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Research Article

The Potential of Moringa Leaf Nanoparticles (*Moringa oleifera*) on the Expression of TNFα, IL10, and HSP 27 in Oral Cavity Cancer

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Abstract
Oral cancer is currently the sixth leading malignancy in the world, with over 330,000 cases resulting in death. Several cytokines and proteins protect the survival of cancer cells, such as *TNF-α*, HSP27, and IL-10. *Moringa oleifera* is an herbal medicine with anti-cancer properties. Nanoparticles of *M. oleifera* have the property to be easily absorbed by water-soluble cells, so only small doses are needed to be used as anti-cancer ingredients. This research aims to prove the ability of *M. oleifera* nanoparticle extract against oral cancer through the expression of tumour necrosis factor α (TNF-α), heat shock protein (HSP27), and interleukin 10 (IL-10). Rat as non-human experimental subjects were divided into four groups: control group (K), treatment group 1 (P1), treatment group 2 (P2), and treatment group 3 (P3). Cancer induction was carried out by injecting with benzo[a]pyrene, and then *M. oleifera* nanoparticle extract was administered in three forms of treatment doses of 125 µg/mL (P1), 250 µg/mL (P2), and 500 µg/mL (P3). Immunohistochemical examination was analysed through TNF-α, HSP27, and IL-10's expression. The expression of TNF-α and HSP27 between control and treatment groups was significantly different. P2 had the lowest expression of TNF-α and HSP27. The expression of IL-10 between control and treatment groups was also significantly different. P1 had the lowest expression of IL-10. *M. oleifera* nanoparticle extract can reduce oral cancer progression by decreasing the expression of TNF-α, HSP27, and IL-10.

Keywords: nanoparticle, *Moringa oleifera*, oral cancer, TNF-α, HSP27, IL-10, immunology

1. INTRODUCTION

Cancer is still a disease that needs serious attention because the frequency of cases continues to increase. In addition, cancer is also the most common cause of death, even though various treatments have been developed. In 2020, the World Health Organization (WHO) reported 19.3 million new cases and 10 million deaths [1]. Oral squamous carcinoma (OSCC) is the most common type of oral cavity cancer. Many factors cause this disorder, one of which is carcinogenic materials such as benzo[a] pyrene [2].

Carcinogenesis is the process of cancer occurrence caused by various molecular changes, including heat shock protein (HSP27), one of the proteins found in breast and ovarian cancers. HSP27 protein can also be bound to antiapoptotic proteins and regulated at the transcriptional and post-transcriptional stages. HSP27 protein is also widely associated with cancer aggressiveness and metastasis [3]. Cancer growth is also influenced by various cytokines, including tumour necrosis factor α (TNF-α), where the molecule has a dual role as a factor associated with inflammation and is also related to cancer cell growth. TNF-α often causes a debate because, on the one hand, it can trigger inflammation, but on the other hand, it suppresses the regulation of several growth molecules, such as vascular endothelial growth factor and fibroblast growth factors [4].

The immune system is one thing that needs to be considered in the process of carcinogenesis because the role of immune surveillance is vital in cancer growth. One of the interleukins that is expected to inhibit cancer growth is interleukin 10 (IL-10). As we know, IL-10 is an anti-inflammatory cytokine whose role is not only to suppress inflammation but also to regulate several growth factors. In addition, IL-10 can activate T helper, increasing cytolytic T lymphocytes' ability [5]. The incidence of cancer is very complex, and many proteins are involved, whose functions are often the cause of cancer growth. This is what makes cancer treatment difficult. Therefore, a study was conducted on a natural substance, *M. oleifera*; its leaves have some ingredients that have roles as anti-cancer agents.

Table 1. Immunohistochemistry expression of TNF-α.

Group	(x ± SD)	Kruskal-Wallis *P*-value
K	9.00 ± 0.894*	0.000
P1	7.00 ± 0.894*	
P2	5.00 ± 0.894	
P3	5.50 ± 0.548	

Notes: Mann-Whitney analysis between groups found significance between groups K and P1 in the expression of TNF-α. *$P < 0.05$ significant difference between groups.

