ORIGINAL ARTICLE

Potential of *Moringa oleifera* Leaf Extract on IL -8 Expression in Benzo(a)pyrene-Induced-Oral Cancer of *Rattus norvegicus*

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ABSTRACT

Introduction: Cancer is a disease caused by abnormal growth and uncontrolled cells. Moringa leaf extract can prevent or destroy tumors and cancer cells because it contains benzyl isothiocyanate. Many scientific studies have shown that these chemicals have anti-cancer and chemoprotective capabilities. IL-8 is a chemotactic factor expressed in endothelial cells. This study aims to know the potential of Moringa leaf extract as anti-angiogenesis of oral cancer by IL-8 expression in rats induced by benzo(a)pyrene. **Material and methods:** This research was an experimental laboratory with a post-test only control group design divided into four groups, control group (K) and a treatment group was given Moringa extract with a dose of 20mg/kg/BW, 40mg/kg/BW, 80mg/kg/BW. Immunohistochemical analysis was used to determine IL-8 expression. **Result:** ANOVA data analysis revealed that group treatment with 40mg/kg/BW has the lowest number of IL-8 expression in endothelial cells of rats induced by benzo(a)pyrene. **Conclusion:** *Moringa oleifera* extract with a dose of 40 mg/kg/BW is the best dose that can inhibit angiogenesis of oral cancer cells in male rats (*Rattus norvegicus*) induced benzo(a)pyrene.

Keywords: cancer of oral cavity, *Moringa oleifera* leaf extract, IL-8, benzyl isothiocyanate, benzo(a)pyrene

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INTRODUCTION

Cancer is a disease caused by the growth of an abnormal cell. WHO reports that one cancer patient dies every 11 minutes and new cancer cases appear every 3 minutes. Oral cavity cancer is one of the highest death in the world, reaching 13% of all deaths. In Indonesia, oral cancer cases range from 3-4% of all cancer cases. The number of deaths is 2-3% of all deaths from malignancy (1,2). Chemotherapy and surgery have become the gold standard for cancer treatment. But these treatments continue to have a negative effect on the patient, most likely as a result of the side effects that might harm normal cells and organs (22). So, alternative treatments are needed.

Angiogenesis is an essential factor in cancer growth because it could make cancer cells grows beyond two mm^3 in diameter (14). Angiogenesis is stimulated when the tumor tissue requires nutrients and oxygen. Angiogenesis is regulated by vascular endothelial growth factors/receptors (VEGF/R) (3). One of the many proteins that have been found to be angiogenic activators is interleukin-8 (IL-8)(15). IL-8 is a cytokine that promotes angiogenesis and inflammation (12,13). Besides its chemotactic function, IL-8 has a role in cancer proliferation and metastasis. Serum and non-vascular extracellular fluids such saliva, mucus, CSF, and urine from cancer patients contained high concentrations of IL-8 (4). In vivo models of angiogenesis show that IL-8 stimulates angiogenic responses, and it is strongly linked to tumor angiogenesis, including in nasopharyngeal carcinoma, hepatocellular carcinoma, and cervical cancer (5,6,7,18).

Moringa oleifera contains unique compounds named isothiocyanate (ITC) and glucosinolate. ITC has the potential to be a chemoprotective and anti-cancer agent, according to studies. By concentrating on the hypoxia-inducible factor (HIF) transcription factor, ITC can reduce the expression of pro-angiogenic molecules like VEGF (8). It could potentially inhibit the angiogenesis of tumors by blocking certain factors. By downregulating matrix metalloproteinase (MMP) and upregulating matrix metalloproteinase inhibitors (TIMP), the ITC

and its derivatives are designed to limit cell adhesion, invasion, and migration in vitro and reduce metastasis in vivo. According to studies, *Moringa oleifera* can act as an anti-neoproliferative agent, preventing the proliferation of cancer cells. It has been demonstrated that soluble extract and leaf solvent are powerful anticancer substances (8,9,10,16).

Based on this theory, this study was prepared to know the potential of *Moringa oleifera* leaf extract (MOE) on the IL-8 expression in endothelial cells of oral cancer in rats that were induced with benzo(a)pyrene.

MATERIALS AND METHODS

500 grams of Moringa leaves were obtained from Kebun Kelor Lawang, Malang. It was dried and then macerated with 96% ethanol. After that, phytochemical screening through a Glycoside test was carried out to detect the active compounds. A positive result of the presence of glycoside bonds is indicated by the formation of a purple ring called the Molish Reaction.

