

Fibroblast Viability Test Toward Red Dragon Fruit (*Hylocereus polyrhizus*) Peel Ethanolic Extract

Annabella Natasha Kylanel¹, Vinna Kurniawati Sugiaman², Natallia Pranata^{2*}

¹Faculty of Dentistry, Maranatha Christian University, Bandung, Indonesia

²Department of Oral Biology, Faculty of Dentistry, Maranatha Christian University, Bandung, Indonesia

Abstract

The nutritional content of red dragon (*Hylocereus polyrhizus*) fruit is not only limited to the flesh, the peel contains polyphenols and antioxidant activity along with the antiproliferative effects that are higher than the flesh. Plant bioactive constituents is belief to have various benefits with minimum side effects but yet not standardized. Phytochemical and viability test are needed to analyze the toxic concentration and 50% inhibition concentration (IC₅₀) from *Hylocereus polyrhizus* peel 70% ethanolic crude extract.

Materials and methods: Qualitative phytochemical test was determined using modified Farnsworth method. Fibroblast cells (3T3BALB/C) given seven different extract (500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg / mL) concentrations. Analysis using colorimetric MTS assay with triplicate. The value of formazan absorbance indicated the viability of the cells.

Results: Flavonoids, saponin, phenols, tannins, triterpenoids, terpenoids, and alkaloids were presence. Statistical data analysis obtained using ANOVA where each group has significantly different effect ($p < 0.05$) and IC₅₀ value was obtained 1576.867 µg / mL, higher than the concentration group that was planned before.

Conclusion: RDFP 70% ethanol extract proven to be not toxic, particularly at a concentration below 250 µg / mL. Meanwhile, the IC₅₀ higher than the 100% concentration.

Keywords: IC₅₀, MTS Assay, Recurrent Aphthous Stomatitis, Red Dragon Fruit, Toxicity.

Corresponding author: Natallia Pranata, drg., M.Kes. Department of Oral Biology, Faculty of Dentistry, Maranatha Christian University, Bandung, Indonesia
Jl. Prof. Drg. Surya Sumantri 65, Bandung 40164, West Java, Indonesia

Email: natallia.pranata@dent.maranatha.edu

INTRODUCTION

Herbal medicines are mainly used to promote healing by accelerate blood clotting and fight infections.¹ The actual side effects of herbal medicines are not yet known because it is not standardized. Some primary consideration of herbal medicines is still used today are because its plant crude extract are widely available with different nutritional content yet economically cheaper compared to the results of purification of specific compounds.² The use of crude extracts which contain several elements of different active substances is believed to work synergistically so that the impact is greater than the effect of each bioactive constituent and its toxicity will also decrease.³ Therefore phytoconstituents from various plants in the form of crude extract need to be further identified and screened so it is standardized with the aim of healing management, in which red dragon fruit peel (RDFP) is one of the plants with the potential to be extracted as a crude extract which later can be processed into herbal medicines.¹

The nutritional content of red dragon fruit is not only limited to the flesh, the RDFP which comprises only one-third of the fruit is often thrown away in the process, especially in the food and beverage production industry.^{1,4} But it turns out that RDFP contains polyphenols and antioxidant activity along with the antiproliferative effects on B16F10 melanoma cancer cells that are higher than the flesh.⁵ Although it can be said to be a 'leftover' product, RDFP contain vitamin C and antibacterial effect on *Salmonella typhi* while its extract of chloroform has an antibacterial effect on Gram-positive and Gram-negative which is higher than dragon fruit peel species *Hylocereus undatus* (Haw.) Britton & Rose (Cactaceae).^{5,6}

The main compounds of *H. polyrhizus* peel are chlorogenic acid, gallic acid, and quercetin.⁵ Quercetin, is one of the flavonoid groups and well absorbed by the body, is known to inhibit mast cell degranulation, basophil histamine release and formation from other inflammatory mediators, which in histopathological analysis of male Wistar groups treated with quercetin, fewer inflammatory cells, an increase in microvessel density, more proliferation of fibroblasts, more regular collagen deposition and epithelialization were found.^{7,8} Disodium cromoglycate (anti-allergic drug) has a structure and function similar to quercetin effective in treating recurrent aphthous stomatitis.⁸ RCT studies in the management of minor aphthous ulcers using topical quercetin, resulting in a total recovery in 35% of cases in 2-4 days and 90% of cases in 4-7 days compared to patients receiving topical benzydamine hydrochloride mouthwash.⁹ Therefore quercetin can be used to reduce the frequency of recurrences by eliminating mild symptoms.⁸ Chlorogenic acids is considered a fairly economical therapeutic agent since it can be found abundant in common plants. The antioxidant activity of chlorogenic acids can contribute to the success of wound healing by increasing the effect of collagen production and capillary density, antioxidant and free radical scavenger effects on oxidative parameters, and anti-inflammatory effects on MMPs in wound tissue. Study revealed that gallic acid a viable wound healing agent which in normal and hyperglycemic conditions, gallic acid has powerful antioxidants that directly increase the expression of antioxidant genes and also accelerate the migration of keratinocyte and fibroblast cells which leads to activation of growth factors responsible for wound healing, such as focal