However, the benefits of HSP27, TNF-α, and IL-10 in oral cancer mouse models induced with benzo[a] pyrene are not yet known [5]. This research used *M. oleifera* in the nanoparticle form because nanoparticles can significantly impact cancer treatment; lowering the dose needed makes the cancer treatment less expensive and minimises the use of heavy operations [6]-[9].

2. MATERIALS AND METHODS

2.1. Materials

This study is an *in-vivo* experimental laboratory research with a post-test-only control group design. Moringa leaf extract (*M. oleifera*) was prepared at the Faculty of Pharmacy, Widya Mandala Catholic University, Surabaya. Moringa leaf extract nanoparticles were processed and manufactured at the Chemistry Laboratory, Faculty of Science and Technology, Universitas Airlangga, Surabaya. Experimental animals were treated at the Unit of Experimental Animals – Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga.

2.2. Methods

The process of immunohistochemistry staining, examining, and analysing was carried out at the Research Center of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya. This research was conducted from April to December 2022. This study was ethically cleared by Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission, No. 738/ HRECC.FODM/XI/2022.

The population of this study was male Wistar rats (*Rattus norvegicus*) aged two months with the same body weight of 160 g. They were kept in the same place, given the same food, and adapted for one week. Samples were taken from the population randomly, as many as 24 individuals were to be used in the study. The samples were induced with benzo[a]pyrene two times a week for four weeks and divided into four groups: the control group, which was given a placebo, and the treatment group, which was induced by benzo[a]pyrene and given Moringa leaf extract nanoparticles with concentrations of 125 µg/mL (P1), 250 µg/mL (P2), or 500 µg/mL (P3) once a day for 14 days. Each group had six male rats, so there were 24 male rats used in this research with they were 2-month-old mice. This old was chosen based on the immunology system. Their immunology was still good, so it could enhance this research's efficacy. The justification for using these three concentration levels was based on previous research conducted by Nararya et al. [10]. They found these concentrations were nontoxic for the cells tested using MTT assay.

2.2.1. Examination of TNF-α, IL-10, and HSP27

TNF-α, IL-10, and HSP27 expression were examined using immunohistochemistry staining. The staining process used mouse monoclonal antibodies of TNF-α, IL-10, and HSP27 from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., SC-7269, California, USA). The assessment of microscopic staining results was also conducted qualitatively and quantitatively. The expression data of TNF-α, IL-10, and HSP27 were determined through qualitative observation of colour degradation where positive samples were indicated by the presence of a brownish colour in the cells (cytoplasm) as a result of a reaction to monoclonal

Table 2. Mann-Whitney analysis between groups revealed a significant difference in TNF-α level between groups K and P1.

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*P*-value
TNF-α	K	P1	2.000*	0.474	0.002
		P2	4.000*	0.474	0.000
		P3	3.500*	0.474	0.000
	P1	K	-2.000*	0.474	0.002
		P2	2.000*	0.474	0.002
		P3	1.500*	0.474	0.023
	P2	K	-4.000*	0.474	0.000
		P1	-2.000*	0.474	0.002
		P3	-0.500	0.474	0.720
	P3	K	-3.500*	0.474	0.000
	P1	-1.500*	0.474	0.023	
	P2	0.500	0.474	0.720	

Notes: *$P < 0.05$ significant difference between groups.

antibodies present in the reagent. The results of the expression examination were then displayed in a quantitative ratio scale based on the number of cells that showed a positive picture, using a counting method quantification by observing the slide using an optical microscope at five different visual fields with 400 times microscopic magnification. Data were documented using the integrated camera available on the microscope set.

2.3 Statistical Analysis

Statistical data analysis was performed using IBM SPSS Statistics version 21. One-way analysis of variance was used to prove the difference in the effect of the Moringa leaf extract nanoparticles on oral cells undergoing malignant transformation through the expression of the TNFα, IL-10, and HSP27 genes. Tukey's HSD test was then performed to analyse the expressions since the data were normally distributed.