This research was an experimental laboratory study with a post-test-only group design that was approved by the Committee of Ethical Clearance of Health Research, Universitas Airlangga (78/KKEPK.FKG/VIII/2015). The experimental animals used were 30 male Wistar rats (*Rattus norvegicus*), weighing 160-200 gram grams, aged 3 months, and acclimated for 7 days in a cage sized 60 cm x 65 cm x 80 cm, bedding with wood shavings, in a room temperature according to the laboratory standards in the Animal Laboratory Unit of Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga. Then, 30 Wistar rats were randomly divided into 4 groups consisting: treatment group 1 (KP1), treatment group 2 (KP2), treatment group 3 (KP3), and control group (K). An additional number of two rats are sacrificed to confirm cancer cell formation by histopathological method HE staining. The K group, also known as the positive control group, consisted of rats that had received benzo(a) pyrene injections but no further treatments. KP1, KP2, and KP3 were groups of rats induced by benzo(a)pyrene and injected with *Moringa oleifera* leaf extract at dose of 20mg/kg, 40mg/kg, and 80mg/kg using 2 ml of stomach tube every day for 1 month.

The samples were induced with Benzo(a)pyrene (Sigma Adrich, Darmstadt Germany) to form oral cancer at a dose of 8 mg/kg dissolved in oleum olivarum with a ratio of 2:1 (21). An injection of 0.7 ml benzo(a)pyrene mixture has done in the buccal mucosa of the rats with 2-3 mm deep using a size thirty-gauge needle twice a week for 4 weeks.

Rats were sacrificed using 10% ketamine hydrochloride (150 mg/kg) associated with 2% xylazine (15mg/kg) as euthanasia after being treated for 4 weeks. Tumor tissues were collected from each sample and made into paraffin blocks for further histological analysis. The paraffin blocks were deparaffinized using xylol and absolute alcohol, then were cut (4 µm) with a microtome (Sakura, Japan) and put into slides. The slides were subsequently incubated in hydrogen peroxide 3% for 10 minutes, followed by incubated in anti-IL-8 monoclonal antibody (Santa Cruz, America) for 2 hours. The slides were then incubated in a biotinylated secondary antibody for 30 minutes, followed by streptavidin–HRP for 30 minutes. A DAB chromogen was added for 6-10 minutes and counterstained by means of Haematoxylin Meyer.

Data were collected by observing IL-8 expression in 5 different fields using a microscope (Olympus) with 400 times magnification. To identify significant differences between groups of variables, data were analyzed using the ANOVA Test and the Post Hoc Tukey HSD Test with the Statistical Package for the Social Science (SPSS) application.

RESULT

The findings of the *Moringa oleifera* leaf extract (MOE) on increased angiogenesis of oral cancer cells by detecting IL-8 (Interleukin 8) expression in the cancer endothelium cells are shown in Figure 1, and its mean is shown in Table I.

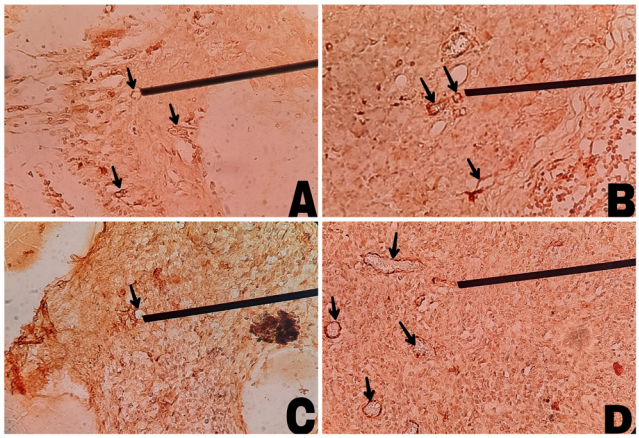

Fig. 1: Expression of IL-8 on group K (A), group KP1 (B), group KP2 (C), and group KP3 (D).

In Table I, the total number of IL-8 expression in the endothelial cells of cancer was higher in group K with a mean 34,2, compared to group KP1 with a mean 19,2, KP2 with a mean 11,2, and KP3 with a mean 19,2.

