



The Effect of Roselle (*Hibiscus sabdariffa* L.) Flowers Extract on the Apoptosis of Fibroblast Proliferation in Keloids

Imam Budi Putra^{1*}, Hardyanto Soebono², Sumadio Hadisahputra³, Adang Bachtiar⁴

¹Department of Dermatology and Venereology, Faculty of Medicine, Universitas Sumatera Utara, Indonesia; ²Department of Dermatology and Venereology, Faculty of Medicine, Universitas Gajah Mada, Indonesia; ³Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia; ⁴Department of Health Policy and Administration, Faculty of Public Health, Universitas Indonesia, Indonesia

Abstract

Edited by: Ksenija Bogoeva-Kostovska

Citation: Putra IB, Soebono H, Hadisahputra S, Bachtiar A. The Effect of Roselle (*Hibiscus sabdariffa* L.) Flowers Extract on the Apoptosis of Fibroblast Proliferation in Keloids. Open Access Maced J Med Sci. 2020 Sep 20; 8(A):611-614. https://doi.org/10.3889/oamjms.2020.4602

Keywords: Fibroblast; Keloid; Roselle; *Hibiscus sabdariffa* L.

*Correspondence: Imam Budi Putra, Department of Dermatology and Venereology, Faculty of Medicine, Universitas Sumatera Utara, Indonesia.
E-mail: imam_65@yahoo.com

Received: 08-Jul-2020

Revised: 01-Aug-2020

Accepted: 06-Sep-2020

Copyright: © 2020 Imam Budi Putra, Hardyanto Soebono, Sumadio Hadisahputra, Adang Bachtiar

Funding: This research did not receive any financial support

Competing Interest: The authors have declared that no competing interest exists

Open Access: This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0)

BACKGROUND: Keloid is a benign fibroproliferative dermal disorder as a result of dysregulation wound healing process in susceptible individuals. The pathogenesis is not clearly known yet, but upregulation of transforming growth factor- β 1 (TGF- β 1) was found to have a significant role in inducing hyperproliferation of fibroblast cells. Roselle (*Hibiscus sabdariffa* L.) flower extract has been found to have high content of polyphenols. Some studies have shown inhibition effect of *H. sabdariffa* polyphenols extract on TGF- β 1, and as result affects fibroblast proliferation. Therefore, roselle flower extract might have a significant role in the prevention of keloid formation.

AIM: The objective of the study was to determine the effect of roselle flower extract on fibroblast cells proliferation in human keloid.

METHODS: An experimental controlled trial of 10 different concentrations (1.96 μ g/ml, 3.91 μ g/ml, 7.81 μ g/ml, 15.63 μ g/ml, 31.25 μ g/ml, 62.50 μ g/ml, 125 μ g/ml, 250 μ g/ml, 500 μ g/ml, and 1000 μ g/ml) of roselle (*H. sabdariffa* L.) flower extract was done on cultured fibroblast cells originated from human keloid biopsied tissue. Tunnel assay was done to evaluate the apoptosis rate of the cultured fibroblast cells on each concentration. Determination of TGF- β 1 titer of the cultured human keloid fibroblast cells in and cytotoxicity assay of the extract on cultured normal human fibroblast cells in each concentration were done with enzyme-linked immunosorbent assay method. All the assays were done in triple repetition. Statistical analysis using linear regression test was done to determine the association between the concentration of roselle flower extract with apoptosis rate and TGF- β 1 titer. One-way ANOVA was used to analyze the results of cytotoxicity assay.

RESULTS: Apoptosis rate of the cultured fibroblast cells was found to be increased dose dependently with roselle flower extract concentration ($r^2 = 0.797$; $p < 0.05$). TGF- β 1 titer was inversely related with the extract concentration ($r^2 = 0.501$; $p < 0.05$). Cytotoxicity assay revealed that no differences in absorbance value and viability cells were found in each concentration.

CONCLUSION: Roselle (*H. sabdariffa* L.) flower extract was found to induce apoptosis of the cultured fibroblast cells and reduction of TGF- β 1 titer in dose-dependent pattern, without cytotoxicity effect against human fibroblast cells.

