

## The Comparative Toxicity of Xanthoness and Tannins in Mangosteen (*Garcinia mangostana* Linn.) Pericarp Extract against BHK-21 Fibroblast Cell Culture

### Abstract

**Objective:** The objective of this study is to compare the toxicity level of xanthoness and tannins derived from mangosteen pericarp extract at specific concentrations against BHK-21 fibroblast cell cultures. **Methods:** Mangosteen was extracted using a maceration method with ethanol 96%. Xanthoness were isolated from the chloroform extract, whereas tannins were isolated using acetone alcohol and serial diluted to 100% concentration. Toxicity levels were monitored after 24 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay technique by ELISA reader at 620 nm. **Results:** Viable cells of BHK-21 against xanthone concentration began to decrease (40.24%) at 3.98% xanthoness, whereas viable cells of BHK-21 against tannin concentration began to decrease (68.06%) at 2.2% tannins. **Conclusion:** It is suggested that tannins were more toxic than the xanthoness derived from mangosteen pericarp.

**Keywords:** BHK-21 fibroblast cell, mangosteen pericarp extract, tannins, toxicity, xanthoness

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### Introduction

Irreversible pulpitis is an inflammatory condition of the dental pulp that requires root canal treatment intended to eliminate the microbial infection from the root canal system itself and/or the periradicular area. According to Chandra and Gopikrishna,<sup>[1]</sup> root canal treatment consists of three steps: root canal preparation which includes cleaning and shaping the root canal system, disinfection or sterilization, and root canal obturation. The application of medicament agents aims to disinfect the root canal between appointments and reduce interappointment pain.<sup>[2]</sup> These medicaments are expected to penetrate the dentinal tubules, entering the root canal and inhibiting the growth of the bacteria within.<sup>[3,4]</sup>

Currently available root canal disinfection materials tend to produce side effects because they fall within the category of therapeutic agents, consist of active chemical agents, and are typically toxic. An alternative herbal-based agent is used to reduce the previous dependence on such agents. Herbal remedies have been widely

accepted in almost all countries throughout the world. The World Health Organization also recommends the use of traditional medicine in public health care, especially for the prevention and treatment of chronic diseases, degenerative diseases, and cancer cases.<sup>[5]</sup> Several studies have shown that the use of herbal medicine is considered less toxic than the modern medicine/chemical synthesis as it has relatively limited side effects.<sup>[6,7]</sup>

Mangosteen (*Garcinia mangostana* L.) is one of the fruit trees native to Indonesia that grows easily throughout Southeast Asia in countries such as Malaysia, Thailand, and Myanmar. Phytochemical research showed that the mangosteen pericarp contains the most active ingredients such as xanthoness, flavonoids, saponins, and tannin.<sup>[8-10]</sup> Xanthoness was reported to produce pharmacological effects, including antibacterial, antifungal, and anti-inflammatory. Xanthoness from mangosteen pericarp have been proven to be nontoxic to mice when administered orally at a dose of 100 mg/kg of body weight/day for 7 days. On the other hand, in the course of his research, Kaomongkolgit also found that alpha-mangostin at certain

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dosages proved nontoxic to human gingival fibroblasts for 480 min.<sup>[11]</sup>

Tannins are widely distributed in many species of plants, often being found in the bark, stems, leaves, and fruit, and play important roles in the protection and growth regulation of cells. Tannins are a derivative of the polyphenolic compounds contained in mangosteen pericarp extract, which is about 11.8%.<sup>[12]</sup> The chemical activity of tannins includes several elements, namely apoptosis, antitumor, antibacterial, and antiplasmin.<sup>[13]</sup> Banso proved that the antimicrobial activity of tannins in relation to certain microbes within the range of minimum inhibitory concentration is around 4–5.5 mg/ml, whereas minimum bactericidal concentration is 4.5–6 mg/ml.<sup>[14]</sup> However, at high concentrations, tannin can cause irritation of the mucous membrane.<sup>[15]</sup> The active compound content of mangosteen pericarp extract offers encouraging potential to support the success of root canal treatment, but any materials used in dentistry must fulfill the requirements of biocompatibility.