Table I: Multiple IL-8 expression data on cancer cells per treatment

No	Group	Mean	Standard Deviation	P Value
1	Control Group	34,2	8,1	
2	KP1 (20 mg/kg/bw)	19,2	6,4	
3	KP2 (40 mg/kg/bw)	11,2	2,8	0,00
4	KP 3 (80 mg/kg/bw)	19,2	5,5	

Notes: * The average difference is significant at 0.05

The normality (Shapiro-Wilk) and homogeneity test (Levene's) were performed on all data. Data were normally distributed and homogeneous ($p > 0.05$). Then, a parametric test was performed using the ANOVA Test and a multiple comparison test was performed using the Post Hoc Test to prove significant differences between each group which can be seen in Table II.

Table II has shown that each group have differences are marked value. KP2 group (value <0.05) has a significant difference compared to group K, KP1, and KP3. Meanwhile KP1 and KP3 (value >0.05) has no significant differences between each other. KP2 is the treatment group that expressed the lowest expression of IL-8 in cancer cells.

Table II: Differences of IL-8 expression on Control and Treatment Group

(I) Group	(J) Group	Sig.
Control	KP 1	.000
	KP 2	.000
	KP 3	.000
KP 1	Control	.000
	KP 2	.032
	KP 3	1.000
KP 2	Control	.000
	KP 1	.032
	KP 3	.032
KP 3	Control	.000
	KP 1	1.000
	KP 2	.032

Notes: *: The average difference is significant at 0.05

DISCUSSION

The cellular damage brought on by free radicals, particularly cellular DNA damage, may contribute to the emergence of cancer and other diseases. Free radicals are reactive molecules. The increased production of free radicals is caused by an imbalance between generating and scavenging free radicals. The presence of an imbalance of free radicals is caused by a state of hypoxia, resulting in increased oxidative stress induced during metabolic activity. Oxidative stress promotes the initiation and progression of cancer through mutations caused by DNA damage. Oxidative stress causes the production of angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) (23). It has been suggested that IL-8 has an autocrine and/or paracrine tumor-promoting role in modulating tumor cell survival and proliferation. The biologic activity of IL-8 in tumors and the tumor microenvironment might contribute to tumor progression through regulation of angiogenesis, cancer cell growth and survival, tumor cell motility, leukocyte infiltration, and modification of immune responses. IL-8 was initially identified as a chemoattractant for neutrophils that release angiogenic growth factors, stimulating angiogenesis as part of cancer development. IL-8 has a direct role in angiogenesis through increased proliferation and survival of endothelial cells and expression of matrix metalloproteinases in endothelial cells expressing CXCR-1- and CXCR-2. IL-8 stimulates endothelial proliferation and capillary formation. A recent study demonstrated that IL-8 stimulates VEGF expression in endothelial cells via CXCR-2, thus promoting autocrine activation of the VEGF receptor. IL-8 has been shown to increase the production and secretion of matrix metalloproteinases MMP-2 and MMP-9 by tumor cells suggesting that it may modulate extracellular matrix invasion and/or remodeling in the tumor environment. Since cell proliferation, angiogenesis, migration, and aggression are all involved in the metastatic process, the expression of IL-8 by tumor cells may affect their metastatic ability (24). Therefore, IL-8 inhibition can be an essential therapy for the inhibition of cancer invasion and metastasis.

The extract used in this research is Moringa leaf extract (MOE) which is made by the maceration method using methanol solvent which is able to dissolve all classes of secondary metabolites (10). Its leaf contains a unique chemical substance such as ITC, glycocyanate, carbamate, glycoside mat, phenolic, niazimicin, and flavonoids which in previous studies showed biological activity as an anti-inflammatory, antioxidant, and anti-tumor (8,9). ITC is particularly useful as a chemopreventive agent for cancer cells. In nature, ITC is founded in the form of benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), and phenyl isothiocyanate (PITC). ITC will be formed by the action of the enzyme myrosinase after the plant cell is damaged when the leaves are chewed. Some research proved the potential of Moringa leaf as an anti-cancer agent by stating that BITC in vitro is able to induce apoptosis against ovarian cancer cells (9,11,20). As an immune response, NF-κB directs the expression of tumor necrosis factor (TNF) cytokines, IL-1, IL-6, and IL-8. However, hyper-activation of NF-kB has contributed to cancer cell proliferation. Therefore, NF--κB is thought to play a "double-edged role" in the immune system. On the one hand, NF--κB stimulation directs leukocytes to sites of inflammation as part of innate immunity. On the other hand, tumors can principally form NF-kB activity, which is increased by several intrinsic and extrinsic factors. Especially NF-kB mutations and constant release of cytokines contribute significantly to cancer development. BITC is known to inhibit NF-kB activity through increased induction of Nrf2. Induction of Nrf2 attenuates NF-kB suggesting that BITC-mediated pathways are involved with ROS generation, antioxidants, and other stress mechanisms of the immune response. NF-κB is a redox-sensitive complex and can be inhibited by higher antioxidants. Suppression of NF-kB activation is considered a central mechanism in preventing tumor growth. Decreased

Nf-kB activity can lead to decreased IL-8 expression in tumor areas (25).

