

The potential of ethanolic extract of *Moringa oleifera* leaves on HSF1 expression in oral cancer induced by benzo[a]pyrene

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ABSTRACT

Background: Oral cancer is the sixth most common malignancy that occurs in the world, with more than 330,000 deaths a year. In cancer, mutations occur in proteins, accompanied by unfolding proteins, caused by the unstable micro-environment in cells. To stabilise this condition, protein protectors called heat shock proteins (HSPs) are needed. HSPs are activated by a group of transcription factors known as heat shock factor 1 (HSF1). HSF1 is a considered target in cancer therapy. *Moringa oleifera* leaves are known to have anti-cancer properties because of bioactive compounds called flavonoid and isothiocyanate and are used as herbal therapy for cancer. **Purpose:** To investigate the potential effect of ethanolic extract of *Moringa oleifera* on HSF1 expression in oral cancer induced by benzo[a]pyrene. **Methods:** This study used 25 male Wistar rats divided into five groups consisting of the negative control group (K-), which was only given aquadest; the positive control group (K+), which was induced with benzo[a]pyrene and given aquadest; and treatment groups that were induced with benzo[a]pyrene and given *Moringa oleifera* leaf extract at concentrations of 3.125% (P1), 6.25% (P2), and 9.375% (P3). Examination of HSF1 expression was carried out by immunohistochemistry staining. Data were analysed using the Kruskal–Wallis test and post-hoc Tukey HSD. **Results:** HSF1 expression in the P1, P2, and P3 groups decreased significantly compared to the K+ group. There were no significant differences between the P1, P2, and P3 groups ($p > 0.005$). **Conclusion:** Ethanolic extract of *Moringa oleifera* leaves in three concentrations can decrease expression of HSF1 in oral cancer induced by benzo[a]pyrene.

Keywords: ethanolic extract of *Moringa oleifera*; flavonoid; HSF1; isothiocyanate; oral cancer

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INTRODUCTION

Oral cavity cancer is a cancer that can be found throughout the world. According to the World Health Organization (WHO), there are an estimated 657,000 new cases of oral and pharyngeal cancer each year and more than 330,000 deaths. Oral cancer can occur due to mutations of the p53 protein, which are triggered by a build-up of carcinogenic substances in the human body.¹

The p53 protein will continue to mutate along with the proliferation of oral cancer cells, and this can be followed by the occurrence of unfolding proteins caused by an unstable cell microenvironment. To stabilise the protein, protein protectors called heat shock proteins (HSPs) are needed.

HSPs are activated by a group of transcription factors known as heat shock factors (HSFs).¹ The HSFs that play the leading role in regulating the chaperones transcription process are HSF1. HSF1 protects the proteome homeostasis of cancer cells through the activation of HSPs so that the stabilization of oncoproteins is maintained and the cancer cells continue to grow and develop.²

Due to its important role in carcinogenesis, HSF1 is targeted as a consideration in cancer therapy. Flavonoid and isothiocyanate bioactive compounds are known to reduce the expression of HSF1.³ Nagai et al.⁴ reported that the compound quercetin, which is a class of flavonoid, can reduce HSF1 expression in HeLa cells. Yang et al.⁵ also stated that quercetin can significantly reduce HSF1

expression. Sarkars et al.⁶ stated that isothiocyanates can reduce HSF1 and HSPs expression so that the apoptosis of breast cancer cells can be induced.

The gold standard for treating cancer nowadays is chemotherapy and surgery. But those therapies are known to have a negative impact on the patient due to their side effects, which can cause damage to normal cells and organs.⁷⁻⁹ Therefore, there are several alternative treatments, one of which uses herbal plants, namely *Moringa oleifera*. *Moringa oleifera* leaves are a source of flavonoid and isothiocyanate components. The leaves have antioxidant, anti-inflammatory and anticancer benefits. Several studies have shown these leaves can be used as an anti-neoproliferative agent that can inhibit the growth of cancer cells. *Moringa oleifera* leaves have also been shown to be effective as safe anticancer agents at certain concentrations.^{10,11}

Based on this theory, further research is needed to see the potential of *Moringa oleifera* leaf ethanol extract on HSF1 expression in oral cancer cells. The purpose of this study was to determine the potential of *Moringa oleifera* leaf ethanol extract against HSF1 expression in oral cancer cells induced by benzo[a]pyrene.

