

The potential effect of *Moringa oleifera* leaves extract on vascular endothelial growth factor expression in Wistar rat oral cancer cells

Dwicha Rahma Nuriska Hartono, Theresia Indah Budhy Sulisetyawati, and Edhi Jularso
Department of Oral and Maxillofacial Pathology
Faculty of Dental Medicine, Universitas Airlangga
Surabaya – Indonesia

ABSTRACT

Background: Cancer is a disease characterized by abnormal and uncontrolled growth of tissue cells. In Indonesia, cancer ranks as the fifth largest cause of mortality, while it is the second largest worldwide. Cancer affected by angiogenesis, the process of forming new blood vessels to provide the nutrient and oxygen necessary for tumor growth. Vascular endothelial growth factor (VEGF) represents a pro-angiogenic factor. *Moringa oleifera* leaf extract can be used as an anticancer agent by reducing the expression of VEGF. **Purpose:** The study aimed to prove that *Moringa oleifera* leaf extract can reduce VEGF expression in benzopyrene-induced oral cancer cells of Wistar rats. **Methods:** This was an experimental laboratory research with posttest-only control group design. All experimental subjects presented symptoms of cancer following induction with 8 mg/KgBW of benzopyrene. The sample consisted of 28 Wistar rats, divided into four groups, namely; a control group (K) whose members were administered with only aquadest and three treatment groups (P) treated with *Moringa oleifera* leaves extract at percentages of 3.125% (P1), 4.6875% (P2) and 6.25% (P3) respectively. Observation of VEGF expression was undertaken by means of immunohistochemical staining. **Results:** A decrease in VEGF expression occurred in all treatment groups when compared with the control group. A significant difference existed between the control group (K) and the treatment group (P1), while there were no significant differences between the treatment groups (P1, P2 and P3). **Conclusion:** *Moringa oleifera* leaf extract with a percentage of 3.125% proved most effective at reducing VEGF expression in oral cancer cells in Wistar rats.

Keywords: isothiocyanate; *Moringa oleifera* extract; oral cancer; VEGF expression

Correspondence: Theresia Indah Budhy Sulisetyawati, Department of Oral and Maxillofacial Pathology, Faculty of Dental Medicine, Universitas Airlangga. Jl. Mayjen. Prof. Dr. Moestopo no. 47 Surabaya 60312, Indonesia. Email: terebudhy@gmail.com

INTRODUCTION

Oral cancer represents one of the six most common malignancies in Asia with 274,300 new cases annually.¹ Cancer ranks as the fifth most common cause of mortality in Indonesia and the second largest worldwide with 7.6 million deaths in 2014, a figure projected to increase to 13.1 million in 2030.^{2,3}

Most cancer-related deaths occur due to its progressive development and metastasis,⁴ both of which can be affected by angiogenesis. Therefore, analyzing the formation of new blood vessels can play a role in determining the prognosis and physiological and pathological management of cancer

patients.^{5,6} Research has indicated that vascular endothelial growth factor (VEGF) is the main factor involved in angiogenesis and that its expression in neoplastic epithelium is higher than dysplastic and normal epithelium.⁷ Termination of VEGF signaling pathway is now approved and used as an alternative cancer therapy.⁵

Cancer treatment often involves the use of chemotherapy⁸ which has the disadvantages of being expensive and producing several side effects such as dizziness, nausea, fatigue, mucositis, nerve damage, neutropenia and infection.⁹ These side effects are caused by chemotherapy drugs which do not selectively destroy cancer cells, but also the surrounding normal cells such as hair follicle cells,

gastrointestinal mucosa cells and bone marrow cells that proliferate rapidly.¹⁰ For these reasons, herbal medicines tend to be taken as a form of cancer treatment. Not only because of its affordable price, but also its convenience and minimal side effects.³ One herbal treatment product is *Moringa oleifera*, also known as kelor, which has high nutrient content in all its parts, ranging from the leaves, stems, flowers and fruit to the roots. *Moringa oleifera* leaves contain isothiocyanate which the research conducted by Gupta *et al.*³ showed to have anticancer properties. These properties render it effective as a treatment for cancer.¹¹

Isothiocyanate, as an anticancer treatment, possesses a mechanism capable of inhibiting the process of angiogenesis.¹² During the development of a cancer, angiogenesis can be induced by various factors, one of which is hypoxia due to limited oxygen diffusion.⁵ A low concentration of oxygen induces the expression of various vasculogenic and angiogenic factors, including VEGF.¹³

The research conducted by Boreddy *et al.*¹⁴ stated that isothiocyanate can inhibit HIF-1 α signal transduction activity and decrease VEGF expression, while that carried out by Nararya¹⁵ also asserted that *Moringa oleifera* leaf extract demonstrates biocompatible properties at concentrations between 0.406% and 3.125%. These concentrations can be interpreted as non-toxic and *Moringa oleifera* leaf extract can, therefore, be used as a herbal medicine.¹⁵

Based on the data above, the authors intend to undertake further examination of the potential of *Moringa oleifera* leaf extract to treat VEGF expression in oral cancer. The study reported here was conducted using a glycoside test to observe the decrease in VEGF expression after administration of *Moringa oleifera* leaf extract that contained 6.92% isothiocyanate.