Fibroblast Viability Test Toward Red Dragon Fruit (*Hylocereus polyrhizus*) Peel Ethanollic Extract

adhesion kinases (FAK), c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK).⁷

At least 20% of the populations are affected by recurrent mouth ulcer also known as RAS (Recurrent Aphthous Stomatitis) which is a multifactorial common mouth disease that disrupts the normal balance condition due to the increased aggression or decreased mucosal resistance.^{10,11} RAS usually self-limits however, because it often interferes with the activity of the oral cavity mainly causes pain when talking, eating, drinking or brushing teeth, prevention of recurrence frequency, duration and pain reduction of the disease are the main goals and objectives in dentistry.¹¹⁻¹³ Some golden treatments for the management of ulcers used among others are anti-inflammatory, antiseptics, analgesics, local anesthetics, corticosteroids, antibiotics, lasers and herbal medications.¹² However, the use of conventional drugs such as topical or systemic antibiotics, anti-inflammatory, immunomodulation, or other symptomatic treatments are not entirely reliable because long-term use and frequent exposure to certain medications can cause undesirable side effects, so the treatment of "natural" ingredients or herbs are often considered to be an alternative because bioactive components from plants are considered low risk and widely available.¹¹⁻¹³ This RDFP state with various health benefits and little utilization has increased the interest of many researchers in converting peel into products that are easier to use with a longer shelf life, especially in its use as an alternative RAS therapy.⁵ Viability test as a preliminary study needs to be done to ensure the safe consumption of herbal medicines as a form of standardization.

MATERIAL AND METHODS

Red dragon fruit peel extract preparation

Red dragon fruit is obtained from the red dragon fruit garden in Cijambe Village, Subang, Indonesia and has been identified by the Bandung Institute of Technology (ITB) herbarium staff. RDFP extract was obtained by using maceration with ethanol solvent. A total of 5 mg extract was dissolved in 10 mL of 10% DMSO which become seven different extract concentrations (500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 $\mu\text{g} / \text{mL}$) hereafter filtered using a 0.22 μm tissue culture pore syringe, so that sterile samples can be obtained.¹⁴

Phytochemical test

The qualitative phytochemical component analysis was carried out on RDFP extract using a modified Farnsworth method to identify flavonoids, phenolics, saponins, triterpenoids, steroids, terpenoids, tannins, and alkaloids.¹⁵

Viability test using MTS assay

Fibroblast cells 3T3 BALB/C (ATCC CL-163) were obtained from the *Aretha Medika Utama BBRC* Laboratory, Indonesia. Cells were then cultured in a complete medium consisting of 10% Fetal Bovine Serum (Gibco, 10270106), 1% Antibiotic-Antimycotic (Gibco, 1772653), and DMEM (Gibco, 11995065) which then incubated at 37°C and 5% CO₂ until cells become confluent 70-80%. Cells were then rinsed with PBS (Gibco, 1740576) and inserted 0.25% EDTA trypsin (Gibco, 25200072). 5000 cells were plated into 96 well plate (Corning, 3596), and treated with seven concentrations of ethanol extract of RDFP hereafter incubated for 24 hours. MTS reagent (Abcam, AB197010) was added, and each well absorbance was measured by using a spectrophotometer. There are two control groups, cell control and 10% DMSO control¹⁶. The extract can be classified as toxic or nontoxic based on ISO 10993-5. The number of viable cells obtained from the viability tests if under 70%, the extract can be classified as toxic.

Statistical analysis

All analyses used SPSS version 20.0 software. The mean \pm standard deviation was determined by analysis of variant ANOVA followed by Tuckey HSD to see significant differences between groups. Inhibition concentration (IC₅₀) values were determined by PROBIT calculations.