Introduction

Keloids are defined as raised fibrous scars that overgrow the boundaries of the original wound. Its name originated from Greek word "cheloides" which means crab [1]. Patients can experience pain and itchiness, also cosmetic aspect that greatly affect quality of life of keloid patient [2]. Keloid is thought to be caused by imbalance between increase synthesis of collagen and extracellular matrix with decreased degradation of these components. There is increased activity of fibroblast, which results from upregulation of transforming growth factor- β 1 (TGF- β 1) and TGF- β 2 with decreased expression TGF- β 3. TGF- β 1 has also known to increase tissue inhibitors of metalloproteinases and decrease matrix metalloproteinases. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are also contributes in

increase collagen production [3]. Treatment options of keloids are varied, including pressure therapy, silicone gel, intralesional injection (corticosteroid, 5-FU, methotrexate, and bleomycin), cryotherapy, laser, and surgical excision [1]. However, successful therapy in keloids still remains challenging and no single effective modality has been proven yet. Therefore, more studies and development of new modalities are necessary.

Herbal medicine has been part of our society for centuries. Until now, only few studies have been done to explore the potential of natural resources in the treatment of keloid. Recently, roselle (*Hibiscus sabdariffa* L.) has become popular due to high anthocyanin content that found in the leaves, flowers, and fruits. This antioxidant is known to have liver protective effect and might play a role in the prevention of cancer and heart disease. According to Chang *et al.*, *Hibiscus* anthocyanin can induce apoptosis of promyelocytic leukemia cells [4].

Furthermore, Huang *et al.* also reported that anthocyanin can inhibit proliferation and migration of vascular smooth muscle cells, which involves connective tissue growth factor signaling pathway [5]. Therefore, this antioxidant might have potential effect on fibroblast proliferation. The aim of this study is to determine the effect of roselle flower extract on fibroblast proliferation and apoptosis in keloid.

Methods

This is an *in vitro* experimental study of roselle (*H. sabdariffa* L.) flower extract on human keloid fibroblast cultured cell. The flowers of identified and certified roselle plant by Indonesian Science Center were dried at 35°C for 5–7 days and mashed with blender into simplicia. Then, 200 g of the simplicia was soaked in ethanol 96% for 3 h, evaporated at 50°C, and freeze dried for 24 h to produce the roselle flower condensed extract. Human keloid tissue was excised and washed with sterile phosphate buffer saline (PBS). Then, it was cut into small pieces and put into Petri discs with PBS/Roswell Park Memorial Institute medium and gentamycin 2%. Dulbecco's Modified Eagle's Medium 10% was added as culture media and put in CO₂ incubator at 37°C until the Petri discs full of cells that can be harvested and subcultured into flash.

Tunnel assay was done to detect apoptosis of the cultured fibroblast cells in different concentrations (1.96 µg/ml, 3.91 µg/ml, 7.81 µg/ml, 15.63 µg/ml, 31.25 µg/ml, 62.50 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml) after incubated at 37°C for one night. All the mediums were carefully removed using PBS and the cells were fixated on slides using formaldehyde 4% in PBS with pH 7.4 at 4°C for 25 min then immersed in PBS for 5 min at room temperature for washing process with two repetitions. The slides then immersed in 0.2% Triton X-100 for 5 min and washed with PBS twice. After carefully dried, the slides were buffered and put under plastic cover with wet tissue paper underneath to maintain the humidity. The slides were inserted to humidified chamber and covered with aluminum foil, then incubated at 37°C for 60 min for tailing reaction. The reaction was stopped with immersion of the slide in 2 × SSC solution for 15 min in room temperature and washed with PBS 3 times for 5 min. Then, staining process was done with propidium iodide 1 µg/mL for 15 min at room temperature in the dark and then washed with aquadest 3 times for 5 min. The slides were carefully dried and sealed for fluorescence microscopic evaluation.

Enzyme-linked immunosorbent assay (ELISA) was done to evaluate TGF-β1 level of the cultured human keloid fibroblast after given roselle flowers extract. Ten series with different concentrations of the extract were added to supernates of the cultured fibroblast in 96 wells

and diluted with 50 µL RD1-21 followed by incubation for 2 h at room temperature. Each well was aspirated and buffer washed, then 100 µL TGF-β1 conjugates were added and incubated for another 2 h at room temperature. Aspiration and buffer wash repeated before adding 100 µL substrate solution in each well followed by 30 min incubation at room temperature and covered from lights. Diluted hydrochloric acid solution was added to stop the reaction and ELISA reading with 450 nm wavelength followed to determine the TGF-β1 titer.