Toxicity assay is carried out during the initial phase of a material biocompatibility assessment and constitutes part of dental material evaluation; it is one of the procedures required for standard screening. Toxicity assay is used to study the effect of a chemical substance or toxic pollutant on specific organisms. As both xanthone and tannin are the most active ingredients and promote antimicrobial activities, the authors were interested in testing the toxicity of xanthones mangosteen pericarp via BHK-21 fibroblast cell culture (Baby Hamster Kidney-21) assay.

The purpose of this study was to compare the toxicity of xanthone and tannins mangosteen pericarp to BHK-21 fibroblast cell culture. The hypothesis being tested was that xanthones were less toxic than the tannins of mangosteen pericarp.

## Materials and Methods

### Preparation of xanthones

Mangosteen (*G. mangostana* L.) pericarp powder was bought from Materia Medica, Batu, East Java Province, Indonesia. Crude mangosteen extract was obtained by a maceration method using 96% ethanol (Friendemann Schmidt, Parkwood, WA, USA). Hexane extract was immersed in alcohol acetate (Friendemann Schmidt, Parkwood, WA, USA) and, subsequently, evaporated. The immersion and evaporation process was repeated once more, before the hexane extract was soaked in hexane alcohol acetate and evaporated to obtain 50 ml of 31.82% pure xanthones. Purified xanthones were diluted by adding sterile distilled water to obtain a variety of xanthone concentrations: 0.99%, 1.99%, 3.98%, 7.96%, and 15.91%.

### Preparation of tannins

Tannins obtained from the mangosteen pericarp extract of *G. mangostana* types by maceration method were dissolved

in a solution of 90% alcohol, before being isolated by means of acetyl alcohol as a solvent. As a result of the isolation process, 35.22% of tannins were obtained. Dilution with Eagle's media and fetal bovine serum was required to obtain a range of tannins concentrations: 35.22%, 17.61%, 8.81%, 4.40%, 2.20%, 1.10%, 0.55%, and 0.28%.

### Ethical consent

The procedure reported here was performed with the approval of the Ethical Review Committee of the Faculty of Dental Medicine, Universitas Airlangga.

### Cell culture

For the cytotoxicity test, BHK-21 fibroblast cell culture (ATCC, Rockville, MD, USA) was used according to ISO 17025.<sup>[16]</sup>

### Cytotoxicity test

A toxicity test of xanthones and tannins was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma Aldrich, Germany). This assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystal formation represents the viable cells of BHK-21. Five replications of each concentration of xanthones and tannins were placed in a 96-well microplate, which was then incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C, for 24 h. After the incubation process, the cell media was removed and washed with PBS, before being replaced with new media. MTT was added directly to the microplate to a maximum of 10 µl. The microplate was incubated for approximately 4 h at 37°C. Before terminating the experiment, the supernatant was aspirated. After that, 50 µl dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The microplate was then stirred mechanically by Plate Shaker (Dynatech, England) for 5 min. The optical density value of formed formazan crystals was measured by ELISA reader (Sigma Aldrich, Germany) at a wavelength of 620 nm. The concentration of viable cells was calculated by means of the formula below:

$$\% \text{ Viable cell} = \frac{\text{OD extracts} - \text{OD medium}}{\text{OD control} - \text{OD medium}} \times 100\%$$

Cell viability was measured according to method proposed by Sjogren *et al.*:

- More than 90% viable cells: noncytotoxic
- 60–90% viable cells: slightly cytotoxic
- 30–59% viable cells: moderately cytotoxic
- Less than 30% viable cells: severely cytotoxic.<sup>[17]</sup>

### Statistical analysis

Collected data were analyzed with a significance level of  $P < 0.05$ , using SPSS statistical analysis (Version 21) (IBM, New York, USA). The statistical test used to determine

the normality of data distribution was a one-sample Kolmogorov–Smirnov test, whereas the homogeneity test data were analyzed with a Levene test. Furthermore, a Mann–Whitney test was also used to compare the differences in toxicity between various concentrations of xanthone and tannin mangosteen pericarp during the growth of BHK-21 fibroblasts cells.

