells)

For the MTT assay, Jurkat T cells were seeded at a density of 1×10^5 cells/well (200 µl/well) in 96 well-plates and the processes as described above were done.

For the trypan blue exclusion test, Jurkat T cells were seeded at a density of 1×10^6 cells/well (200 µl/well) in 24 well-plates, and the processes as described above were carried out.

Cytotoxicity rating

Cytotoxicity was rated based on cell viability relative to controls as; non-cytotoxic (more than 90 % cell viability), slightly cytotoxic (60 - 90 % cell viability), moderately cytotoxic (30 - 59 % cell viability), and severely cytotoxic (less than 30 % cell viability) [9].

Statistical analysis

Data are expressed as a mean ± SEM of at least 3 independent samples. Comparisons were performed by using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test. Differences were considered significant when $p < 0.05$.

Results

Cytotoxicity studies in HepG2 cells

MTT assay

HepG2 cells were plated at 2×10^4 cells/well in 96 well-plates. They were exposed to 0.01 % DMSO in medium as a control and exposed to starch solutions at concentrations of 0.001, 0.01, 0.01, 0.1, 1, 5, 10, 50 and 100 µg/ml for 24, 48 and 72 h. After incubation, cell viability was assayed by the MTT. The results showed that cell viability when cells were exposed to starch solutions at concentrations of 0.001, 0.01, 0.1, 1, 5, 10 and at 50 µg/ml were not significantly different from the control; therefore, these concentrations were not toxic to HepG2 cells after 24 h incubation. However, incubation with starch solution 50 µg/ml for 48 and 72 h, cell viability was significantly different ($p < 0.005$) when compared

with the control. At 100 µg/ml, cell viability showed a significant difference ($p < 0.005$) from the control and was slightly toxic after 24, 48 and 72 h incubations (**Figure 1**).

Trypan blue exclusion

HepG2 cells were seeded at 1×10^6 cells/dish in 60 mm culture dishes. For control culture dishes, cells were exposed to 0.01 % DMSO in medium. Experimental culture dishes were exposed to starch solutions at concentrations of 0.001, 0.01, 0.01, 0.1, 1, 5, 10, 50 or 100 µg/ml for 24, 48 and 72 h. After cells were detached from culture dishes by trypsinization, they were tested using the trypan blue exclusion test. The results showed that starch solutions at concentrations of 0.001, 0.01, 0.01, 0.1, 1 and 5 µg/ml were non-toxic after being exposed to cells for 24, 48 and 72 h (**Figure 2**). Starch solutions at 10 and 50 µg/ml resulted in significantly different ($p < 0.005$) cell viability compared with the control after being exposed to cells for 24, 48 and 72 h, indicating that they were slightly toxic, and at 100 µg/ml, cell viability was significantly decreased, and was shown to be slightly toxic after 24, 48 h exposure and was shown to be moderately toxic (52.67 ± 4.33 cell viability) after 72 h exposure.

Cytotoxicity studies in Jurkat T cells

MTT assay

Jurkat T cells were plated at 1×10^4 cells/well in 96 well-plates, exposed to 0.01 % DMSO in medium as a control, and exposed to starch solutions as an experiment at concentrations of 0.001, 0.01, 0.01, 0.1, 1, 5, 10, 50 and 100 µg/ml for 24, 48 and 72 h. After incubation, cell viability was assayed by MTT. The results showed that starch concentrations at 0.001, 0.01, 0.01, 0.1, 1, 5 and 10 µg/ml were non-toxic to Jurkat T cells when exposed to the cells for 24, 48 and 72 h (**Figure 3**). Starch concentrations at 50 and 100 µg/ml cell viability were significantly different ($p < 0.005$) from the control and were shown to be slightly toxic after 24 h exposure, and were shown to be moderately toxic after time exposure of 48 and 72 h.

Trypan blue exclusion

Jurkat T cells were seeded at 1×10^6 cells/well in 24 well-plates. Control wells were exposed to 0.01 % DMSO in medium. Experimental culture wells were exposed to starch solutions at concentrations of 0.001, 0.01, 0.01, 0.1, 1, 5, 10, 50 or 100 µg/ml for 24, 48 and 72 h. After incubation, cells were tested by using the trypan blue dye exclusion test. The results showed that starch concentrations at 0.001, 0.01, 0.01, 0.1, 1, 5 and 10 µg/ml were non-toxic, whereas starch solutions at 50 and 100 µg/ml caused cell viability to be significantly decreased and were shown to be severely toxic after 24, 48 and 72 h exposure time (**Figure 4**).