MATERIALS AND METHODS

This research was an experimental laboratory study with a posttest only group design that was approved by the Committee of Ethical Clearance of Health Research, Faculty of Dentistry, Universitas Airlangga (592/HRECC.FODM / IX / 2019). The experimental animals used were 25 male Wistar rats (*Rattus norvegicus*), with the addition of four male Wistar rats examined histopathologically, weighing 160 grams, aged three months, and acclimated for seven days in a cage measuring 60 x 65 x 80 cm according to the laboratory standards in the Animal Laboratory Unit of Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga.

Twenty-five male Wistar rats were randomly divided into 5 groups consisting of a negative control group (K-), a positive control group (K+), treatment group 1 (P1), treatment group 2 (P2), and treatment group 3 (P3). In addition, four mice were sacrificed and examined clinically and histopathologically to establish whether cancer had formed or not. The K- group of rats were not injected with benzo[a]pyrene and *Moringa oleifera* leaf ethanol extract therapy. The K+ group was injected with benzo[a]pyrene but not given *Moringa oleifera* leaf ethanol extract therapy. The P1, P2, and P3 groups were groups of rats induced with benzo[a]pyrene and injected with *Moringa oleifera* leaf ethanol extract at concentrations of 3.125%, 6.25%, and 9.375%, respectively.

Five hundred grams of *Moringa oleifera* leaves were obtained from Kebun Kelor Lawang, Malang, East Java and dried for three months. The dried leaves were incubated at 60°C for 24 hours and then soaked in 96% ethanol

solvent to soften them. The macerated mixture was put into a container, which was sealed and left for two days. After that, the mixture was filtered to obtain a clear liquid. This was then evaporated using a vacuum rotary evaporator at a temperature of 400°C. The *moringa oleifera* leaf extract was then subjected to phytochemical screening to detect the active compounds of the plant. The making of the phytochemical extracts and the screening were carried out at the Surabaya Laboratory of Industry Research and Consultation.

Cancers in the K+, P1, P2 and P3 sample groups were induced by benzo[a]pyrene (Sigma Aldrich, Saint Louis, USA). Benzo[a]pyrene induction was carried out in the Biochemical Laboratory Experimental Unit of the Faculty of Medicine, Universitas Airlangga. Benzo[a]pyrene was used in the form of a solid powder with a dose of 8 mg / kgBB dissolved in the olivary oleum at a ratio of 2:1. Induction was done by the injection of 0.2 ml of benzo[a]pyrene, using a syringe, 2–3-mm deep into the buccal mucosa of the Wistar rats, twice a week for one month.¹² Then, a clinical examination was performed to check the success of the induction, by looking for signs of bumps on the buccal site.¹³ An HPA examination was also carried out by sacrificing four additional mice. Histologically, cancer cells were characterized by anaplastic cell signatures, which are enlarged cell nuclei, varying core shapes, abnormal mitosis, nucleus:cytoplasmic ratio (1:1), hyperchromatic, irregular shape, and chromatin appear coarse and lumpy, that found in malignancy cases.¹⁴

Moringa oleifera leaf ethanol extract was given to the treatment sample group in the following concentrations, 3.125% (P1), 6.25% (P2), and 9.375% (P3), and the rats were given ad libitum using 2 ml of insulin sonde every day for one month. After one month, the Wistar rats were sacrificed to make histopathological preparations.

