Indonesian Dental Association

Journal of Indonesian Dental Association

http://jurnal.pdgi.or.id/index.php/jida
ISSN: 2621-6183 (Print); ISSN: 2621-6175 (Online)

Research Article

Effect of Ethanol Extracts from *Persea americana* Leaves on HSC-3 Proliferation

Svetlana Tessalonica[1], Moehamad Orliando Roeslan[2§]

[1] *Undergraduate Student, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia*
[2] *Department of Histology, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia*

Received date: April 20, 2020. **Accepted date:** July 10, 2020. **Published date:** October 30, 2020.

KEYWORDS

HSC-3;
Persea americana leaves;
ethanol extracts;
proliferation inhibition

ABSTRACT

Introduction: Oral cancer has one of the highest death rates of all cancer types due to lack of treatment. *Persea americana* leaves consist of phenolic compounds that are known to have anticancer effects to inhibit the proliferation of human squamous carcinoma (HSC-3) cell lines. Ethanol extracts from *P. americana* leaves can inhibit the cell cycle in the G1/S and G2/M phases to give the cells time to repair damaged DNA. **Objective:** The aim of this study was to investigate the effect of ethanol extracts from *P. americana* leaves on HSC-3 proliferation.
Methods: This study was conducted using experimental research and in vitro laboratory testing. Groups of extract consisted of four concentrations (100%, 50%, 25%, and 10%). Cytotoxic activity of the extracts was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) assay**.** **Result:** *P. americana* leave ethanol extracts with concentrations of 100% had a significant difference ($p < 0.05$) compared to negative control. **Conclusion:** Ethanol extracts from *P. americana* leaves could inhibit HSC-3 proliferation.

§ Corresponding Author
E-mail address: orliando.roeslan@trisakti.ac.id (Roeslan MO)

DOI: 10.32793/jida.v3i2.464

Copyright: ©2020 Tessalonica S, Roeslan MO. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium provided the original author and sources are credited.

INTRODUCTION

Cancer is a non-infectious disease with one of the highest death rates in the world.[1] There are 12.7 million new cancer cases reported every year resulting in 7.6 million deaths.[2] Based on the WHO database, in 2018, new cancer cases rose to 18.1 million with 9.6 million ending in death. One body part that is most frequently attacked by cancer is the oral cavity.[3]

Oral cancer is one of the most common and deadliest diseases in the world with a 50% rate of survival over the last five years.[4] It is most often found in males over 45 years old.[5] Oral cancer is a disease that is frequently diagnosed too late, can receive an unfavorable prognosis without specific biomarker, and the treatment is relatively expensive.[6]

The treatments most frequently used for oral cancer are surgery, radiotherapy, and chemotherapy.[5] Unfortunately, some of these treatments impair healthy cells inside the body[4] and, over the last ten years, traditional medicine has become more important for treating cancer.[5] Traditional substances, like fruits and leaves from plants, have been used as medication to decrease the risks from conventional cancer treatments.[1]

Recently, many medications have been derived from plants for reasons of safety, quality, and efficacy.[5] Many plants contain compounds that have health benefits, and some medicinal plants are the source of new bioactive compounds to cure cancer.[6] *Persea americana,* known commonly as avocado, is one plant with chemo-prevention potential for cancer.[7] This plant is in the Lauraceae family, and it comes from tropical areas in South America.[1,8]

Previous studies have shown that the leaves of *P. americana* have antioxidant and anti-inflammatory properties.[7] Moreover, this plant has been used to cure hypertension, diabetes, hemorrhage, dysentery, and as an aphrodisiac drug.[8] Flavonoid, tannin, quinon, steroid/ triterpenoid, saponin, alkaloid, polyphenol, quercetin, and sugar alcohol are compounds that have been found in *P. americana*.[9] Flavonoid is the most abundant compound in this plant.[10] Flavonoid, alkaloid, and saponin in *P. americana* have anticancer activity by inhibiting HeLa (cervical cancer cell line)[7] and MCF-7 (breast cancer cell line)[8] proliferation. With the knowledge of anticancer activity of *P. americana*, we try to explore its anticancer activity on oral region cell lines, namely human squamous carcinoma-3 (HSC-3) cell lines. Therefore, the aim of this study was to evaluate the effect of ethanol extracts of *P. americana* leaves from Indonesia on human squamous cell-3 (HSC-3) proliferation.

