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Research Paper

In vitro cytotoxicity of *Jatropha curcas* in epithelial and fibroblast cells

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ABSTRACT

The latex of *Jatropha curcas* is herbal medicine that is traditionally used as dental pain relief. To abolish the pain, the latex was dripped to the dental cavity, which may be exposed and may cause toxic effect to oral mucosa to happen. The aim of this study is to detect cytotoxicity of extracted latex of *J. curcas* to epithelial and fibroblast cells. The cells ($1x10^5$) were cultured in 96 well plates and allowed to attach for 5 days before treatment with serial concentration of *J. curcas* for 24 h period. The cytotoxicity assay of epithelial and fibroblast cells was performed using MTT assay to determine inhibition concentration 50 (IC_{50}). In both of cells toxicity were detected dose-dependent, and IC_{50} value for epithelial and fibroblast cells were 4339µg/ml and 3876µg/ml respectively. Morphologically both of treatment cells were smaller than control cells. Thus *J. curcas* was toxic to ephithelial and fibroblast cells

Keywords: *Jatropha curcas*; Cytotoxicity; Epithelial and fibroblast cells.

INTRODUCTION

Dental pain is a common illness suffered by the society. To overcome the dental pain people in rural area often use herbal medicine instead of visiting the dentist. The latex of *Jatropha curcas* is herbal medicine that is traditionally used as dental pain relief (Handayani, 2003). To abolish the pain, traditionally the latex dripped to the dental cavity, which may be exposed and may cause toxic effect to oral mucosa to happen Scientific data regarding analgesic effect of latex *J. curcas* on the dental pain have been widely studied (Siregar, 2000; Matulada, 2005; Irmaleny, 2010). Phytochemical content and simple standardized herbal medicine are recognized (Siregar, 2000; Irmaleny, 2010). The local safety based on the effect of latex *J. curcas* on the gingival fibroblast cells and periapical tissue has been studied (Siregar, 2000). However, *J. curcas* in latex preparation is categorized as empirical based herbal medicine. To develop latex *J. curcas* to be scientific based herbal medicine, herbal medicine must be prepared as extract. The study of standard parameter of extract *Jatropha curcas* (specific and non specific) was determined. The systemic safety based on the acute and sub-chronic toxicity test of extracted *J. curcas* were reported; it was safe for short term usage but be careful in long term usage (Irmaleny, 2010). Analgesic effect of extracted *J. curcas* as well as substance P (SP) and cyclocxygenase 2 (COX2) have

been reported (Irmaleny, 2010). Local safety to oral mucosa of extracted *J. curcas* has not been reported yet. Local safety of extracted *J. curcas* to oral mucosa could be evaluated through cytotoxicity assay. The aim of our study was to detect cytotoxicity of extracted latex of *J. curcas* to epithelial and fibroblast cell.

The MTT assay is the most common employed for the detection of cytotoxicity of cell viability following exposure to toxic substance and to determine IC_{50} value. The other is investigation of cytotoxic effects due to the cells alteration of cell morphology and cytoskeleton by light and electron microscopy. Finally the protein assay is an indirect measurement of cell viability since it measures the protein content of viable cells.

Here we evaluate cytotoxic of *Jatropha curcas* on epithelial and fibroblast cells based on IC_{50} value, cell morphology, and the protein content of viable cells.

MATERIAL AND METHODS

***Jatropha curcas*:** Latex of the *Jatropha curcas* (Family-*Euphorbiaceae*) was collected in June of 2008, identified by Dr. Eko Baroto Waluyo from Indonesian Institute of Science Research Center for Biology, and their voucher speciment number is 778/IPH.1.02/If.8/2008. The latex of the *Jatropha curcas* was collected from Balai Penelitian Tanaman Obat dan Aromatik (BALITRO), Bogor, Indonesia. The stem of the tree was cut and a bottle was hanged to collect the fresh latex. The plants used for latex collection were one and half years old. Latex was collected into vials containing a few drops of 95% ethanol to prevent browning and oxidation (Osoniyi and Onajobi, 2003). To obtain the extract of *Jatropha curcas*, the collection of latex was done by cold maceration, and was stored at – 20°C.