RESULTS

Phytochemical test

Based on the results of phytochemical tests, ethanol extract of RDFP contains components as can be seen in Table 1.

Viability test

The data seen in Figure, there was a decrease in the number of viable cells with increasing concentrations ranging from the lowest concentration of 7.81 $\mu\text{g} / \text{mL}$ to the highest concentration of 500 $\mu\text{g} / \text{mL}$. Based on ISO 10993-5,¹⁷ the cell viability classification, concentrations of 250, 125, 62.5, 31.25, 15.63 and 7.81 $\mu\text{g} / \text{mL}$ are nontoxic. In this study, the IC₅₀ was 1576.867 $\mu\text{g} / \text{mL}$, higher than the concentration group that was planned before.

DISCUSSION

The result of phytochemical test obtained are slightly different from the studies conducted previously in which research the RDFP extract did not contain alkaloid compounds but contained saponins, whereas, in this study, RDFP extract did not contain saponins and terpenoids.¹⁸ These differences in results can be influenced by several factors related to discrepancy in edaphology and climate because the secondary metabolites of plants can differ depending on the season or location of plants (diversity in nutrients and soil conditions) and bioactive compounds synthesis is also depending on time so the sampling time could be one of the determining factors.^{19,20}

Flavonoid content in RDFP extract can be used as an anti-inflammatory therapy because flavonoids are known to accelerate proliferation process by shortening the time of inflammation. Flavonoids have a function to stall bleeding by increasing the number of platelets, so that when there is bleeding, platelets will rupture and produce thromboplastin or thrombokinase enzymes that will work and activate prothrombin which helps by Ca²⁺ and vitamin K contained in blood plasma can activate thrombin which will eventually change fibrinogen molecules to form fibrin monomers.²¹

Triterpenoids are known to work as antiviral, antimicrobial, anti-inflammatory, antitumoral agents, and become immunomodulatory compounds. Many medicinal plants in traditional medication with pharmacological effects that play a role in fighting the disease of the immune system such as allergies and hypersensitivity (especially types I and IV), have triterpenoids, although further research needs to be done on the specifications of the triterpenoid type and the appropriate dosage in this crude extract study.²²

The content of phenols, alkaloids, and tannins can act as an antibacterial. Alkaloids will disrupt the peptidoglycan of the bacterial cell, so that the cell wall layer is not fully formed which will eventually cause death in these cells, whereas tannins will shrink cell walls or cell membranes that can disrupt cell permeability so that bacterial cells cannot carry out living (growth is obstructed or even dead). The presence of anthocyanins on the RDFP can increase tannin content, so in addition to its antibacterial properties according to Iranian Traditional Medicine (ITM), tannins are used in RAS treatment because they have astringent effects.²³⁻²⁵ Gallic acid, which is one of the groups of tannins found in RDFP, has a migration effect on fibroblasts and is a potential agent for treating damage to wound healing, both acute and chronic

Fibroblast Viability Test Toward Red Dragon Fruit (*Hylocereus polyrhizus*) Peel Ethanol Extract

skin disorders.^{7,26} Treatment with gallic acid does not lead to cytotoxicity in normal mouse fibroblast and endothelial cells, besides there is visible potential for anti-apoptotic effects on normal human lymphocytes.²⁷

Regardless of its dosage, other factors that play a role in viability include the duration and mechanism of exogenous agents against cells. Cells exposed to exogenous agents can react with different results. An exogen agent can be metabolized without any effects that can be observed in certain cell lines, but the opposite can also occur. Exogenous agents that are cytotoxic can affect various cell functions through a variety of different mechanisms, such as prevention of protein synthesis, destruction of cell membranes, inhibition of elongation of polydeoxynucleotide, irreversible binding to receptors, and other enzymatic activities can make cells experience necrosis or apoptosis. Necrosis occurs when cells experience hypothermia or are exposed to conditions that are very different from their physiological conditions so that the cell membrane becomes damaged. Necrosis interferes with the cell's ability to maintain homeostasis, which is essential for ATP production, enzyme activity, and the influx of water and extracellular ions so that intracellular organelles of cells such as mitochondria, can swell and rupture (cell lysis). As a result of the disruption of the plasma membrane, cytoplasmic contents, including the lysosome enzyme, will be released into the extracellular fluid which can be used to measure the level of necrosis.^{28,29}

In this study, agents that have the potential to play a role in cell death are (including) alkaloids. Due to their toxicity, plants alkaloids efficient in protecting against predators by causing changes in the central nervous system, impaired protein function after consumption and metabolism. Alkaloids also has bitter taste as defense. But again in general, toxicity effect can be dangerous and beneficial depend on the specific dose, time of exposure, and individual characteristics such as developmental stage, sensitivity, site of action, and depending on the pharmacological context.³⁰ As a tropical plant of the family of Cactaceae red dragon fruit has low toxicity index of alkaloid. Therefore, further research is needed to identify the amount of content and type of alkaloid or purification to remove toxic substances in the RDFP extract.

