

## ORIGINAL ARTICLE

# The Role of *Moringa Oleifera* L. Leaves Extract in Increasing Caspase 3 Expressions in Carcinoma of Oral Squamous Cells

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## ABSTRACT

**Introduction:** Oral cancer also affects the oral epithelial tissue, known as the carcinoma of oral squamous cells. *Moringa oleifera* L leaves (MOL) is an alternative herbal medicine for use as an anticancer agent. The purpose of this study is to ascertain the function of *Moringa oleifera* L. Leaves extract to increase caspase 3 expressions in carcinoma of oral squamous cell. **Methods:** This study used 25 *Rattus norvegicus* that were randomly divided into five groups; K-(not given MOL extract and benzopyrene), K+(benzopyrene exposed and untreated), P1 (benzopyrene exposed and given MOL extract of 3,125%), P2(benzopyrene exposed and given MOL extract of 6,25%), P3 (benzopyrene exposed and given MOL extract of 9,375%). The caspase3 expression was obtained by immunohistochemistry techniques. The data were analyzed statistically with the One way Anova and Least Significance Difference(LSD) test. **Results:** One way anova test showed there was a significance difference of Caspase3 expression ( $p=0.000$ ) between the groups. LSD test showed that P3 group has the most significant increased of caspase3 expressions. **Conclusions:** *Moringa oleifera* L.leaves extract with an optimum concentration of 9.375 percent could increase Caspase 3 expressions in oral squamous cell carcinoma.

**Keywords:** *Moringa oleifera* L., Caspase3, Oral squamous cell carcinoma

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## INTRODUCTION

In 2018 there were 18.1 million new cases, and 9.8 million deaths from cancer (1). The prevalence of cancer in Indonesia has increased from 1.4 per mile in 2013 to 1.8 per mile, based on findings from the 2018 Basic Health Research. In all these patients 5% are diagnosed with oral cancer. Of all these patients, 5% of them are oral cancer sufferers (2). Carcinoma of oral squamous cell can occur in the tongue, lips, and buccal mucosa while buccal mucosal carcinoma occurs most commonly in Southeast Asia (3). Caspase is a cysteine protease that has an important role in apoptosis and is essential to induce of apoptosis or programmed cell death (4). In particular, caspase 3 converts cytoplasmic DNase into an active form that can break down nuclear DNA and induce apoptosis of cancer cells(5). Caspase 3 is expressed in the cytoplasm and nucleus of the oral carcinoma cell. In oral squamous cell carcinoma, there is decreased expression of caspase 3 in cancer cells (6).

At present, the gold standard therapy for oral cancer is surgery, radiotherapy, and chemotherapy. Surgery is often mutilating, while radiotherapy has limited efficiency (7). Chemotherapeutic agents also have a cytotoxic ability that often leads to side effects in intolerance cancer patients. Several herbal components have been used for thousands of years for the human disease treatment (8). *Moringa* leaves have been widely used in several developing countries as vegetables, dietary supplements, and as ingredients in traditional medicine. Almost all parts of the *moringa* plant can be used for the treatment of several diseases such as diabetes, obesity and cancer. *Moringa* leaves was chosen because it contains the higher concentration of bioactive compounds than the other parts of the plant. This extract has been shown anti-cancer effects on MDA-MB-231 (breast) cancer cells, A549 lung cancer cells, and HCT-8 colorectal cancer cell cultures (9), but it has not been fully researched in oral cancer.

*Moringa oleifera* L. contains isothiocyanate bioactive components that can inhibit cancer cells (10). Isothiocyanates help to prevent cancer of various types, especially lung cancer and esophageal cancer (11). Therefore the aim of this study was to decide

*Moringa oleifera* L.'s Leaves extract to improve caspase 3 expression in carcinoma of oral squamous cell.

## MATERIALS AND METHODS

This research was an experimental study with only a control group with a post-test design (12) and had been accepted with ethical clearance by the Health Research Ethical Clearance Committee, Faculty of Dental Medicine, Universitas Airlangga. The twenty-five *Rattus norvegicus* of this study were taken from the Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. The kind of the rats used in this study are healthy *Rattus norvegicus* Wistar strain, 3 months old,  $\pm$  160 grBW, male(13). All of the rats were equally divided into 5 groups, namely negative control group K-(not given MOL extract and benzopyrene), positive control group K+(benzopyrene exposed and untreated), treatment group P1 (benzopyrene exposed and given MOL extract of 3.125%), P2 (benzopyrene exposed and given MOL extract of 6.25%), P3 (benzopyrene exposed and given MOL extract of 9.375%). In this study, 5 *Rattus norvegicus* were added to anticipate drop out and to check cancer formation (12).