## Results

Based on optical density data, the percentage of viable cells can be calculated. The higher the degree of optical density, the more numerous the viable fibroblasts or proliferates. The calculation of the percentage number of viable cells obtained using the aforementioned formula is shown in Figure 1.

Figure 1 shows the percentage of the viable fibroblasts exposed to xanthenes and tannins from mangosteen pericarp extract at various concentrations, those of xanthenes being 0.99%, 1.99%, 3.98%, 7.96%, 15.91%, and 31.82%, whereas those of tannins were 1.10%, 2.20%, 4.40%, 8.80%, 17.61%, and 35.22%.

As shown in Figure 1, the xanthone concentrations between 0.99% and 1.99% showed a slight reduction followed by an extreme decrease before reaching 40.24% at 3.98% xanthone concentrate. The graph between 3.98% and 15.91% increases gradually and then decreases slightly to 31.82% xanthone concentrate.

In the tannins groups, it appears that at concentrations of 1.1%, the percentage of viable cells is high but begins to decrease at concentrations of 2.2%. At concentrations of 8.8%, 17.61%, and 35.22%, a false positive was shown. The statistical test used to determine normality results indicated that all groups had  $P$  values greater than  $\alpha$  5% ( $P > 0.05$ ), indicating that the data were normally distributed, whereas a Kruskal–Wallis test showed that  $P$  was equal to 0.000. This, in turn, indicated the

differences in the number of fibroblasts BHK-21 in each group.

## Discussion

A toxicity test is an indicator used to determine the effect of a certain concentration of exposure to a substance. Noordin *et al.* in their research identified the toxicity effect on embryos at concentrations of 250, 125, and 62.5  $\mu\text{g/ml}$ ,<sup>[18]</sup> while Morada found that the LC50 values for the tannin-containing extract from the mangrove tree crude extract were 817.5 ppm and 515.8 ppm, respectively, for acute and chronic exposures, indicating mild toxicity.<sup>[19]</sup> In the research reported here, it was found that xanthone at a concentration of 3.98% shows moderately cytotoxic activity with a rate of fibroblast proliferation as high as 40.24%. The results for tannin concentration of 2.20% indicate slight toxicity with a proliferation ability as high as 68.06%.<sup>[17]</sup> At concentrations of 7.96%, 15.91%, and 31.82% and 8.8%, 17.61%, and 35.22%, respectively, xanthenes and tannins are known to demonstrate higher proliferative ability compared with the toxic concentration, indicating the existence of an uncommon state. In general, the higher the concentration, the lower the proliferation of cells. As outlined in previous research into the cytotoxic effects of epigallocatechin gallate on cell flavin and RL-34, all polyphenols will induce cytotoxicity at high concentrations.<sup>[20]</sup> This is probably caused by the xanthone and tannin from mangosteen pericarp having a color that was also recorded at the time of reading. The higher the concentration, the greater the influence of the color contained in the extract of mangosteen. This is shown by the high absorbance value at concentrations of 8.8%, 17.61%, and 35.22% tannins and at concentration of 7.96%, 15.91%, and 31.82% xanthenes. This can lead to false positives. A false-positive test is a condition where the result contains an incorrect value because the test failed to identify certain conditions or findings.

