In vitro cytotoxicity of Artocarpus lakoocha aqueous extract and oxyresveratrol in SH-SY5Y cells

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Abstract. Artocarpus lakoocha is a traditional plant found in South/Southeast Asia, which has been used for centuries as an antelmintic in the form of an aqueous extract. This extract, also called puag haad in Thailand, contains oxyresveratrol, a stilbene, as a major active compound of puag haad. To evaluate biological activity of these tested compounds, cytotoxicity study was conducted to determine proper doses of the tested compounds in SH-SY5Y neuroblastoma cells. SH-SY5Y cells are a neuroblastoma cell line which can be used to study several neurodegenerative diseases in cell cultural models. This study aimed to examine cytotoxicity of A. lakoocha aqueous extract and oxyresveratrol in SH-SY5Y neuroblastoma cells by XTT and LDH assays. The result of this study demonstrated that ≤25µg/ml of puag haad and ≤200 µM of oxyresveratrol did not show toxicity to SH-SY5Y neuroblastoma cells. The concentrations at ≤25µg/ml of puag haad and ≤200 µM of oxyresveratrol could be used to investigate neuroprotective effect using SH-SY5Y neuroblastoma cells. Further studies are required to investigate the effect of A. lakoocha aqueous extract and oxyresveratrol related to neurodegenerative disease in cell culture system.

1. Introduction
Artocarpus lakoocha Roxb. is a tropical medicinal plant belonging to the Moraceae family found in South and South East Asian countries such as India, Nepal and Thailand [1]. The characteristics of A. lakoocha are its green leathery leaves with rough under surface. The tree grows to 15–20 m in height with brownish grey or dark brown scaly bark. The fruits are generally eaten fresh [2].

A. lakoocha heartwood extract has been traditionally used in Thailand as an antelmintic treatment for many centuries. Methanolic extracts from the leaf of A. lakoocha has various biological activities as an anti-inflammatory, an analgesic, a CNS depressant, and antidiarrheal action [3]. Additionally, an in vitro study by Raghavendra, H., et al [4] showed that A. lakoocha Roxb acts as an anticarcinogenic, pancreatic lipase), inhibitor and is an antioxidant, tyrosinase inhibitor [5, 6], with anticanidial and antibiofilm [7] and antelmintic and insecticidal actions [8] Many phytochemicals have been identified in extracts from A. lakoocha suggesting it might have clinical applications. One such constituent is, oxyresveratrol, which is a major active component of this plant, has been reported to have several biological activities, including being a tyrosinase inhibitor [9], an anti-inflammatory [10], and is a treatment against the herpes simplex virus (HSV-1) [11], as well as demonstrating anti-adipogenesis activity [12]. Additionally, oxyresveratrol has been indicated as a potential compound for treating several neurodegenerative diseases such as Alzheimer’s disease, Parkinsons disease [13] and
brain traumatic injury [14]. Treatment might be achieved by oxyreveratrol mediating of various signaling pathways, as demonstrated in cell culture studies.

Cytotoxicity screening tests of the extract or of single compounds have been performed to determine the concentrations of the test compounds which delineate biological actions, without interfering toxic actions. To date, there have been no cytotoxicity studies of puag haad and oxyresveratrol in SH-SY5Y neuroblastoma cells, and such testing is the focus of our study reported here.

Specifically, this was an in vitro cytotoxicity study of A. lakoocha aqueous extract and oxyresveratrol in SH-SY5Y neuroblastoma cells.

2. Materials and Methods

2.1. Materials
Dulbecco’s modified Eagle’s medium (DMEM)/F12 and retinoic acid were purchased from Sigma (St. Louis, MO): Fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were purchased from Gibco (Grand Island, NY). 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay kit was purchased from Roche Diagnostics (Mannheim, Germany). Hardwood aqueous extract of A. lakoocha so called puag haad was obtained from Origin Plant Co., Ltd (Bangkok, Thailand). SH-SY5Y cells preparation
SH-SY5Y cells were grown in DMEM /F-12 supplemented with L-glutamine, 10% FBS, 1% penicillin-streptomycin. Cell cultures density was 1×10^6 cells/cm^2 in 75 cm^3 flasks at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The medium was replaced with new medium every 3-4 days and there were no more than 30 cell passages to ensure cell uniformity and reproducibility. The cells were plated into 96-well plates at densities of 20,000 cells/well and then differentiated by adding low serum culture medium (2% FBS) containing retinoic acid (10µM) simultaneously, at least for 10 days.