***Cell cultures and treatments*:** The protocol for the present research was approved by research ethics committee at the Faculty of Dentistry University of Indonesia (No.31/ethical Clearance/FKGUI/VI/2011).

Epithelial cells lines (Hacat cells) were obtained from Section of Molecular Embryology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Life Technologies, California, USA), supplemented with 10% fetal bovine serum (FBS, Caisson laboratories Inc, Utah, USA), 1% penicillin-streptomycin (10,000 units of penicillin and 10mg of streptomycin, Caisson laboratories Inc, Utah, USA) in humidified atmosphere of 5% CO_2, 95% air at 37°C.

Tissue collection and cell culture of primary human gingival fibroblast (Sabaliauskas, V., 2011): Human gingival subepithelial tissues were obtained from a healthy patient undergoing odontectomy procedure in the third molar region. Immediately after the biopsy, the tissue (2-3 mm^3 in size) was placed in Dulbecco's modified Eagle's medium (DMEM) enriched with 250U/ml penicillin, 0.25mg/ml streptomycin, 0.05mg/ml gentamycin and 200U/ml Nystatin for transportation. Then the tissue specimen was minced under sterile conditions and placed into the six wells plate with DMEM as referred to Hacat cells culture. The process of the cell movement from the explants commenced at the 10^{th} to 14^{th} day. After the monolayer was completely formed, the cells were subcultured by using tripsin/EDTA mixture and maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Caisson laboratories Inc, Utah, USA), 1% penicillin-streptomycin (10,000 units of penicillin and 10mg of streptomycin, Caisson laboratories Inc, Utah, USA) in humidified atmosphere of 5% CO_2, 95% air at 37°C.

The passage number range for both cells was maintained between 5 and 9. The cells were cultured in 75cm^2 cell culture flasks. For experimental purposes, cells suspension containing 1X10^{5}cell/200µl was inoculated in 96 well flat-bottom plates (200µl of cell solution/well). Cells were allowed to attach for 5 days before being exposed to different concentrations of extracted *Jatropha curcas*.

The stock solution of extraxted *Jatropha curcas* was made in DMSO 10% and filtered with Minisart Filters (0.45µm). A working solution in the corresponding media was prepared. Cell monolayers were incubated for 24h in experimental medium containing *Jatropha curcas* at final concentration of within a range of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 5%. The particular treatment dose was based on the preliminary study.

***Protein measurement*:** Determination of the protein content is based on the Bradford method (Bradford, 1976) using the Coomassie Protein assay reagent (Thermo scientific, Pierce, Rockford). Inhibitory Concentration 50% of extracted *J. curcas* was calculated and exposed to culture of Hacat and Fibroblast cells for 24h. Cells were washed with PBS and tripsinize cells with tripsin-EDTA in HBSS for 5 min and followed by adding complete media culture. Centrifuge in a sterile 15ml falcon tube at 1000rpm for 10min. Protein cell was isolated by trizol reagent. Aliquots of standard bovine serum albumin or protein cells samples were pipetted in 96 polystyrene wellplate and mixed with equal volume of Coomassie reagent. The absorbance was measured at 595nm using microplate reader (BioRad, Benchmark, California, USA). Results are presented as µg/ml protein.

***MTT assay*:** The MTT assay is based on the protocol of MTT (Sigma, Aldrich). Briefly, for the purposes of experiments at the end of the incubation time (24h), remove media culture carefully, and add 20µl of 5 mg/ml MTT solution to each well (96 well plate), incubate for 3h at 37°C in incubator. Add 150µl acidified isopropanol, cover with tinfoil, and the plate was then placed on a shaker for 1h at room temperature. The absorbance was measured spectrophotometrically at 490nm using a microplate reader (BioRad, Benchmark, California, USA). Each experiment was performed in triplicate. The same cells without exposure to *J. curcas* were served as controls. The percentace of cell viability was calculated using the following formula: (mean experimental absorbance/mean control absorbance) X 100%. The cytotoxicity of *J. curcas* was stated as a value of IC_{50}.

IC_{50} value was obtained by analysis of the percentage of inhibition by *J. curcas* at eight different concentrations and was calculated from the cytotoxicity curves (Graph Pad Software, Inc, California, USA).