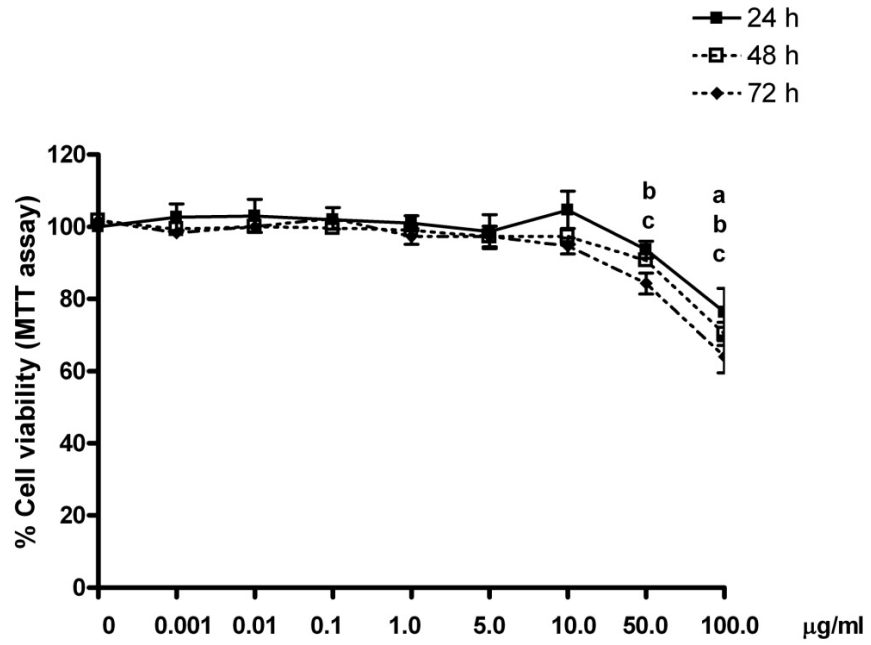

Figure 1 Effects of starch solution at various concentrations on HepG2 cell viability by using MTT assay after co-incubation for 24, 48 and 72 h. Data representative of 3 independent experiments were expressed as mean ± SEM; a, b, c letters are significantly different ($p < 0.05$) for 24, 48 and 72 h, respectively.

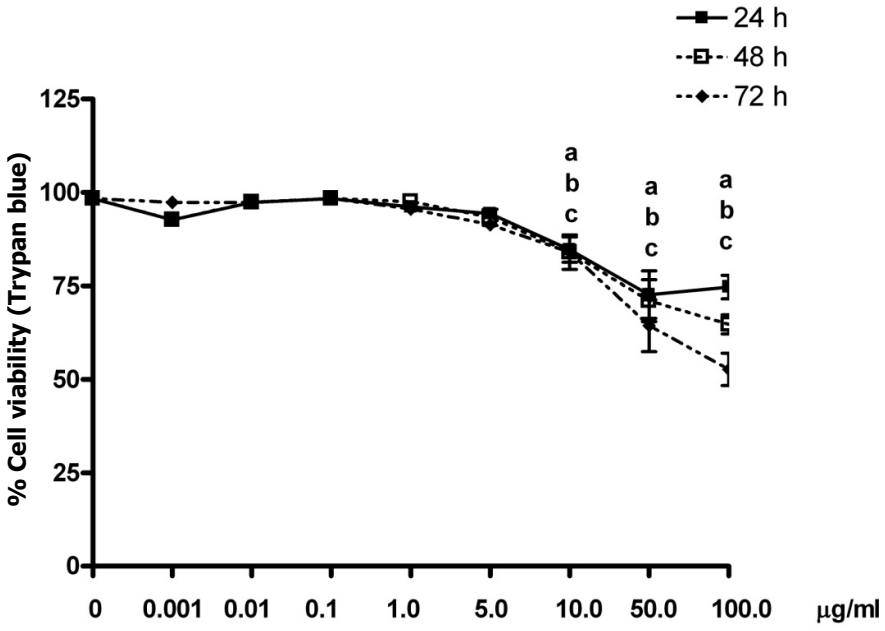

Figure 2 Effects of starch solutions at various concentrations on HepG2 cell viability by using trypan blue exclusion after co-incubation for 24, 48 and 72 h. Data representative of 3 independent experiments were expressed as mean ± SEM; a, b, c letters are significantly different ($p < 0.05$) for 24, 48 and 72 h, respectively.