Cytotoxicity assay was also done to determine viability of the cultured fibroblast cells in each concentration using ELISA. The fibroblast cells from normal human skin were cultured and 10 concentrations of roselle flower extract were added, then incubated for 24 h. Dimethylthiazol diphenyltetrazolium in 5 mg/mL PBS was added to each well and followed with incubation for 4–6 h. Stopper solution (SDS 10% in HCL 0.1 M) was added overnight, and the absorbance results were read with 550 nm wavelength.

The assays were done in triple repetition and all documented data were statistically analyzed. Linear regression test was done to determine the relation between apoptosis of the cultured fibroblast cells and TGF-β1 titer with different concentration of roselle (*H. sabdariffa* L.) flower extract. One-way ANOVA test was done to analyze the differences of absorbance value in each concentration.

Results

Tunnel assay result revealed that the apoptosis rate of the cultured fibroblast cells from human keloid increased with higher concentration of roselle (*H. sabdariffa* L.) flower extract (Table 1).

Table 1: Association between the concentration of roselle (*Hibiscus sabdariffa* L.) flower extract with mean apoptosis rate and TGF-β1 titer of the cultured human keloid fibroblast cells

Concentration (µg/mL)	Mean apoptosis rate (%)	TGF-β1 titer (pg/mL)	Absorbance (OD) (Mean ± SD)	Cell viability (%)
Control	0	1072.54	1.019 ± 0.015	93.1
1.96	13.22	741.66	1.002 ± 0.0032	91.4
3.91	17.68	688.98	1.045 ± 0.007	89.4
7.81	20.43	637.69	0.970 ± 0.011	94.7
15.63	21.85	638.40	1.011 ± 0.051	90.2
31.25	26.17	622.94	1.022 ± 0.019	89.0
62.5	28.65	454.34	0.998 ± 0.018	91.6
125	40.68	353.88	0.984 ± 0.016	93.2
250	43.49	375.66	0.914 ± 0.021	99.2
500	58.32	378.47	0.874 ± 0.013	94.9
1000	71.69	190.20	0.826 ± 0.026	89.7

TGF-β1: Transforming growth factor-β1.

Linear regression test (Figure 1) also showed strong association between the increase of concentration of roselle (*H. sabdariffa* L.) flower extract and the elevation in apoptosis percentage of the cultured human keloid fibroblast cells ($r^2 = 0.797$; $p < 0.05$).

ELISA evaluation revealed that TGF-β1 titer reduction was resulted by the increase in roselle (*H. sabdariffa* L.) flower extract concentration (Table 1).

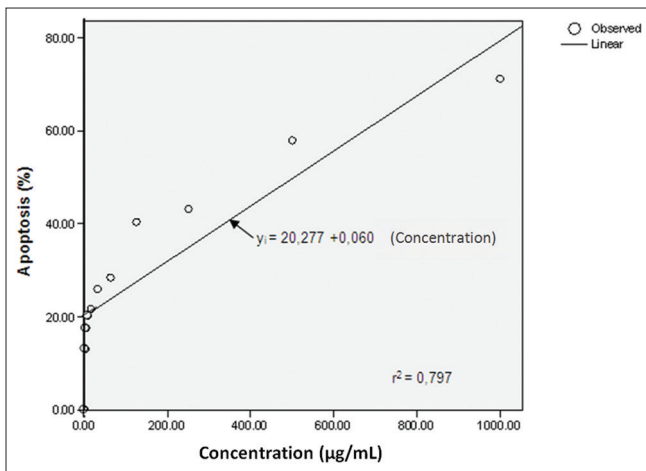


Figure 1: Linear curve of the association of roselle (*Hibiscus sabdariffa* L.) flower extract concentration with percentage of apoptosis rate

In linear regression test (Figure 2), higher concentration of roselle flower extract is associated with lower TGF- β 1 titer ($r^2 = 0.501$; $p < 0.05$). In cytotoxicity assay, mean viability of the normal human fibroblast cells was 92.33%. One-way ANOVA analysis showed that there were no differences in absorbance value of each concentrations. As result, roselle (*H. sabdariffa* L.) flower extract did not show any cytotoxicity effect against human fibroblast cells.