In this research, cancer cells were obtained from mucosal tissue of a rat's oral cavity that have been induced with benzo(a)pyrene. Benzo(a)pyrene is a carcinogenic material that is able to enter the DNA and interfere with the transcription process. The induction was performed by injection of a benzo(a)pyrene solution at a dosage of 8 mg/kg BW. The incidence of cancer was characterized by the presence of tumors in the oral mucosa and a histological method picture through HE staining (21).

Data analysis revealed that samples from treatment groups had lower levels of IL-8 expression in the endothelial cells of oral cancer patients than the control group. The number of IL-8 expression in KP2 was lower than the expression of IL-8 in KP1 and KP3 in oral cancer endothelial cells. These results prove that the active ingredients in *Moringa oleifera* leaf extract could decrease the number of IL-8 expression. IL-8 is pro-angiogenic for forming new vascular, it can make cancer spread progressively (15,17). Endothelial cells have a big contribution to malignancy (19). The microvessel would represent the angiogenic potential of neoplasia. According to this study, a dose of 40 mg/kg/BW of moringa leaf extract can suppress the expression of IL-8, which is not present in neo-vascular tissue. We assume that IL-8 increases the growth of capillaries and endothelial cells. IL-8 has been demonstrated in oral cancer with poor prognosis and high metastatic potential in the control group. The treatment group with a dose of 40mg/kg/BW (KP2) is better than the other groups.

CONCLUSION

It can be concluded that MOE with dose 40 mg/kg/BW is the best dose that can inhibit angiogenesis of oral cancer cells in male rats (*Rattus norvegicus*) induced by benzo(a)pyrene.

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REFERENCES

1. Saifudin J, Iwan H, Adiastuti E. Prevalence of Oral Mucositis in Head and Neck Cancer Patients with Radiotherapy at RSAL Dr. Ramelan Surabaya. Oral Medicine Dental Journal. 2016;8(2):9-17. Available from: http://repository.unair.ac.id/id/eprint/58631.
2. Hwang SL et al. 2006. Cancer risks in a population with prolonged low dose-rate gamma-radiation exposure in radiocontaminated buildings, 1983-2002. Int J Radiat Biol. 2006;8(12):849-58. doi:10.1080/09553000601085980.
3. Chavan P. 2015. Peran Interleukin untuk Prognosis Kanker. IOSR Journal of Dental and Medical Sciences. 2015;14(6):44-47. doi:10.9790/0853-14644447.
4. Bapat R, Supriya K, Mohit K, Tanya S, Archana AG. 2016. Role of interleukin 8 in oral squamous cell carcinoma: A systematic review. Research Journal of Pharmaceutical, Biological, and Chemical Sciences. 2016;7(2):436-442. Available from: https://www.researchgate.net/publication/301556823_Role_of_interleukin_8_in_oral_squamous_cell_carcinoma_A_systematic_review.
5. Sales KJ, Suterland JR, Jabbor HN, Katz AA. Seminal plasma induces angiogenic chemokine expressionin cercvical cancer regulate vascular function. Biochim Biophys Acta. 2012;1823(10): 1789-1795. doi:10.1016/j.bbamcr.2012.06.021.
6. Waugh DJ, Wilson C. The Interleukin 8 pathway in cancer. lin Cancer Res. 2008;14(21):6735-6741. doi: 10.1158/1078-0432.CCR-07-4843.
7. Nomura T, Morishita A, Jian G. Expression of angiogenic factors in hepatocarcinogenic: Identification by antibody arrays. Oncol. 2013;30(5): 2476-2480. doi: 10.3892/or.2013.2674.
8. Ullah MF. 2015. Sulforaphane (SFN): An Isothiocyanate in a Cancer Chemoprevention Paradigm. Medicines (Basel). 2015;2(3):141-156. doi: 10.3390/medicines2030141.
9. Anwar F, Latir S, Ashraf M, Gilan A. *Moringa oleifera* a food plant with multiple medicinal uses. Phytother Res. 2007;21(1):17-25. doi: 10.1002/ptr.2023.
10. Luqman S, Suchita S, Ritesh K, Anil KM, Debabrata C. Experimental Assesment of *Moringa oleifera* Leaf and fruit for its Antistress, antioxidant, and Scavenging Polential Using In Vitro and In Vivo Assays. Evid Based Complement Alternat Med. 2012;2012(519084): 1-12. doi: 10.1155/2012/519084.
11. Navarro SL, Fei li, Johanna WL. Mechanisms of action of isothiocyanates in cancer chemoprevention: an update. Food Funct. 201;2(10):579-587. doi: 10.1039/c1fo10114e.
12. Akiba J, Yano H, Ogasawa S, Higaki K, Kojiro M. Expression and Function of Interleukin 8 in human hepatocellular ca. Int J Oncol. 2007;18(2):257-264. doi: 10.3892/ijo.18.2.257.
13. Koch AE, Polverini PJ, Kunkel SL. Interleukin8 as a macrophage derived mediator of angiogenesis. Science. 1992;258(5089):1798-801. doi: 10.1126/science.1281554.
14. Nishida, Naoyo, Hirohisa Y, Takashi N, Toshiharu K. Angiogenesis in Cancer. Vascular Health and Risk Management. 2006;2(3):213-219. doi: 10.2147/vhrm.2006.2.3.213.