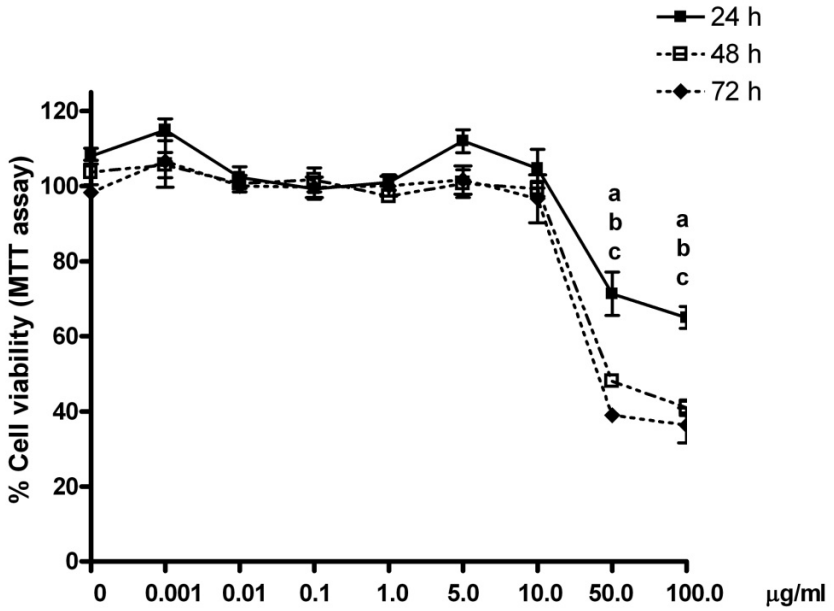

Figure 3 Effects of starch solutions at various concentrations on Jurkat T cell viability by using MTT assay after co-incubation for 24, 48 and 72 h. Data representative of 3 independent experiments were expressed as mean ± SEM; a, b, c letters are significantly different ($p < 0.05$) for 24, 48 and 72 h, respectively.

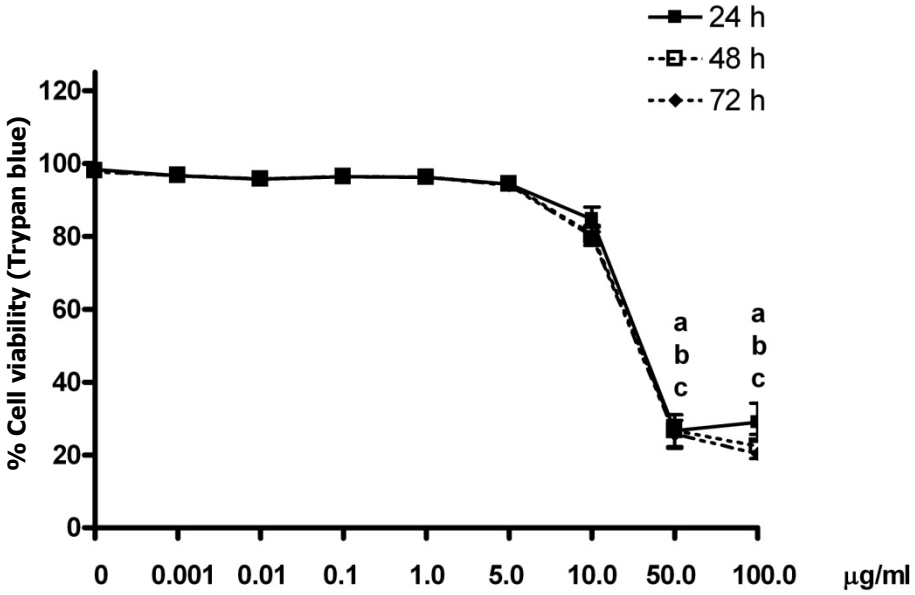

Figure 4 Effects of starch solutions at various concentrations on Jurkat T cell viability by using trypan blue exclusion after co-incubation for 24, 48 and 72 h. Data representative of three independent experiments were expressed as mean ± SEM; a, b, c letters are significantly different ($p < 0.05$) for 24, 48 and 72 h, respectively.

Discussion

The rhizomes of the Zingiberaceae family are natural resources that provide many useful products for food in many Asian countries, and some species used constitute a great part of Thai traditional medicine. In addition, their rhizomes have been reported to have a high starch content. *C. comosa* is an indigenous Thai medicinal plant, the rhizome of which is widely used for treatment of inflammation in postpartum uterine bleeding. Recent research has found anti-inflammatory properties of polar and non-polar extracts of *C. comosa* and its diarylheptanoids. However, there are no reports of using starch isolated from *C. comosa* rhizome in pharmaceutical applications. If the starch form *C. comosa* is proven to be safe and has appropriate properties, it can be used as an alternative source of cheap starch for pharmaceutical applications, and therefore increase the value of *C. comsa*.