The buccal mucosa tissue of the male Wistar rats was fixed with 10% Neutral Buffered Formalin (NBF) for further processing of the tissue by the paraffin method through several stages, namely dehydration, clearing, impregnation, and embedding. Then the tissue was cut 4-mm thick with a rotary microtome. The network processing procedure was carried out at the Anatomy Pathology Laboratory of the Faculty of Medicine, Universitas Airlangga, according to its Standard Operating Procedures.

Immunohistochemical staining was carried out in the Electron Microscope Laboratory of the Faculty of Medicine, Universitas Airlangga and was carried out in accordance with applicable procedures. Immunohistochemical staining was used to check HSF1 expression with HSF1 monoclonal antibodies (Santa Cruz Biotechnology, INC., USA) as a binder, which was then diluted (1:100). The chromogen used was DAB chromogen and the staining was done with Meyer's Hematoxylin.

Data collection was carried out by the counting technique, which observed HSF1 expression in five different fields, using a microscope (Olympus Microscope, Tokyo, Japan) with 400-times magnification. Data analysis

used the Kruskal–Wallis test and the post-hoc Tukey HSD test with the help of the Statistical Package for the Social Sciences (SPSS) (IBM SPSS, New York, USA) to find out significant differences between groups of variables.

RESULTS

Figure 1 shows the preparations using a light microscope with a magnification of 400 to count the number of HSF1 expressions from each group on the cytoplasm designated by the black arrows. Based on the research data, the positive control group (K+) showed high HSF1 expression compared to the negative control group (K-) and the treatment groups (P1, P2, and P3), while the treatment group 3 (P3) showed lower HSF1 expression than the other treatment groups (P1 and P2) (Table 1).

All the data were tested for normality (Shapiro–Wilk) and homogeneity (Levene), but the data obtained were not normally distributed and not homogeneous because p was significantly less than 0.05. Furthermore, a non-parametric test was performed using the Kruskal–Wallis test. The results showed that there was a significant difference of $p = 0.000$ ($p < 0.05$) between the sample groups, with HSF1 expression.

Table 1. Mean and standard deviation of HSF1 expression in the five groups

Group	Sample	Mean	SD
K-	5	0.480	0.109
K+	5	9.400	3.016
P1	5	3.712	0.326
P2	5	2.920	0.729
P3	5	2.560	0.517

After non-parametric tests using the Kruskal–Wallis test, a multiple comparison test was performed using the post-hoc Tukey HSD Test to identify significant differences between each sample group. Table 2 shows that there were significant results concerning the K+ group and K- group. The K+ group was significant for the P1, P2, and P3 groups and vice versa. The K- group did not show significant results with the P2 and P3 groups, nor did the P1, P2, and P3 groups show significant differences from each other.

DISCUSSION

Based on the results of the study, the mean expression of HSF1 in the K+ group was significantly higher compared to the K- group. This contrast was due to the fact that HSF1 is over-expressed in an abnormal cell (e.g. cancer) compared to a normal cell. HSF1 is activated when there are stressors, such as changes in temperature, chemistry, or nutrition, that affect the cell environment. HSF1 in the form of monomers will be released under these circumstances and translocated to the nucleus. HSF1 monomers will be phosphorylated by protein kinase C, so HSF1 will be in active trimer form. Trimer HSF1 will induce HSPs that act as chaperones so that cellular homeostasis can be maintained.^{15,16}

Table 2. Post Hoc Tukey HSD test for HSF1 expression in each group

Groups	p value				
	K-	K+	P1	P2	P3
K-		0.000	0.013	0.085	0.179
K+			0.000	0.000	0.000
P1				0.899	0.702
P2					0.994
P3					