Five hundred grams of dried *Moringa* leaves are incubated at 600 ° C for 24 hours then immersed in 96% ethanol solvent, then filtered to obtain clear liquid, then evaporated using a rotary vacuum evaporator at 40 ° C. *Moringa* leaves extract was tested to detect the content of bioactive compounds and to screen the glycoside content. The formulation of *Moringa* leaves extract was made using dilution method with aquadest to a concentration of 3.125%, 6.25%, 9.375%. The concentration of 3.125% was obtained by taking 3.125 ml of *Moringa* leaves extract then added with aquadest to produce 100 ml of *Moringa* leaves extract. A concentration of 6.25% was obtained by taking 6.25 ml of *Moringa* leaves extract then added with aquadest to produce 100 ml of *Moringa* leaves extract. A concentration of 9.375% was obtained by taking 9.375 ml of *Moringa* leaves extract and then adding it with aquadest to produce 100 ml of *Moringa* leaves extract. All of extract groups were stored in a closed dark glass bottle.

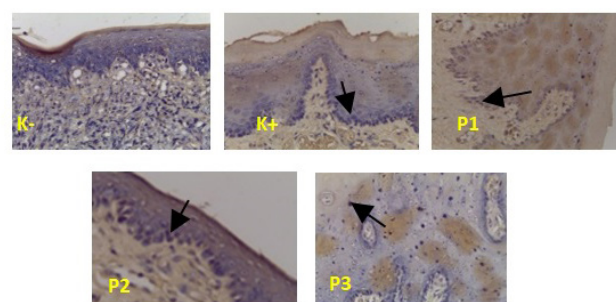
Benzo[a]pyrene was used in solid powder with a dosage of 8 mg / kgBW dissolved in olive oil at a ratio of 2: 1. Cancer induction in positive control groups and treatment groups was performed by injection of 0.2 ml of benzo[a]pyrene, twice a week for a one month (13). The clinical examination of tumor lumps at the end of the fourth week is needed for an indication that cancer cells have grown in these rats. The presence of tumor lumps is characterized by the presence of hard immovable nodules (4). Buccal mucosa tissue of addition rats was taken and stained by Hematoxylin Eosin staining method. The cancer cells were signed by anaplastic cells, varied nuclei, abnormal mitosis,

abnormal nucleus:cytoplasmic ratio (1: 1), multiple nuclei, and hyperchromatic (5).

The buccal mucosa tissue of the samples was fixed with 10% Neutral Buffered Formalin (NBF), with a pH of 6.5 -7.5. Then the tissue was cut 4-mm thick with a rotary microtome and put in the water bath. After that, the incision in the slide is placed on a hot plate at 60°C for 10-15 minutes (14). To detect caspase 3 expression using a caspase-3 monoclonal antibody (Santa Cruz Biotechnology), with the indirect IHC method. The caspase-3 positivity was observed as a brown color, both in the cytoplasm and the nucleus. The caspase 3 expression was then analyzed and measured by Leica Microsystem Europe with 400 magnification in 10 different fields using an optical microscope. Firstly, the data was checked with the Saphiro-Wilk normality test and the Levene homogeneity test. Then the data were analyzed statistically using the Parametric One Way Anova test and the LSD (Least Significant Difference) test to determine its significance in each group.

## RESULTS

Based on Figures 1, the arrows indicate the expression of caspase 3 as a brown color, both in the cytoplasm and the nuclei. Caspase 3 expression in the K-group was zero. In the K+ group, there was a positive caspase 3 expression, and caspase 3 expressions in oral cancer cells were increased after administration of *Moringa oleifera* (L.) leaves extract therapy. The highest positive expression of caspase 3 occurred in P3, namely in the group treated with *Moringa oleifera* (L.) leaves extract therapy at 9.375%.



**Figure 1: Expression of Caspase 3 on squamous epithelial cells in the oral cavity of control groups (K-,K+) and treatment groups (P1, P2, P3).**

Table I showed that there was a significant difference in caspase 3 expressions between the groups,  $p = 0.000$  ( $p < 0.05$ ). Significant differences existed between groups P3 with K+, P3 with P1 (0,000), and groups P3 with P2 (0.000). This means that the P3 group with a concentration of 9.375% had a more significant difference in the expression of caspase 3 than the P1 group with 3.125% concentration and P2 with 6.25% concentration. The significance value between

P1 and P2 is 0.095 so that P1 is not significantly different from P2. It means that the concentration of 3.125% and 6.25% ethanol extract of moringa leaves had almost same effect in increasing the expression of caspase3 in oral squamous cell carcinoma.

**Table I : Comparison of Caspase 3 Expressions Between The Groups**

Group	Mean $\pm$ SD	One Way Anova
		(p)
K-	0 $\pm$ 0	0,000 (p<0,005)
K+	1,8 0,84	
P1	3,0 0,71	
P2	3,8 0,84	
P3	6,8 0,84	

**Table II : Least Significant Difference Test of Caspase 3 Expressions**

	K-	K +	P1	P2	P3
K-	-	0,001*	0,000*	0,000*	0,000*
K +	0,001*	-	0,016*	0,000*	0,000*
P1	0,000*	0,016*	-	0,095	0,000*
P2	0,000*	0,000*	0,095	-	0,000*
P3	0,000*	0,000*	0,000*	0,000*	-

\* $\alpha$  = 0,05 (5%)

## DISCUSSION

Based on the results of the One Way Anova statistical test, p value was <0.005. It means that there is a role for the ethanol extract of Moringa leaves on the expression of caspase 3. Based on table II, the P1 and P2 group were significantly different from the K-, K +, and P3 groups. It means that the group treated with MOL 3.125% and 6.25% could increase caspase3 expression in the cancer group, but this ability was not as well as the group treated with MOL 9,375%.