***Morphology of cell monolayer after* exposure to *Jatropha curcas*:** The intensity of cell growth including cells morphology was observed visually with an inverted microscope (Axiovert 40 CFL, Carl Zeiss) by assessing the quality of cell monolayer exposed to 24h to *J. curcas* and cell control, with original magnification 100X.

Statistical analysis: All experiments were performed in duplicate of three different experiments and statistical analysis was performed by Student t-test with GraphPad Prism 5 Software. Significance was determined at $P < 0.05$.

RESULTS

Epithelial cell and oral Fibroblast cell were treated with eight different concentrations of extracted *J. curcas* for 24h, and cytotoxicity was determined with the protein assay and the MTT assay and also morphologically. Based on three independent experiments, IC_{50} values were determined by using Non linear regression: Dose

response-inhibition (log {inhibitor}) vs. normalized response (variable slope) in GraphPad Prism 5 software. Tables 1 and 2 represent dose-effect relationship of extracted *J. curcas* on epithelial and fibroblast cells respectively, after exposure to eight different concentrations of extracted *J. curcas* for 24h. Cell viability data are presented as percentage of control, and dose is presented as percent (n=3).

Concentration causing cytotoxic activity was dose dependent on both of the cells tested. The IC_{50} values of epithelial and fibroblast were 4339µg/ml and 3876µg/ml, respectively. Figure 2 presents epithelial cell monolayers, non exposed control culture (A) and exposed with IC_{50} extracted *J. curcas* for 24 h (B), human primary gingival fibroblast monolayer non exposed control culture (C), and exposed with IC_{50} extracted *J. curcas* for 24h (D). Cells monolayers epithelial and fibroblast were none exposed with extracted *J. curcas,* revealing normal morphology. Meanwhile, cells monolayers epithelial and fibroblast were exposed with IC_{50} extracted *J. curcas* for 24h revealing that a part of cells is morphologically smaller than control cell. Table 3 shows the total protein cells decreasing significantly ($P<0.05$) in both cells exposed to IC_{50} extracted *J. curcas* for 24h in compared to control cells.

DISCCUSSION

To abolish the dental pain, traditionally the latex of *J. curcas* was dripped to the dental cavity, which may be exposed and may cause toxic effect to oral mucosa to happen. An objective of the present study is to evaluate the cytotoxic effecs of extracted *J. curcas* on epithelial dan fibroblast cells. Herbal medicine is categorized as Empirical based herbal medicine, scientific-based herbal medicine and clinical herbal medicine (Chaerunissa, 2011). Extracted *J. curcas* is categorized as scientific-based herbal medicine. Extract is one of the herbal medicine preparations where its quality and its chemical composition have been known (Ansel, 1989). The effectiveness and safety extracted *J. curcas* as scientific-based herbal medicine are based on Acute toxicity (LD_{50}), subchronic toxicity, and the effect to substance P (SP) and COX-2 in animal models has been reported (Irmaleny, 2010). Oral mucosa is the tissue that covers mouth surface, in spite of the fact that extracted *J. curcas* has been used directly to dental cavity, and locally safety of extracted *J. curcas* to oral mucosa could be evaluated through cytotoxicity assay.

In Vitro cytotoxicity assays can be used to predict human toxicity and for general screening of chemical substances (Clemedson and Ekwall, 1999; Scheers, et al., 2001). It has been previously reported that different results depending on the test agent used and the cytotoxicity assay employed (Weyermann, et al., 2005). MTT assay being the most sensitive in detecting cytotoxic event, causing a cytotoxicity assay based on mitochondrial respiratory activity, would give early signs of toxicity following exposure to a mitochondrial toxicant (Fotakis, et al., 2006). Cell cultures derived from humans and animals have been used during the past 30 years for determination of cytotoxic effect caused by dental materials. Permanent cell lines and primary cells have been used as a model (Sabaliauskas, 2011). Various biological endpoints are used for the investigation of cytotoxic effects due to the cells, evaluation of enzyme activity, alteration of cell morphology and cytoskeleton by light and electron microscopy and determination of cell growth inhibition and of ED_{50} (effective dose which causes a 50% reduction of cell proliferation) (Geurtsen, 2001).