MATERIALS AND METHODS

The research constituted a laboratory-based experimental investigation with posttest-only control group design, approved by the Ethical Clearance for Health Research Committee, Faculty of Dental Medicine, Universitas Airlangga (No.121/HRECC.FODM/VIII/ 2018). The research sample comprised 2-3 month old, healthy, male, *Rattus norvegicus* with a body weight between 130 and 150 grams. The 28 Wistar rats in the sample were divided into four groups, namely: the control group (K) which was given only aquadest and three treatment groups (P) treated with *Moringa oleifera* leaves extract at respective percentages of 3.125% (P1), 4.6875% (P2) and 6.25% (P3).¹⁵

Moringa oleifera leaf extract was produced by the Research Institute and Industrial Consultation Laboratory in Surabaya. *Moringa oleifera* leaves were dried for 24 hours before being mashed in a blender. The resulting powder was then soaked in 96% ethanol in a closed container over a period of two days. The resulting solution was filtered

with maceration subsequently carried out until clear results were obtained and evaporated at 40°C in a rotary vacuum evaporator. Furthermore, *Moringa oleifera* leaves extract was diluted with aquadest to obtain concentrations of 3.125%, 4.6875% and 6.25%.¹⁵

The maceration result subsequently underwent phytochemical screening using a glycoside test to determine the amount of its isothiocyanate phytochemical content. The first step involved producing a trial solution. The filtrate was filtered three times with a 20 mL mixture of chloroform P and isopropanol P at a volume of 3:2. Sodium sulfate hydrate was added to the solution, filtered and evaporated at 50°C with the remaining filtrate being diluted with 2 mL of methanol P.¹⁵

Approximately 0.1 mL of trial solution was evaporated in a water bath and the remaining solution diluted in 5 mL of anhydrous acetic acid P. Sulfuric acid P was dripped into the solution ten times. The resulting blue or green color indicated the presence of glycosides (Liebermann reaction).

Another 0.1 mL of trial solution was inserted into a test tube and subsequently evaporated in a water bath. The remaining solution was added to 2 mL of water, two drops of Molish LP and 2 mL of sulfuric acid P. A purple ring was formed at the edge of the liquid indicating the presence of sugar bond (Molish reaction).¹⁵

Oral cancer was induced to all the experimental subjects by administering a dose of benzopyrene in powder form amounting to 8 mg/kg of bodyweight which was dissolved in oleum olivarum at a ratio of 2:1.¹⁶ The administration of 0.2 mL benzopyrene was performed through extraoral injection into the right cheek of the Wistar rat subjects three times a week over a period of four consecutive weeks.

After having been induced with cancer, all experimental subjects in the treatment group were administered with *Moringa oleifera* leaf extract in the following approximate amounts: the treatment group 1 (P1) at a concentration of 3.125%; treatment group 2 (P2) at a concentration of 4.6875% and treatment group 3 (P3) at a concentration of 6.25%. Based on the research of Nararya,¹⁵ the 2 mL extract was managed intraorally by means of an insulin sonde, once a day for 20 days. Meanwhile, the control group was administered only with aquadest. All experimental subjects were sacrificed through chloroform inhalation. The tissue was excised using surgical scissors and blades, then fixed using 10% neutral buffered formalin (NBF) solution (pH 6.5-7.5). The tissue was processed and 4 μ m-thick paraffin blocks were produced. VEGF expression was examined by immunohistochemical (IHC) staining involving the use of VEGF antibodies. The counterstain employed was haematoxylin mayer with the result that the positive cell was indicated by the presence of a brownish color.¹⁵ The data obtained was analyzed using one-way ANOVA and post-hoc Tukey HSD tests to determine its respective significance in each group.

RESULTS

Observation and calculation of VEGF expression using a light microscope at 400X magnification in five fields of view produced the following results. Based on the contents of Table 1, the respective VEGF expressions of P1, P2 and P3 had lower numbers than the control (K). The result of the VEGF expression in a field of view using a light microscope is also shown in Figure 1. The significance of VEGF expression is presented in the P1 group and the control group (K) which have p values lower than 0.05 (Table 2).