Note: * $p < 0.05$

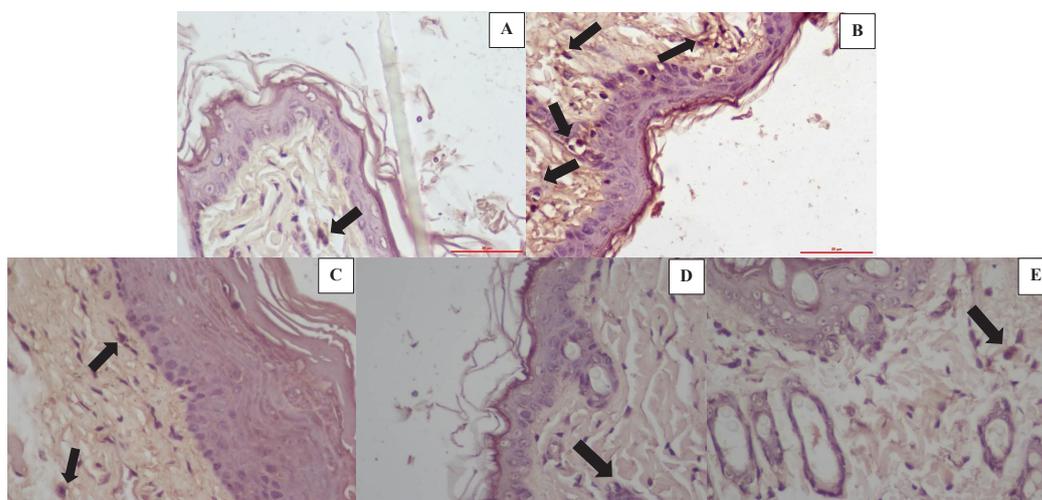


Figure 1. Immunohistochemistry staining to see the expression of HSF1 in cytoplasm (indicated by arrows) (A) K- group; (B) K+ group; (C) P1 group; (D) P2 group; (E) P3 group.

When the cancer cell is still growing rapidly, there are many stressors that can affect its survival. In this case, HSF1 is widely expressed with the aim of maintaining the stability of cancer proteostasis, so that cancer growth can continue. This is in accordance with research showing that HSF1 expression is over-expressed in oral cancer cells compared to normal cells.¹⁷

HSF1 expression in the treatment groups (P1, P2, P3) was significantly lower compared to the K+ group. This is because the treatment groups were given *Moringa oleifera* leaf ethanol extract while the K+ group was not given it. *Moringa oleifera* leaf ethanol extract has isothiocyanates and flavonoids, which can inhibit HSF1 expression^{5,18}, as its natural compounds. These can inhibit the phosphorylation of protein kinase C (PKC). This is a serine/threonine kinase that plays a role in several cellular activities, including cell proliferation, survival and apoptosis. PKC is an important mediator for cell survival in solid tumors.¹⁹ In cancer cells, PKC also regulates protein transcription, one of which is HSF1. If PKC is inhibited, the activation of HSF1 is inhibited and its expression decreases.⁵

The results of the treatment groups, P1, P2, and P3, showed no significant differences between them. There was no significant difference in the mean expression of HSF1 between the treatment groups presumably because *Moringa oleifera* leaf ethanol extract has a very strong impact on cancer cells, so most of the concentration used can cause the death of cancer cells. The mean expression of HSF1 in group P1 (3.125%) compared with P2 (6.25%) and P3 (9.375%) showed that with high concentrations of *Moringa oleifera* leaf ethanol extract, the expressions of HSF1 were lower. This was probably because many cancer cells had died. Nararya²⁰ stated that concentrations above 3.125% are toxic. It is suspected that in the P2 and P3 groups the expression of HSF1 reached lower amounts than the P1 group as a result of cancer cell death, along with other normal cell death.

The conclusion of this study is that ethanolic extracts of *Moringa oleifera* leaf concentration 3.125%, 6.25%, and 9.375% can reduce HSF1 expression in cancer cells in oral cancer. Further studies are required to find the optimal concentration of *Moringa oleifera* leaf ethanol extract that can decrease the expression of HSF1 in oral cancer.

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