Our study revealed that based on MTT assay, extracted *Jatropha curcas* is toxic to epithelial and fibroblast cells in dose-dependent. Epithelial cells appear to be

more sensitive than fibroblast cells as indicated by IC_{50} value as well as dose- effect relationship presented in Table 1 and Table 2. MTT assay is mainly based on mitochondrial respiratory activity or on the enzymatic conversion of MTT in the mitochondria. It is thought that the inhibition of the mitochondrial respiration induces active oxygen related cell death. Reactive oxygen species can be generated within the mitochondria which can also damage mitochondrial components (Koizumi, et al., 1996). On the other hand, cells reveal morphological changes in both cells after exposure to IC_{50} value *Jatropha curcas*, presented in Figs 2(A-D). The fact that cells morphological changes in size compared to control cells indicated that *Jatropha curcas* contains toxic materials, which may affect the cell membrane. Our result is also supported by the work of Lewis and Van den Berg in 1995. Curcain a protease phytotoxin has been extracted and purification from latex of *J. curcas* (Nath,L.K., 1991). Curcacylin A a novel cyclic actapeptide is also isolated from the latex of *J. curcas* that inhibits proliferation human cell T (Van den Berg AJ, et al., 1995). Irmaleny (2010) reported that one of the chemical content of extracted *J. curcas* is diterpene. Devappa in 2011 reported that curcasones C dan D are diterpenes in *J. curcas* that have cytotoxic biological activities. Siregar (2000) reported that latex *J. curcas* in 300µg/ml 24 h observation caused decreased gingival fibroblast cell viability by 50%; however, in this study IC_{50} value has not been reported yet. In our study IC_{50} value extracted *J. curcas* for gingival fibroblast investigated is based on Graph Pad Software, Inc, California, USA was 3876µg/ml. The differentiation of the results may be caused by different biological effects of latex and extracted preparation of *J. curcas*.

The results from our studies are in accordance with these data concerning the 24h exposure of both cells to *J. curcas*. However, further time-course experiment analyzing the optimal action time of extracted *J. curcas* and expression of cell death-related molecules are needed to investigate, to understand more detailed mechanisms of action of extracted *J. curcas* as toxic material.

Scientific data regarding analgesic effect of *J. curcas* latex have been widely studied; it was reported that the latex had the analgesic effect and had the ability to decrease the level of PGE2 in inflamed tooth pulp of *Macaca nemestrina* (Matulada, 2005). It was also reported that either the latex or the extract had analgesic effect through the writhing test. Dose effective of *J. curcas* for analgesic is 500mg/kg BW, and the phytochemical content and standardized parameters are recognized. It can decrease the concentration of substance P (SP) in vivo, but the effect to COX-2 is not clear (Irmaleni, 2010). It is also considered safe for short-term usage but should be careful in long-term usage. This study determines toxicity to the oral mucosa as preparation to be performed as scientific-based herbal medicine.

CONCLUSION

We can conclude that *Jatropha curcas* extracts toxic to ephitelial and fibroblast cells in dose dependent. This study is hoped to give the contribution in developing *J. curcas* as standardized herbal medicine, in determining safety to the gingival, as preparation to performed clinical test as pain killer and tooth devitalized in dentistry.