MATERIALS AND METHODS

Plant Material Preparation

Fresh leaves of *P. americana* were collected from Indonesia. The specimens were authenticated by the Indonesian Institute of Science. The leaves were dried in the sun and separated from the stem. The dried sample was ground to powder. One hundred grams of powder were extracted with 500 mL of ethanol absolute (Merck, USA) by the maceration methods. After three days, the solvent and residue of powder was filtered with no.1 filter paper (Whatman Int. Ltd., Maidstone, UK). The residue continued to another stage of maceration. This process was repeated three times. The solvent was then evaporated from the extracted leaves with a rotary evaporator (Buchi, Switzerland). The temperature of the water in the evaporator water bath was maintained at 30°C.

Cell Culture

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA), 250 IU/mL penicillin (Invitrogen, USA) under standard culture conditions at 37°C in 5% CO_2 humidified incubator (Memmert, Germany). The medium was changed regularly and the cells were sub-cultured every 3–4 days or after reaching 80% confluents in the culture flasks.

Viability Test

The mitochondrial-dependent reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (Sigma Aldrich, USA) to formazan was used to measure cell respiration as an indicator of cell proliferation as previously described.[11] Briefly, 1×10^5 HSC-3 cells/well were incubated overnight in 96-well plates. The cells were then washed with phosphate-buffered saline (PBS). The cells were treated with various concentrations (100%, 50%, 25% and 10%) of *P. americana* extract for 24 hours. Doxorubicin 3 μM was used as positive control. After changing the medium, MTT was added to a final concentration of 0.5 mg/ml and the cells were incubated for four hours at 37°C and 5% CO_2. The medium was then removed, and the formazan precipitate was solubilized in dimethyl sulfoxide (Sigma Aldrich, USA). The absorbance was measured at 570 nm using a microplate reader (Tecan, Salzburg, Austria). The cell viability (%) relative to the control wells containing cell culture medium without test samples as a vehicle was calculated using $[A]_{test} / [A]_{control} \times 100$. Where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of the control.

Statistical Analysis

The differences between experimental groups were analyzed using one-way ANOVA. A *p*-value ($p < 0.05$) was considered statistically significant.

RESULTS

Based on qualitative phytochemical assay, *P. americana* contains flavonoid, steroid, tannin, and quinone (Table 1). The results of the cell proliferation assay indicated that *P. americana* extract at 100% concentration (53.56% ± 15.813%) and positive control (34.18% ± 6.825%) are significantly different compared to untreated cells. In other experiment groups, 50% concentration (90.24% ± 6.739%), 25% concentration (90.5% ± 4.95%), and 10% concentration (95.72% ± 7.255%) had no significant difference compared to untreated cells (Fig. 1).

Table 1. Qualitative phytochemical assay of ethanol extracts from *P. americana* leaves

Assay	Results
Flavonoid	+
Steroid	+
Tannin	+
Quinone	+

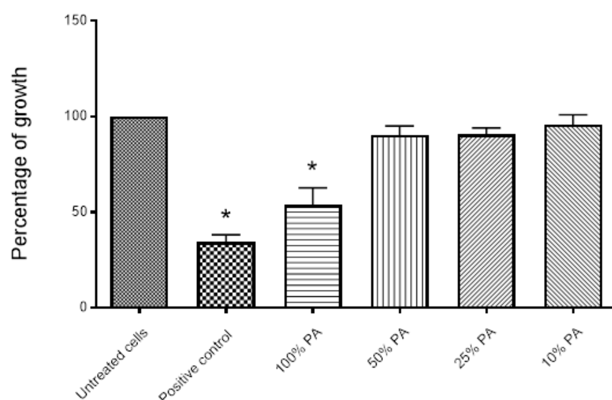

Figure 1. Cell proliferation after being treated with ethanol extracts from *P. americana* leaves with varying concentrations (100%, 50%, 25%, and 10%). Doxorubicin 3 μM served as a positive control. The data are expressed as mean ± SD (n = 3).
*Indicates a significant difference compared to untreated cells ($p < 0.05$).

DISCUSSION

Abnormal and uncontrolled cell growth and division are distinct characteristics of cancer cells. HSC-3 is a cell line that has local invasion ability.[12] Cancer cells are also capable of metastasis by invading and destroying healthy tissue and organs, moving from one part of the body to another.[13] A previous study indicated that *P. americana* can be used as a chemo-preventive agent on cancer cells.[7]