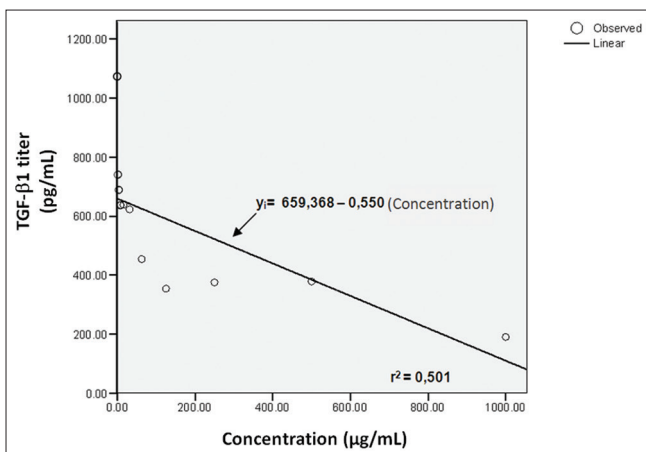


Figure 2: Linear curve of the association of roselle (*Hibiscus sabdariffa* L.) flower extract concentration with TGF- β 1 titer

Discussion

As a fibroproliferative disorder, dysregulation of wound healing pathway plays a crucial role in keloid with initiating activation of keloid fibroblasts and promotes extracellular matrix (ECM). Keloid fibroblasts are thought to be the main inductive cells which initially found highly infiltrating lesion tissue and subsequently mediate elevation of ECM deposition [6], [7]. Therefore, novel therapy targeting these pathogenesis is required to provide treatment option with better efficacy and successful rate. In this study, 10 escalating

concentrations of roselle flower extract were tested on the cultured human fibroblast. Apoptosis rate of the keloid fibroblast cells was found to be increased in accordance with the extract concentration. High anthocyanin content in roselle flower extract is thought to play a significant role in inducing fibroblast apoptosis. *In vitro* studies have shown that anthocyanin-rich extracts exhibited pro-apoptotic effects in multiple cell types through both intrinsic (mitochondrial) and extrinsic (FAS) pathways [8]. In the treatment of cancer, anthocyanin has antitumor effect that inhibited cancer cell growth by targeting on receptor tyrosine kinases, such as epidermal growth factor receptor, PDGF receptor, and VEGF receptor. It also induces cancer cell apoptosis that mediated by ROS and JNK/p38-MAPK [9]. In this study, we did not measure the level of anthocyanin content in our roselle flower extract.

In the past decades, TGF- β 1 pathway is recognized as a pivotal inducer the progress of tissue fibrosis and an important growth factor. There is higher expression of TGF- β 1 in hypertrophic scar and keloidal tissues that promote proliferation and differentiation of dermal fibroblast, also increased production of collagen [10]. A study showed that TGF- β 1 production increases according to scar severity [11]. Subsequently, it becomes an important pharmacological target in keloids treatment, with successful antifibrotic strategies based on TGF- β signaling inhibition or elimination at various transduction steps. A study by Wang *et al.* showed promising result of TGF- β antagonist peptide in prevention and reduction of hypertrophic scar [12], [13]. In this study, TGF- β 1 titer was found dose-dependently decrease with roselle (*H. sabdariffa* L.) flower extract. The previous study by Yang *et al.* also reported that *H. sabdariffa* polyphenol extract (HPE) exhibit reduction of TGF- β 1 in renal of diabetic rats [14]. With these findings, it is confirmed that extract of *H. sabdariffa* L. flower extract has anti-TGF- β 1 potential that might be useful keloid prevention and treatment.

Cytotoxicity assay in this study revealed that roselle flower extract has no toxicity effect on normal skin human fibroblast, with 89.7% viable cells in the extract concentration of 1000 μ g/mL (1 mg/mL). In accordance with this result, Yang *et al.* also reported that cell viability was not significantly altered by 0.05–1 mg/mL of HPE. However, when the concentration increased to 5 mg/mL, only 20% of the cells were viable [14]. Therefore, maximum concentration of 1 mg/mL is considered safe without any cytotoxicity effect.

This is the first study to acknowledge the effect of *H. sabdariffa* L. flower extract on human keloid fibroblast cells. With the promising effect of inducing fibroblast apoptosis and TGF- β 1 reduction, the application of roselle flower extract might be able to provide good alternatives in the treatment of keloids.

Conclusion

Roselle (*H. sabdariffa* L.) flower extract was found to induce apoptosis of the cultured fibroblast cells and reduction of TGF- β 1 titer in dose-dependent pattern, without cytotoxicity effect against human fibroblast cells. With these promising effects, it has a great potential in the novel treatment strategies of keloids. Further studies are required to determine the efficacy of roselle flower extract in human keloid lesion either as therapy or prevention.