P1 (concentration 3.125%) was not significantly different from the P2 group (concentration 6.25%). Previous research on the potential of Moringa leaf extract concentrations of 3.125%, 4.6875%, 6.25% against Bcl-2 expression in oral cancer cells, proved effective in reducing the expressions of the anti-apoptotic gene Bcl-2 at concentration 3.125%. Potential of Moringa Leaves extract to Ki-67 expression in wistar rats with oral cancer cells, which used a concentration of 3.125%, 4.6875%, 6.25% also showed a concentration of 3.125% was better in reducing Ki-67 expression (cell proliferation biomarker) (15-16). This results were different from this study which showed a concentration of 9.375% was better in increasing the expression of caspase 3 than the concentrations of 3.125% and 6.25%. It means that the concentration of 3.125% is good for inhibiting cancer cell proliferation and decreasing the expression

of Bcl 2 which plays a role in inhibiting pro-apoptosis genes, but it is not good enough in increasing caspase 3 expression.

Caspase 3 is an executor caspase that converts cytoplasmic DNase into an active form that can break down the nuclear DNA and induce cell apoptosis. The apoptotic process occurs due to changes in mitochondrial permeability and the release of pro-apoptotic molecules into the cytoplasm. The release of this molecule is controlled in a balanced manner through members of the Bcl protein family between pro and anti-apoptosis (4). There are 2 groups of Bcl-2, the first is pro-apoptotic protein (BAX, Bak, Bad, Bcl-x, Bid, Bik, Bim, and Hrk) and the second is anti-apoptotic (Bcl-2, Bcl-xl, BBcl-1). Pro-apoptotic and anti-apoptotic protein balance will determine whether the cell dies or not. Cytochrome c released into the cytosol will bind to Apaf-1 (Apoptosis activating factor-1), forming a cytochrome-c-apaf-1 complex. The formation of the cytochrome-c-Apaf-1 complex in the cytoplasm will activate pro-caspase 9 to become caspase 9. Activation of caspase 9 will then activate caspase 3 (5).

The ability of the ethanol extract of Moringa leaves to increase the expression of caspase 3 can be due to the presence of the isothiocyanate content. Sulforaphene, a type of isothiocyanate can inhibit the growth of breast cancer cells by increasing the expression of the pro-apoptotic protein Bax and caspase 3 and decreasing the expression of the anti-apoptosis gene Bcl-2 and Bcl-xl (17). The other study found that the Benzylisothiocyanate (BITC) and Phenethylisothiocyanate (PEITC), family of the isothiocyanate can cause cell cycle arrest and induces apoptosis via activation of caspase-3, mitochondria dysfunction and Nitric Oxide (NO) in human osteogenic sarcoma U-2 OS Cells(18). BITC and PEITC not only increased the active form of caspase-9 and caspase-3 but also decreased the levels of mitochondrial membrane potential, thus causing the release of caspase-9 and then activated the caspase-3 for apoptosis.

In addition to isothiocyanates, the ability of Moringa oleifera (L.) Lamk leaf extract to induce apoptosis of cancer cells also depends on the antioxidant ability of the phenolic compounds. Phenol induces apoptosis in many cancer cells by activating caspase 3 and 7 and inhibiting Bcl2 from preventing the release of cytochrome C from mitochondria. The role of the active ingredient Phenethyl Isothiocyanate (PEITC, a type of isothiocyanate) on oral cancer cell cultures with variations in the p53 gene mutation, showed that PEITC therapy can cause damage to cancer cell DNA by stopping the cancer cell cycle in the G2 / M phase (check point), so that it can give the damaged DNA an opportunity to undergo the DNA-repair process(19). In addition, PEITC also induces apoptosis of oral cancer cells by increasing the

expression of wild type p53 accompanied by an increase in the expression of p21, cdc2, an increase in the pro-apoptosis gene Bax and a decrease in Bcl-2, causing an increase in the release of cytochrome c, and finally caspase 3 is activated. Caspase-3 is an executor caspase, which can be activated directly from caspase-8 or-9 before leading to apoptosis. Activated caspase will activate DNase which acts to damage DNA which ultimately causes DNA fragmentation so that abnormal cells (cancer cells) will experience death, which is known as apoptosis.

## CONCLUSION

It can be concluded that the ethanolic extracts of *Moringa oleifera* L. leaves concentration of 3.125%, 6.25%, and 9.375% can increase caspase 3 expressions in oral squamous cell carcinoma.

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