In this study, *P. americana* leaves were extracted using 96% ethanol by the maceration technique. Maceration was chosen because it is simple and can extract many compounds from the plant. Ethanol was chosen because it is a polar solvent, which can extract bioactive compounds, such as flavonoid, steroid, tannin, and quinone.[14] After the extraction process was finished, the extract was divided into four concentrations (100%, 50%, 25%, and 10%). The aim of the cell proliferation assay was to determine if any cells survived after being treated by the substance. In this study, the MTT assay method was used, which is based on conversion of MTT to formazan crystals by active mitochondrial dehydrogenases from surviving cells.[15] The results of this study showed that *P. americana* at 100% concentration could inhibit HSC-3 proliferation by 50%. Doxorubicin 3 μM as positive control killed HSC-3 up to 65.82% ± 6.825%, while *P. americana* at 100% concentration could kill cells up to 46.44% ± 15.813%. Positive control compared to *P. americana* at 100% concentration had no significant difference. This means that doxorubicin 3 μM has the same inhibitive effects on HSC-3 proliferation as *P. americana*; however, concentrations of 50%, 25%, and 10% showed no signs of inhibition because lower concentrations did not have enough active compound extracted to inhibit HSC-3 proliferation.

The results of this study are in line with a previous study that showed cytotoxicity of ethanol extract of *P. americana* leaves against HeLa cells. In this study, *P. americana* at concentration 360 μg/ml could kill 50% of HeLa cells. This study also showed alteration of cell morphology as the concentration of the extracts increased.[7]

In the present study, the 100% concentration killed 46.44% of HSC-3. Concentration of 100% is very high compared to 360 μg/ml, possibly because HeLa and HSC-3 have different resistances to *P. americana.* Therefore, further research is needed to study effective concentrations to inhibit different cancer cells. Moreover, the leaves, seeds,[1] root bark,[16] stem bark,[8] and fruit[17] of *P. americana* have anticancer effects against various cancer cells, and some studies used methanol and water extracts instead of ethanol extract.

A previous study investigated the antioxidant capacity of seven different Mexican avocado leaves. The results showed that Mexican *P. americana* possess various polyphenolic compounds with high antioxidant activity.[18]

Another previous study reported the chemical constituents of *P. americana* leaves by isolating flavonol glycoside along with ten known flavonoids, four megastigmane glycosides, and two lignans. They claimed that megastigmane glycosides and lignans classes have never been isolated from *P. americana*.[19] However, none of those constituents have been investigated for their anticancer activity.

Flavonoid has proven to have analgesic, anti-inflammatory, antipyretic, and even anticancer properties.[20] Flavonoid is also considered to be an antioxidant as a potential cancer prevention phenolic compound.[21] A previous study showed that phenolic compounds also induce apoptosis, halt the cell cycles in G1/S and G2/M phases, and alter signaling pathways involved in cancer.[22] There are many ways for flavonoid to inhibit cancer cell proliferation, such as rendering carcinogenic compound inactive, inhibiting the cell cycles, inducing apoptosis and cell differentiation, inhibiting angiogenesis, and acting as an antioxidant.[23]

P. americana possess a steroid compound that is probably responsible for the inhibition of HSC-3 proliferation. Steroids have been known to have therapeutic value, including against anticancer, because they possess enzyme inhibitors, such as aromatase, sulfatase inhibitors, 5α-reductase inhibitors, and CYP 17 inhibitors. Another type of steroid that fights cancer are receptor modulators clinically used in hormone therapy, such as selective estrogen receptor modulators (SERMs) and selective androgen modulators (SARMs).[24] Natural steroids isolated from plants are proving to be promising anticancer drug treatments for the future.

Tannin has proven to have anticancer properties to fight various cancer cells in many previous studies that have tested against HeLa cells,[25] MCF-7 (breast cancer cell lines), HCT-116 (human tumorigenic colon cell lines), CCD-18Co (non-tumorigenic colon cell lines),[26] M220 (pancreatic cancer cells), JIMT-1 (breast cancer cells),[27] HL-60 (human promyelocytic leukemia cell lines),[28] and many more. Tannin's anticancer mechanisms are possibly due to its ability to prevent the activation of PARP-1, reduce Bax, and increase Bcl-2 expression.[29] Exposure to tannin increased apoptotic activity in prostate and breast cancer cells.[30] In one study on human colon cancer (Caco-2) showed that tannin provoked down regulation of cyclins A and B1, up regulation of cyclin E, cell cycle arrest in the S phase, and induction of intrinsic pathway apoptosis.[31]

Quinone was another compound found in *P. americana* that showed anticancer properties. There were two general mechanisms of quinone that promoted anticancer activity. The first was by mediating through quinone redox cycling. After quinone was reduced to semiquinone, it could be oxidized which first lead to the production of superoxide anion radicals and then reactive oxygen species (ROS).[32] ROS have the capability to damage lipids, protein, RNA, and DNA of cancer cells. This DNA damage can cause apoptosis.[33] ROS also have the power to cause impairment of the mitochondrial membrane that leads to apoptosis activation.[34, 35]