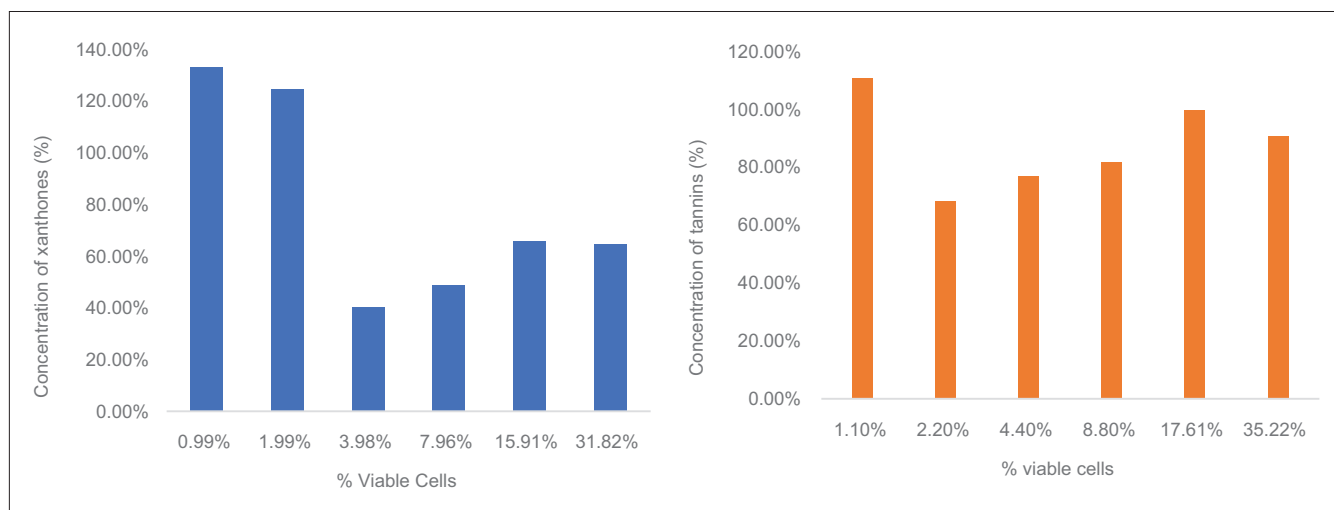


Figure 1: Comparison of xanthone and tannin toxicity in samples at various concentrations

Xanthenes are able to reduce the production of TNF $\alpha$  in inflammatory processes. The mechanism of decreased production of TNF- $\alpha$  started with the blockade process involving kappa-B kinase inhibitor activity. Inhibition of NF- $\kappa$ B distribution of p65/p50 will inhibit the genomic process of NF- $\kappa$ B p65/p50, which is characterized by decreased protein products. The decline in the distribution of NF- $\kappa$ B p65/p50 will cause reduced mRNA expression of TNF- $\alpha$ .<sup>[21]</sup> It is suggested that the inhibition of free radicals and decreased production of TNF- $\alpha$  will support the process of cell proliferation of fibroblasts.

Xanthone derivatives,  $\alpha$ - and  $\gamma$ -mangostin, can significantly inhibit the production of nitric oxide (NO), PGE<sub>2</sub>, and iNOS expression in lipopolysaccharide-stimulated RAW 264.7 cells. Alpha and  $\gamma$ -mangostin can reduce PGE<sub>2</sub> products via inhibition of COX-2 activity and NO production. In addition, NO can activate the form of cyclooxygenase (COX-1 and COX-2) enzymes, which are determinant enzymes for the biosynthesis of PGE<sub>2</sub> during inflammatory process.<sup>[22]</sup>

Apart from its ability to support the proliferation of fibroblasts, xanthenes can also cause fibroblasts to undergo apoptosis. The toxicological properties of xanthenes seem dominant at 3.98% and 7.96% concentrate. A xanthone derivative,  $\alpha$ -mangostin, can result in mitochondrial membrane depolarization and cytochrome *c* release, which can cause cells to undergo apoptosis. The release of cytochrome *c* from the inner mitochondrial membrane causes the accumulation of apoptosome, which consists of cytochrome *c*, Apaf-1, dATP or ATP, and procaspase-9. Procaspase-9 is activated by binding itself to Apaf-1. Active caspase-9 can proliferate itself and activate caspase-3. Caspase-3 and mitochondrial membrane depolarization are expressed as an indicator of apoptosis.<sup>[23]</sup>