Hence to ascertain the effective dose of this peel extract as RAS therapy, a further viability test can be conducted to determine its ED₅₀, LD₅₀, MIC, etc. as appropriate and to know the exact percentage of phytochemical composition and nutritional content of the peel extracts, other phytochemical tests or methods can be carried out in other ways or using different ethanol concentrations or solvents.

CONCLUSIONS

RDFP 70% ethanol extract proven to be not toxic, particularly at a concentration below 250µg/mL. Meanwhile, the IC₅₀ higher than the 100% concentration.

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Tables and Figures

Table 1. Phytochemical Test Result

Phytochemical Components	Qualitative Results
Flavonoid	(+)
Saponin	(-)
Phenol	(+)
Tanin	(+)
Triterpenoid	(+)
Steroid	(-)
Terpenoid	(-)
Alkaloid	(+)

Note: +/- indicates the existence of substances in the extract

Fibroblast Viability Test Toward Red Dragon Fruit (Hylocereus polyrhizus) Peel Ethanolic Extract

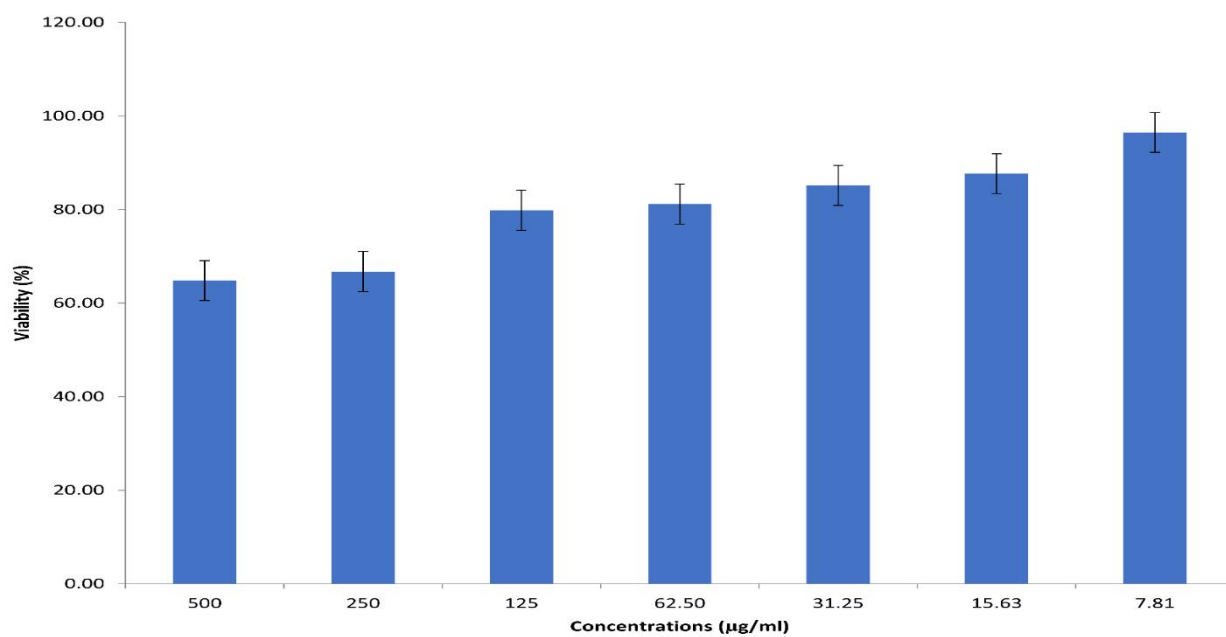


Figure 1. Relationship between concentration of RDFP extract and 3T3BALB/C viability.

There was a decrease in the number of viable cells with increasing concentrations ranging from the lowest concentration of 7.81 µg / mL to the highest concentration of 500 µg / mL.