Given the early successes into the investigation of *P. americana,* it is necessary to continue this study to determine which compounds of *P. americana* are responsible for anticancer activity. One limitation of this study was that the phytochemical assay was qualitative, so we could not find out the amount of dominant chemical compound present in the *P. americana* extract used for the treatment to cells. For future research, it is recommended that quantitative phytochemical assay be used to know the chemical compounds that play a role in inhibiting HSC-3 proliferation. A second limitation was the dose selection. For future research, it is necessary to choose a wider range of concentrations, so the minimum effective concentration can be known.

CONCLUSION

Based on the qualitative phytochemical assay, it was determined that ethanol extracts from *P. americana* leaves contain flavonoid, steroid, tannin, and quinone. At 100% concentration, this extract could inhibit HSC-3 proliferation. These results suggest that *P. americana* may be a potential agent for treating oral cancer, even though further studies are still needed.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Indra Kusuma, M. Biomed from Faculty of Medicine, YARSI University, Jakarta, Indonesia, who provided HSC-3 cells.

REFERENCES

1. Widiyastuti Y, Pratiwi R, Riyanto S, Wahyuono S. Cytotoxic activity and apoptosis induction of avocado Persea americana Mill. seed extract on MCF-7 cancer cell line. Indones J Biotechnol. 2018;23(2):61-7.
2. Al-Dimassi S, Abou-Antoun T, El-Sibai M. Cancer cell resistance mechanism: a mini review. Clin Transl Oncol. 2014;16(6):511-6