DISCUSSION

This research aimed to discover the potential of *Moringa oleifera* leaf extract to decrease the VEGF expression in the oral cancer cells of Wistar rats induced by benzopyrene. Benzopyrene is an organic compound with a specific molecular formula, $C_{20}H_{12}$, which is a member of the extremely toxic class of polycyclic aromatic hydrocarbons

(PAH) and represents a carcinogen proven to cause tumors in experimental subjects.¹⁷ The research conducted by Juliyarsi and Melia¹⁸ indicated that subcutaneous injection of benzopyrene can induce cancer in mice because it constitutes a hydrophobic compound lacking methyl groups or other reactive groups that can be converted into more polar compounds.¹⁸ As a result, the body experiences considerable difficulty in excreting these compounds. Furthermore, benzopyrene demonstrates structural similarities with nucleobases such as adenosine, thymine, guanine and cytosine. This renders inserting itself into DNA strands and potentially disrupting their transcription process relatively straightforward for benzopyrene. Failure to repair the resulting damage caused by this process will culminate in the development of cancerous cells.¹⁹

For the purposes of this research, ethanol was extracted from *Moringa oleifera* leaves by means of a process involving maceration which employs organic solvents to extract the desired compound from a solution.²⁰ This process is extremely beneficial due to being both economic and comparatively straightforward to complete. It causes

Table 1. Differences in the one-way ANOVA test results of VEGF expression

Group	Mean ± SD	ANOVA (p)
K	9.571 ± 5.2782	0.008
P1	3.343 ± 0.8772	
P2	6.657 ± 2.2052	
P3	5.514 ± 2.0359	

Table 2. Post-hoc Tukey HSD test results

Group	K	P1	P2	P3
K	-	0.005*	0.308	0.090
P1	-	-	0.208	0.557
P2	-	-	-	0.897
P3	-	-	-	-

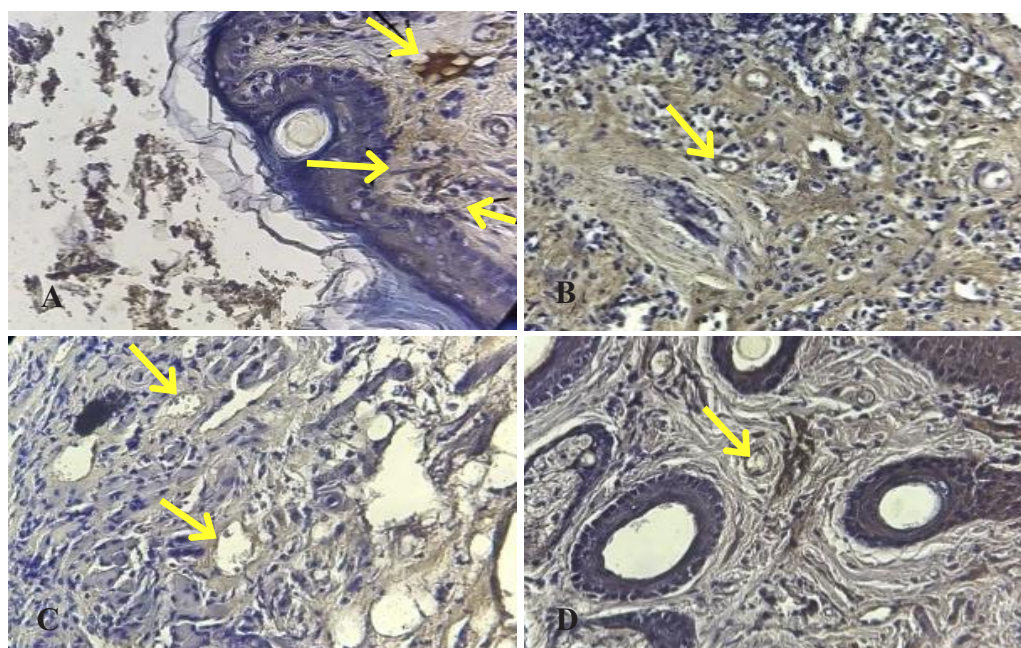


Figure 1. The area designated by an arrow (→) constitutes the VEGF expression in endothelial cancer cells in the control group (A), treatment group 1 (B), treatment group 2 (C), treatment group 3 (D).

cell walls and membranes to rupture because of differences between the internal and external pressure affecting the cell. Consequently, secondary metabolites in the cytoplasm dissolve in organic solvents.²¹

Solvent selection during the maceration process can significantly enhance its effectiveness by prioritizing the solubility of natural material compounds in these solvents.²² Since *Moringa oleifera* leaves contain numerous metabolites which are generally polar, during this research polar solvents such as ethanol were employed.²³ Vongsak *et al.*²⁴ posited that maceration using ethanol represents the recommended extraction method to promote further treatment product development.