Data Availability

All the data used to support the findings of this study are available from the corresponding author on request.

Acknowledgment

The authors would like to thank Nelva Karmila Jusuf for the supports and encouragement.

References

- Dunsky K, Brissett A. Keloids and hypertrophic scars. In: Sataloff RT, editor. *Sataloff's Comprehensive Textbook of Otolaryngology: Head and Neck Surgery: Facial Plastic and Reconstructive Surgery*. London: JP Medical Ltd.; 2015.
- Kouwenberg CA, Bijlard E, Timman R, Hovius SE, Busschbach JJ, Mureau MA. Emotional quality of life is severely affected by keloid disease: Pain and itch are the main determinants of burden. *Plast Reconstr Surg*. 2015;136(4):150-1. <https://doi.org/10.1097/01.prs.0000472474.17120.84>
- Betarbet U, Blalock TW. Keloid: A review of etiology, prevention, and treatment. *J Clin Aesthet Dermatol*. 2020;13(2):33-43. PMID:32308783
- Chang YC, Huang HP, Hsu JD, Yang SF, Wang CJ. *Hibiscus* anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cell. *Toxicol Appl Pharmacol*. 2005;205(3):201-12. <https://doi.org/10.1016/j.taap.2004.10.014> PMID:15922006
- Huang CN, Chan KC, Lin WT, Su SL, Wang CJ, Peng CH. *Hibiscus sabdariffa* inhibits vascular smooth muscle cell proliferation and migration induced by high glucose-A mechanism involves connective tissue growth factor signals. *J Agric Food Chem*. 2009;57:3073-79. <https://doi.org/10.1021/jf803911n> PMID:19301817
- Shih B, Garside E, McGrouther DA, Bayat A. Molecular dissection of abnormal wound healing processes resulting in keloid disease. *Wound Repair Regen*. 2010;18(2):139-53. <https://doi.org/10.1111/j.1524-475x.2009.00553.x> PMID:20002895
- Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG. Hypertrophic scarring and keloids: Pathomechanisms and current and emerging treatment strategies. *Mol Med*. 2011;17:113-25. <https://doi.org/10.2119/molmed.2009.00153> PMID:20927486
- Wang LS, Stoner GD. Anthocyanins and their role in cancer prevention. *Cancer Lett*. 2008;269(2):281-90. <https://doi.org/10.1016/j.canlet.2008.05.020> PMID:18571839
- Lin BW, Gong CC, Song HF, Cui YY. Effects of anthocyanins on the prevention and treatment of cancer. *Br J Pharmacol*. 2017;174:1226-43. <https://doi.org/10.1111/bph.13627> PMID:27646173
- Liu Y, Li Y, Li N, Teng W, Wang M, Zhang T, et al. TGF- β 1 promotes scar fibroblasts proliferation and transdifferentiation via up-regulating MicroRNA-21. *Sci Rep*. 2016;6(1):32231. <https://doi.org/10.1038/srep32231>
- Kim SY, Nam SM, Park ES, Kim YB. Differences in hypertrophic scar fibroblasts according to scar severity: Expression of transforming growth factor β 1 at the mRNA and protein levels. *Arch Aesthetic Plast Surg*. 2015;21(3):116-20. <https://doi.org/10.14730/aaps.2015.21.3.116>
- Wang X, Gao Z, Wu X, Zhang W, Zhou G, Liu W. Inhibitory effect of TGF- β peptide antagonist on the fibrotic phenotype of human hypertrophic scar fibroblasts. *Pharm Biol*. 2016;54(7):1189-97. <https://doi.org/10.3109/13880209.2015.1059862> PMID:26135051
- Lu L, Saulis AS, Liu WR, Roy NK, Chao JD, Ledbetter S, et al. The temporal effects of anti-TGF- β 1, 2, and 3 monoclonal antibody on wound healing and hypertrophic scar formation. *J Am Coll Surg*. 2005;201:391-7. <https://doi.org/10.1016/j.jamcollsurg.2005.03.032> PMID:16125072.
- Yang YS, Wang CJ, Huang CN, Chen ML, Chen MJ, Peng CH. Polyphenols of *Hibiscus sabdariffa* improved diabetic nephropathy via attenuating renal epithelial mesenchymal transition. *J Agric Food Chem*. 2013;61(31):7545-51. <https://doi.org/10.1021/jf4020735>