2.2. LDH assay
SH-SY5Y cell survival was measured by LDH assay, a soluble cytosolic enzyme released from cytoplasm when the membrane becomes leaky after toxic insults. The cells were plated at a density 2 x 10^5 cells/well in a 96-well plate. Cells were treated with the test compounds, puag haad and oxyresveratrol, at concentrations of 25 to 800 µM of oxyresveratrol and 6.25 to 200 µg/ml of puag haad, and incubated for a further 24h. After treatment, 50 µl samples of medium were removed from each well and was and mixed with the LDH reaction solution (0.3 mM NADH and 3 mM pyruvate). The absorbance was monitored at 340 nm OD every 5 min for 20 min.

2.3. XTT assay
XTT assay is used for measuring activity of dehydrogenase enzyme in mitochondria. XTT assay is based on an ability of viable cell to convert 3-[4, 5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide (XTT) into orange formazan dye. This assay was performed to mitochondrial viability and hence survival of SH-SY5Y neuroblastoma cells after treatment with the test compounds. The cells were treated with puag haad at concentration of 6.25 to 200 µg/ml or oxyresveratrol at concentration of 25 to 800 µM for 24h. XTT solution was added to the cells and the absorbance was measured after 4h.

3. Results and Discussion
Cytotoxicity studies were conducted to determine the concentration limits of the compounds appropriate for pharmacological studies. Cytotoxicity of puag haad and oxyresveratrol was tested by XTT and LDH assay. The principle of an XTT assay is based on the ability of mitochondrial dehydrogenase enzymes to convert XTT to yellow formazan, whereas an LDH assay is based on the release of cytosolic LDH through leaky and damaged cell membranes.
SH-SY5Y neuroblastoma cells were treated with oxyresveratrol for 24h and then measured the cell viability with an XTT assay and an LDH assay. Based on these assays oxyresveratrol concentrations ≤200 µM might be regarded as harmless (fig 1A). We drew a similar conclusion for the LDH release assay (fig 1B).

For puag haad, a concentration of ≤25 µg/ml appeared safe while higher concentrations demonstrated cell toxicity (fig 1A and 1B). The ISO 10993-5:2009 test standard for *in vitro* cytotoxicity protocol [15] has set the safe threshold as being a concentration where >70% of cells remain viable.

Several toxicity studies of puag haad have been conducted both *in vivo* and *in vitro*. A 3 g oral dose of puag haad selected for taeniasis treatment in hospital showed adverse effects [16], including nausea and vomiting [17]. In a rat study, an LD₅₀ value of 1,148 mg/200 g body weight was found. As this is 46 times more than the usual therapeutic dose [18], this indicates its probable safety in human treatments. Another study on *Paramphistomum cervi* trematodes showed a cell toxicity of ≥2,000 µg/ml [19] and on *Schistosoma mansoni* trematodes the cell toxicity was ≥250 µg/ml [20]. In addition, toxicity studies of oxyresveratrol by MTT assay in a cell culture model showed that ≥15 µg/ml was toxic to B16 melanoma cells [21]. The toxic concentration of oxyresveratrol was shown in [22] to lead to cell death by autophagy and apoptosis by deactivating of PI3K/AKT/mTOR for autophagy and p38 activation for apoptosis. These results suggest that the toxicity of both oxyresveratrol and puag haad are different depending on cell type, and on the organism, and on the methods used to test toxicity. In conclusion, ≤25 µg/ml of puag haad and ≤200 µM of oxyresveratrol did not show toxicity to SH-SY5Y neuroblastoma cells and may be used to investigate their neuroprotective effect for future study.

**Figure 1** *In vitro* cytotoxicity of puag haad. Mitochondrial metabolism as measured by XTT assay (A) and LDH release as measured by LDH assay (B).

**Figure 2** *In vitro* cytotoxicity of puag haad. Mitochondrial metabolism as measured by XTT assay (A) and LDH release as measured by LDH assay (B).
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