3. Sarode GS, Sarode SC, Maniyar N, Anand R, Patil S. Oral cancer databases: A comprehensive review. J Oral Pathol Med. 2018;47(6):547-56.
4. Danaraddi S, Koneru A, Hunasgi S, Ramalu S. Natural ways to prevent and treat oral cancer. J Oral Res Rev. 2014;6(1):34-9.
5. Yasir M, Das S, Kharya MD. The phytochemical and pharmacological profile of *Persea americana* Mill. Pharmacogn Rev. 2010;4(7):77-84.
6. Bonilla-Porras AR, Salazar-Ospina A, Jimenez-Del-Rio M, Pereanez-Jimenez A, Velez-Pardo C. Pro-apoptotic effect of Persea americana var. Hass (avocado) on Jurkat lymphoblastic leukemia cells. Pharm Biol. 2013.
7. Mardiyaningsih A, Ismiyati N. Cytotoxic activity of ethanolic extract of *Persea americana Mill.* leaves on HeLa cervical cancer cell. Trad Med J. 2014;19(1):24-8.
8. Falodun A, Iyamabo H, Odion EE, Engel-Lutz N. Antiproliferative and Pro-apoptotic activities of the stem bark of Persea Americana (Lauraceae) mill in Human Breast Adenocarcinoma Cell Line. J Appl Sci Environ Manage. 2013;17 (3):433-8.
9. Khoswanto C, Juliastuti WS, Adla KA. The effect of Avocado leaf extract (*Persea americana* Mill.) on the fibroblast cells of post-extraction dental sockets in Wistar rats. Dent J. 2018;51(3):129-32.
10. Kemit N, Widarta IWR, Nocianitri KA. Pengaruh jenis pelarut dan waktu maserasi terhadap kandungan senyawa flavonoid dan aktivitas antioksidan ekstrak daun alpukat (*Persea Americana* Mill). 2016;5 (2):130-41.
11. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. In: Markossian S, Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, et al., editors. Assay Guidance Manual. Bethesda (MD)2004.
12. Medawati A. Karsinoma sel skuamosa sebagai salah satu kanker rongga mulut dan permasalahannya. Insisiva Dent J. 2013;2 (1):87-90.
13. Nafis FDR, Sofian FF. Review jurnal: Aktivitas anti kanker payudara beberapa tanaman herbal. Farmaka. 2018;16 (2):84-95.
14. Mukhriani. Ekstraksi, pemisahan senyawa, dan identifikasi senyawa aktif. Jurnal Kesehatan. 2014;7 (2):361-67.
15. van Tonder A, Joubert AM, Cromarty AD. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. BMC Res Notes. 2015;8:47.
16. Falodun A, Engel N, Kragl U, Nebe B, Langer P. Novel anticancer alkene lactone from Persea americana. Pharm Biol. 2013;51(6):700-6.
17. Khalifa NS, Barakat HS, Elhallouty S, Salem D. Effect of the Water Extracts of Avocado Fruit and Cherimoya Leaf on Four Human Cancer Cell Lines and Vicia Faba Root Tip Cells. J Agric Sci. 2013;5(7):245-54.
18. Castro-Lopez C, Bautista-Hernandez I, Gonzalez-Hernandez MD, Martinez-Avila GCG, Rojas R, Gutierrez-Diez A, et al. Polyphenolic Profile and Antioxidant Activity of Leaf Purified Hydroalcoholic Extracts from Seven Mexican Persea americana Cultivars. Molecules. 2019;24(1).
19. Park S, Nam YH, Rodriguez I, Park JH, Kwak HJ, Oh Y, et al. Chemical constituents of leaves of Persea americana (avocado) and their protective effects against neomycin-induced hair cell damage. Revista Brasilera de Farmacognisa. 2019;29:739-43.
20. Putri EPK, Hamzah B, Rahman N. Analisis kualitatif zat bioaktif pada ekstrak daun alpukat (*Persea americana* Mill.) dan uji praklinis dalam menurunkan kadar glukosa darah pada mencit (mus musculus). J Akad Kim. 2013;2 (3):119-27.
21. Redha A. Flavonoid: Struktur, Sifat Antioksidatif Dan Peranannya Dalam Sistem Biologis. Jurnal Belian. 2010;9 (2):196-202.
22. Jeane M, Asih IARA, Bogoriani NW. Asupan Glikosida Flavonoid Terong Belanda (Solanum betaceum Cav.) Terhadap Aktivitas Superoksida Dismutase Dan Kadar Malondialdehid Tikus Wistar Yang Diberi Aktivitas Fisik Maksimal. Jurnal Media Sains 2018;2(1):32-6.
23. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. Med Res Rev. 2003;23(4):519-34.
24. Salvador JA, Carvalho JF, Neves MA, Silvestre SM, Leitao AJ, Silva MM, et al. Anticancer steroids: linking natural and semi-synthetic compounds. Nat Prod Rep. 2013;30(2):324-74.
25. Yildrim I, Kutlu T. Anticancer Agents: Saponin and Tannin. Int J Biol Chem. 2015;9 (6):332-40.
26. Gonzalez-Sarrias A, Yuan T, Seeram NP. Cytotoxicity and structure activity relationship studies of maplexins A-I, gallotannins from red maple (Acer rubrum). Food Chem Toxicol. 2012;50(5):1369-76.
27. Barrajon-Catalan E, Fernandez-Arroyo S, Saura D, Guillen E, Fernandez-Gutierrez A, Segura-Carretero A, et al. Cistaceae aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells. Food Chem Toxicol. 2010;48(8-9):2273-82.
28. Yang LL, Lee CY, Yen KY. Induction of apoptosis by hydrolyzable tannins from Eugenia jambos L. on human leukemia cells. Cancer Lett. 2000;157(1):65-75.
29. Tikoo K, Sane MS, Gupta C. Tannic acid ameliorates doxorubicin-induced cardiotoxicity and

potentiates its anti-cancer activity: potential role of tannins in cancer chemotherapy. Toxicol Appl Pharmacol. 2011;251(3):191-200.

30. Bawadi HA, Bansode RR, Trappey A, 2nd, Truax RE, Losso JN. Inhibition of Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cell proliferation by water-soluble black bean condensed tannins. Cancer Lett. 2005;218(2):153-62.
31. Larrosa M, Tomas-Barberan FA, Espin JC. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J Nutr Biochem. 2006;17(9):611-25.
32. Verrax J, Delvaux M, Beghein N, Taper H, Gallez B, Buc Calderon P. Enhancement of quinone redox cycling by ascorbate induces a caspase-3 independent cell death in human leukaemia cells. An in vitro comparative study. Free Radic Res. 2005;39(6):649-57.
33. Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol. 2007;47:143-83.
34. Barshteyn N, Elfarra AA. Formation of mono- and bis-Michael adducts by the reaction of nucleophilic amino acids with hydroxymethylvinyl ketone, a reactive metabolite of 1,3-butadiene. Chem Res Toxicol. 2009;22(5):918-25.
35. Briggs MK, Desavis E, Mazzer PA, Sunoj RB, Hatcher SA, Hadad CM, et al. A new approach to evaluating the extent of Michael adduct formation to PAH quinones: tetramethylammonium hydroxide (TMAH) thermochemolysis with GC/MS. Chem Res Toxicol. 2003;16(11):1484-92.