In vitro cell cultures have been widely used to evaluate cytotoxicity of many biomaterials. The tests should be performed using the most appropriate cells [8]. In this study, the researcher used two types of cell lines, HepG2 cells and Jurkat T cells, which are used as models for a preliminary cytotoxicity test, because HepG2 can represent liver cells and Jurkat T cells can represent immune cells; although both cell types are cancer cells, they are used worldwide as an *in vitro* model for toxicity testing, because they are developed from human cells and have similar characteristics to human normal cells [16-21]. There is the possibility that the components of starch are exposed to liver cells as well as immune cells in the body. Further, there are both adherent cells and suspension cells in human body. HepG2 can represent adherent cells, whereas Jurkat T cells can represent suspension cells. Adherent cells are said to be anchorage-dependent, and attachment to a substratum is a prerequisite for proliferation. They are generally subjected to contact inhibition, which means they grow as an adherent monolayer and stop dividing when they reach such a density that they touch each other. Most cells, with the exception of mature hemopoietic cells and transformed cells, grow in this way. In contrast to anchorage-dependent cells, cells cultured from blood, the spleen, or bone marrow adhere poorly, if at all, to the culture dish. In the body, these cells are held in suspension, or are only loosely adherent. For a cell culture system, suspension cultures are easier to propagate, since a subculture only requires dilution with medium. Cultures in which cells grow attached to each other or to a substratum have to be treated by a proteolytic enzyme to break the bond between cells and substratum. The most commonly used enzyme is trypsin [6].

Here, the 2 tests were used to determine cell viability and to evaluate cytotoxicity. The MTT assay assesses cell viability by using a function of mitochondrial succinate dehydrogenase which reduces tetrazolium salt to form a purple formazan product, which is subsequently dissolved by DMSO and the absorbance measured. The trypan blue exclusion test determines the number of viable cells in a cell suspension, based on the principle that live cells possess intact cell membranes that exclude the dyes, and determines viability by cell counting with a hemocytometer. The steps of preparation of each cell type to test by these methods were different because the HepG2 cell is an adherent cell which needs to be detached from culture flask or culture dish by trypsinization, whereas the Jurkat T cell is a suspension cell which does not need trypsinization. However, the outcomes from the 2 *in vitro* cytotoxicity tests are determined in the same way for each cell type. The results of the present study showed that the *C. comosa* starch at low concentrations of 0.001, 0.01, 0.1, 1.0 and 5 µg/ml were not toxic to either cell type after being exposed for 24, 48 and 72 h using either the MTT assay or the trypan blue exclusion test. For the HepG2 cells, starch solutions at concentrations of 10 and 50 µg/ml were non-toxic, and 100 µg/ml was slightly toxic after being exposed for 24 and 48 h and 50, 100 µg/ml of starch solutions exposed to the cells for 72 h were slightly toxic when using the MTT assay, while the trypan blue exclusion test gave results which at 10 and 50 µg/ml were slightly toxic after being exposed for 24, 48 and 72 h, at 100 µg/ml was slightly toxic at 24, 48 h, and was moderately toxic at 72 h. For the Jurkat T cells, 10 µg/ml of solution was non-toxic by using both methods after 24, 48 and 72 h exposure. 50 and 100 µg/ml concentrations were slightly toxic at 24 h and were moderately toxic after exposure time of 48 and 72 h by using the MTT assay, while the trypan blue exclusion test demonstrated that 50 and 100 µg/ml were severely toxic to Jurkat T cells after being exposed for 24, 48 and 72 h. These different outcomes might have occurred because 1) the sensitivity of cell viability assay methods are different [23] and 2) the sensitivity of adherent and suspension cells to each assay are different [28]. The present study showed

that low concentrations (0.001, 0.01, 0.1 and 5 μg/ml) were not toxic to both cell types, while high concentrations (10, 50 and 100 μg/ml) might be toxic. One possible explanation may result from the contamination of other substances used in the process of starch solution, which may have interfered with cell functions, and the higher concentrations of starch solution, the more concentration of contaminants. Therefore, this presumption would need to be clarified before the starch was introduced for development for pharmaceutical applications or use in any other products.

Conclusions

The starch isolated from *C. comosa* rhizomes at low concentrations have no toxicity in HepG2 and Jurkat T cells; this shows that the starch may be quite safe to be developed for pharmaceutical applications in the future. However, further biocompatibility studies are needed, by using appropriate both *in vitro* and *in vivo* models.

Acknowledgments

This work was supported by a research grant from Srinakharinwirot University. It was a part of a research plan entitled "Development of pharmaceutical applications derived from local Thai plants". The author would like to thank Associate Professor Dr. Ornlaksana Paeratakul, head of the research plan, for valuable suggestions, and Associate Professor Dr Weerasak Samee for providing starch powder of *C. comosa*.

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