Certain controversial research reported that xanthenes affect ERK 1/2 and JNK 1/2 pathway by stimulation or inhibition of activation, depending on the cell type. ERK 1/2 and JNK 1/2 is the mitogen-activated protein kinase whose function is to regulate mitosis, cell survival, and apoptosis. Alpha-mangostin can lower p-Akt levels, a protein kinase associated with cell survival. The antiproliferative activity of  $\alpha$ -mangostin in colorectal cancer cells occurs through inhibition of the transcriptional activity of T-cell factor/ $\beta$ -catenin. Limited information is known about the effect of xanthone on the cell cycle, although a number of studies assert that it arrests the G1 phase and decreases cyclin regulation.<sup>[24]</sup>

Tannins are active compounds, which are divided into two types, condensed tannins and hydrolyzed tannins. Both types are found in plants, but the most dominant type in a variety of plants is condensed tannin. Tannins inhibit lipid peroxidation induced by adenine 5'-diphosphate (ADP) and ascorbic acid. Tannins

also inhibit lipid peroxidation induced by ADP and nicotinamide adenine dinucleotide phosphate, both of which can stimulate mitochondria.<sup>[12]</sup> Transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>[25]</sup> through antioxidant activity is capable of blocking the initiation of free radical composition so that TGF- $\beta$  can stimulate the proliferation of fibroblasts. TGF- $\beta$  can also protect healthy tissue and stimulate the production of collagen.<sup>[26]</sup> In addition, the antioxidant effect of tannin is a transition metal ion chelator which prevents oxidative DNA by neutralizing excess metal ions, suppressing the formation of hydroxyl radicals and stabilizing proinflammatory and prooxidative activity.<sup>[27]</sup> One derivate of tannins is proanthocyanidins (condensed tannins), which have a strong affinity with proteolytic enzymes such as elastase, xanthine oxydase,  $\beta$  glucuronidase, collagenase, and hyaluronidase. These are all involved in the destruction of the matrix component. This shows that proanthocyanidins interact with the membrane cell wall by combining the weak energy of hydrogen bonding and hydrophobic interaction types. In such cases, proanthocyanidins neutralize enzymes that decrease degradation by neutralizing the excess of extracellular matrix MMPs (matrix metallo protease). The presence of MMP-2, -3, and -9 can damage the cell matrix, thereby potentially inhibiting cell growth. Counteracting MMPs can help create a favorable environment for the growth of fibroblast cells. This is consistent with the results of studies showing the activity of tannins against cell proliferation of BHK-21.

A decrease in cell proliferation indicated nonviable cells; the astringent of tannins causes mucous membranes to bind stronger and become less permeable. When tannins are administered in high doses, this will cause irritation to the mucous membranes.<sup>[15]</sup> The tannins have a high affinity for the protein in the mucosa and mucosal epithelial cells because, as phenolic compounds, they have molecules able to form complexes with proteins. Consequently, the enzyme and protein will be inactivated which can cause disturbances in the cytoplasm.<sup>[28]</sup>

From the isolation of mangosteen pericarp, 31.82% xanthenes and 35.22% tannins can be obtained, indicating that tannins are present at higher concentrations than xanthenes, whereas the toxicity test indicated that xanthenes are slightly less toxic than the tannins of mangosteen pericarp. Both xanthenes and tannins have their respective advantages and disadvantages in supporting root canal treatment.

Phenol compounds have the potential to promote redox activity, including reactivity with nitroblue tetrazolium.<sup>[29]</sup> This study showed that, at high concentrations, they cannot provide accurate results by using MTT assay. One potential alternative is a luciferin/luciferase assay, which can measure the amount of ATP present in viable cells. There are several other factors that may increase the risk of error in the MTT assay process

such as types of medium culture and filtration (filter set).<sup>[30]</sup> However, these factors can be minimized to the extent that there is no effect influencing results.

## Conclusion

From this study, it can be concluded that 3.98% xanthoness and 2.2% tannins of mangosteen pericarp extract was found to be toxic to BHK-21 fibroblast cell culture. It is suggested that tannins were more toxic than the xanthoness of mangosteen pericarp.

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## Conflicts of interest

There are no conflicts of interest.

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