15. Sahibzada, Haafsa A, Zohaib K, Rabia SK, Mustafa N, Khalid MS, Maria M, Muhammad SZ. Salivary IL-8, IL-6 and TNF-α as Potential Diagnostic Biomarkers for Oral Cancer. Diagnostics (Basel). 2017;7(2):21. doi: 10.3390/diagnostics7020021.
16. Shi J, Pin-Kang W. Interleukin-8: A potent promoter of angiogenesis in gastric cancer. Oncol Lett Journal. 2016;11(2):1043–1050. doi: 10.3892/ol.2015.4035.
17. Strieter RM, Kunkel Sl, Elner VM. Interleukin8 a corneal factor that induces neovascularization. Am J Pathol. 1992;141(6):1279-1284. Available from: https://pubmed.ncbi.nlm.nih.gov/1281615/.
18. Widaningsih Y, William H, Ruland DNP, Uleng B. Kadar Interleukin-8 Kanker Payudara by Juranah. Indonesian Journal of Clinical Pathology and Medical Laboratory. 2017;20(2): 205-209. doi:10.24293/ijcpml.v20i3.471.
19. Wibowo RMD, David SP, dan Eddy HT. Mechanism of Apoptosis Inhibition to Squamous Cell Carcinoma of Oral Cancer in Cisplatin Treatment. Folia Medica Indonesiana. 2017;53(1):1-6. doi:10.20473/fmi.v53i1.5482.
20. Malabed, Raymond S, Marissa GN. Karakterisasi glukosinolat dan isothiosianat di Malunggay (*Moringa oleifera* L.) Ekstrak dan penentuan aktivitas Myrosinase dan sifat antikanker. Presented at the Research Congress 2013 De La Salle University Manila. 2013. Available from: https://www.dlsu.edu.ph/wp-content/uploads/pdf/conferences/research-congress-proceedings/2013/FNH/FNH-I-004.pdf.
21. TI Budhy, HS Watadianto, K Abubakar. Disertasi: Mekanisme Nekrosis Sel Kanker Oleh Sel Imunokompeten Makrofag Karena Pemberian Ekstrak Teh Hitam Pada Mukosa Rongga Mulut yang Terpapar Benzopirene. Airlangga University Library, 2000. Available from: https://opac.perpusnas.go.id/DetailOpac.aspx?id=528337.
22. Pearce, A. et al. Incidence and severity of self-reported chemotherapy side effects in routine care: A prospective cohort study. 2017;12(10):e0184360. doi: 10.1371/journal.pone.0184360.
23. Sheikhpour R. The Role of Interleukin-8 and Its Mechanism in Patients with Breast Cancer: Its Relation with Oxidative Stress and Estrogen Receptor. Int J Cancer Manag. 2017;10(9):e8791. doi: 10.5812/ijcm.8791.
24. Todorović-Raković N, Milovanović J. Interleukin-8 in Breast Cancer Progression. Journal of Interferon & Cytokine Research. 2013;33(10):563-570. doi: 10.1089/jir.2013.0023.
25. Soundararajan P, Kim J. Anti-Carcinogenic Glucosinolates in Cruciferous Vegetables and Their Antagonistic Effects on Prevention of Cancers. Molecules. 2018;23(11):2983. doi: 10.3390/molecules23112983.