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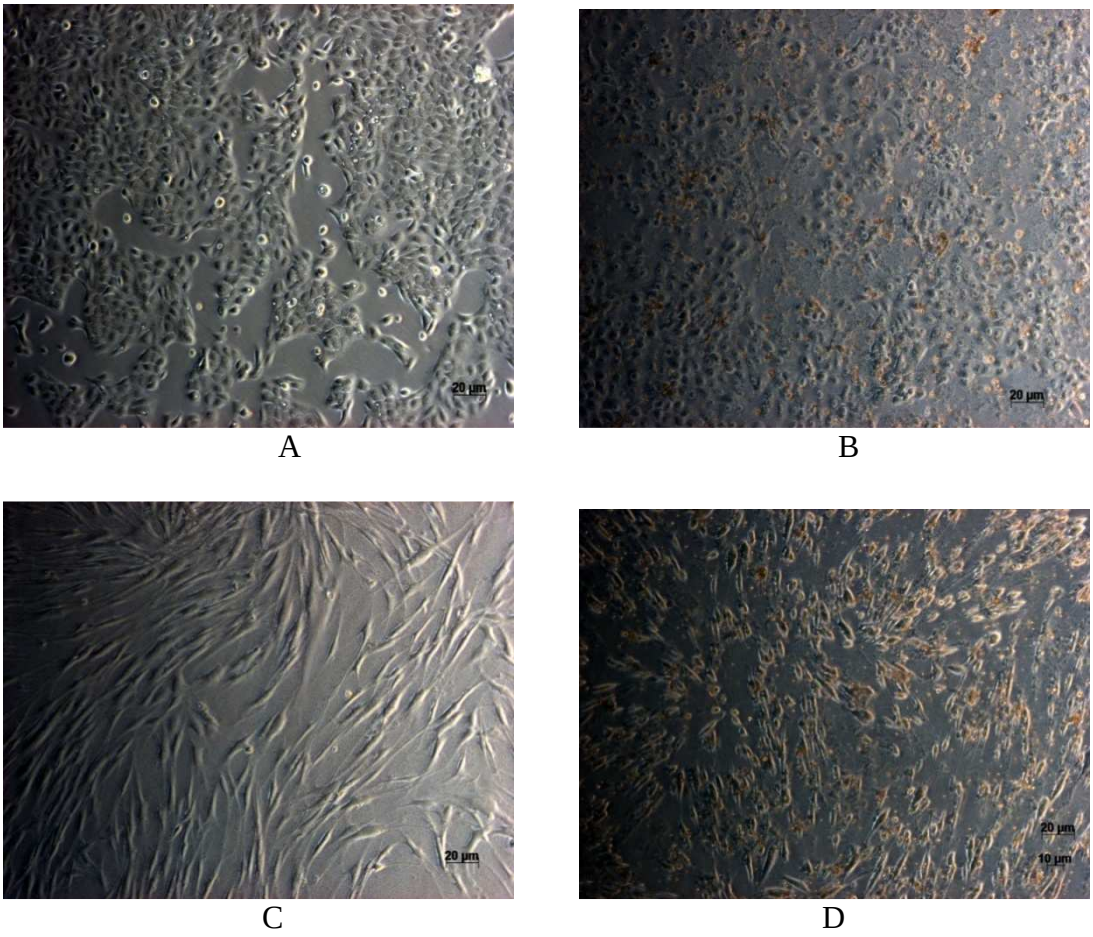

A B

C D

Figure-1: Epithelial cell monolayers control (A) and Human primary cells reveal gingival fibroblast monolayers control (C), cells reveal normal morphology. Epithelial cell line (B) and Human primary gingival fibroblast monolayers (D) threated with IC_{50} of extracted *Jatropha curcas* showed morphological change. Original magnification 100X

Table-1: Effect on epithelial cells after exposure to eight different concentrations of extracted *Jatropha curcas* for 24 h.

Concentration of *J. curcas*	Cells viability ± SD
0	100 ± 0
0.05	75.02 ± 6.80
0.1	66.80 ± 5.52
0.2	62.16 ± 8.65
0.4	51.74 ±10.03
0.8	40.72 ±13.18
1.6	27.99 ± 3.48
3.2	25.25 ± 4.59
5	24.45 ± 3.19

- Cell viability data presented as percentage of control and dose presented as per cent. (n=3)

Table-2: Representation of dose-effect relationship of extracted *J. curcas* on fibroblast cells after exposure to eight different concentrations of extracted *J. curcas* for 24 h.

Concentration of *J. curcas*	Cells viability ± SD
0	100 ± 0
0.05	71.96 ± 15.16
0.1	54.89 ± 3.35
0.2	54.77 ± 3.80
0.4	51.85 ± 5.13
0.8	40.46 ± 8.06
1.6	38.98 ± 6.51
3.2	30.70 ± 10.70
5	19.93 ± 3.11

- Cell viability data presented as percentage of control and dose presented as per cent. (n=3)

Table- 3: Protein content (µg/ml) of Epithel and fibroblast cells, after exposure to IC_{50} *J. curcas* for each cell.

Cells	Control cells	Cell exposed to IC_{50} *J. curcas*
Epithel	1220.60 ±41.95	750.43 ±110.48
Fibroblast	1926.61 ± 88.28	1381.42 ±108.02