Moringa oleifera is a plant rich in chemical compounds, one of them being isothiocyanate which is a substance that can act as a chemopreventive agent in cancer cells.^{11,25} The research of Cavell *et al.*¹² has shown isothiocyanate to possess potential chemopreventive and chemotherapeutic properties through a inhibitive mechanism in both in vitro and in vivo angiogenesis processes. Angiogenesis constitutes the process of new blood vessel formation within the human body and a prerequisite of tumor development.¹²

The main mediator of tumor angiogenesis is VEGF.²⁶ As tumors grow in size, the distance between cells and their closest blood vessels also increases. This causes tumor cells to experience hypoxia resulting in areas with low oxygen levels. In order to compensate for this, tumor cells will produce endothelial growth factors, including VEGF. As a result of this mechanism, VEGF expression increases in tumors, specifically in necrotic areas with low oxygen levels. Therefore, VEGF overexpression can be considered a compensation mechanism that allows tumor tissue to increase oxygen uptake through endothelial proliferation.²⁷ Lalla *et al.*²⁸ state that the increase in VEGF expression is proportional to the greater density of micro blood vessels in a variety of tumors.

According to Smith *et al.*²⁹ VEGF overexpression is the most influential factor in the deficient prognosis of oral squamous cell carcinoma. In addition, the results showed that cancer associated with high VEGF levels had a much shorter recurrence period than those with low levels. These results indicate that VEGF affects not only the development of cancer and angiogenesis but also their prognosis.²⁷

In general, the research results indicate that the average VEGF expression in the group treated with *Moringa oleifera* leaf extract had a lower value than that of the control group. Such results are consistent with those of the research conducted by Cavell *et al.*¹² which posited that the antiangiogenic activity of isothiocyanate is associated with decreased production of proangiogenic factors, including VEGF. Isothiocyanate can reduce the amount of VEGF by means of inhibitory pathways in the transcription of hypoxia inducible factor (HIF).¹²

The results showed that *Moringa oleifera* leaf extract reduce VEGF expression. Cancer cells can produce various proangiogenic factors, including VEGF, which

act to increase the survival and proliferation of endothelial cells, leading to the initiation of angiogenesis. Therefore, administration of substances with the potential to act as antiangiogenic agents can suppress the number of proangiogenic factors such as VEGF. A balance between pro and antiangiogenic factors will occur which reduces the level of angiogenesis.¹²

Data significance analysis using a post-hoc Tukey HSD test indicated the absence of significant differences between groups treated with *Moringa oleifera* leaf extract at concentrations of 3.125%, 4.6875% and 6.25%. This result shows that doses of these three respective concentrations demonstrate almost equal effectiveness.

Oral provision of certain drugs or other substances will result in their absorption, distribution, metabolism and excretion. Drugs reaching target cells will have an effect dependent upon the absorption and distribution processes involved. These processes are influenced by several factors such as the physiological condition of the gastrointestinal tract, absorption mechanisms including active transport, protein carriers (transporters) and individual pathological conditions.³⁰ Polyglyco-protein (PgP) is one of the transporters that functions as a repellent to chemical compounds or drugs in the duodenum and ileum. Within the body, PgP can be found in the cells of the intestines, liver, kidney tubules, and endothelial capillaries.³¹

In organ systems, PgP affects the absorption, distribution and elimination of drugs³² and can reduce the amount of chemical compounds or drugs absorbed. PgP can also prevent anticancer compounds entering cancerous tissue, thereby reducing their effectiveness. In cancer therapy, other substances capable of inhibiting PgP are required to enhance the effectiveness of anticancer compounds.³³ In this research, *Moringa oleifera* leaf extract was administered as a single substance. The role of PgP can still influence the effect of isothiocyanate on target cells, thus producing insignificant results. Therefore, further research is required in this area.³⁴

Non-significant results can also be produced due to the individual immune system which affects the acceleration or retardation of the progression of the cancer. Weakening of the immune system can cause tumor cells to develop more easily and affect the effectiveness of anticancer drug compounds on target cells.³⁵ The conclusion of this research was that *Moringa oleifera* leaf extract at a concentration of 3.125% containing 6.92% isothiocyanate, as confirmed by phytochemical glycoside screening, was the optimum means of decreasing VEGF expression in benzopyrene-induced oral cancer cells of Wistar rats.

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