3. RESULTS AND DISCUSSIONS

3.1. TNF-α Expression Between Each Group

Table 1 shows the immunohistochemistry expression of TNF-α with a *p*-value of 0.000. The Mann-Whitney test that demonstrated the K and P1 groups were the significant groups with their *p*-value < 0.05 (Table 2). Figure 1 shows the immunohistochemistry staining used to assess the expression of TNF-α among the K, P1, P2, and P3 groups. Table 1, 2, and Figure 1 proved that the expression of TNF-α between control and treatment groups was significantly different which P2 had the lowest expression of TNF-α compared to other groups.

3.2. HSP27 Expression Between Each Group

Table 3 shows the immunohistochemistry expression of HSP27 with a *p*-value of 0.000. The Mann-Whitney test results, showing that the K and P1 groups were the significant groups with a *p*-

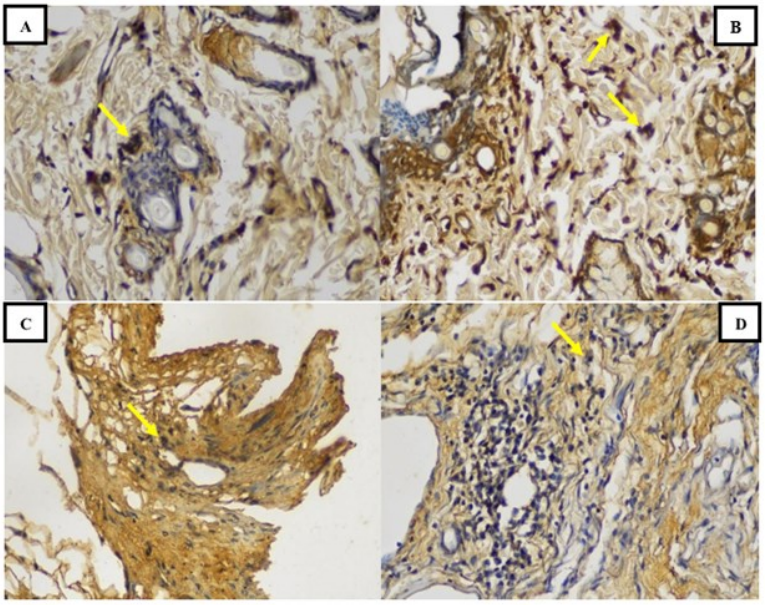

Figure 1. TNF-α expression by immunohistochemistry between the control (K) and three treatment groups (P1, P2, and P3). Both the control group (K), shown in A, and the P1 treatment group, shown in B, displayed the strongest expression compared to the other groups, P2 (C) and P3 (D). All the TNF-α expression was measured by immunohistochemistry staining with a 400x objective lens and was indicated by yellow arrows.

value < 0.05 (Table 4). Figure 2 shows the immunohistochemistry staining used to assess the expression of HSP27 among the K, P1, P2, and P3 groups. Table 3, 4, and figure 2 proved that the expression of HSP27 between control and treatment groups was significantly different which P2 had the lowest expression of HSP27 compared to other groups.

3.3. IL-10 Expression Between Each Group

Table 5 shows the immunohistochemistry expression of IL-10 with a *p*-value of 0.000. Figure 3 shows the immunohistochemistry staining used to assess the expression of IL-10 among the K, P1, P2, and P3 groups. Table 5, 6, and figure 3 proved that the expression of IL-10 between control and treatment groups was also significantly different which P1 had the lowest expression of IL-10 compared to other groups.

3.4. Discussion

The causes of oral cancer are multifactorial. No single causative agent or carcinogen can be explained, as it is likely that more than one factor is required to cause oral cancer [11]-[13]. However, 90% of oral cancer cases are caused by smoking [13][14]. The by-products of cigarette smoke are polycyclic aromatic hydrocarbons (PAH). The most

Table 3. Immunohistochemistry expression of HSP27.

Group	(x ± SD)	Kruskal-Wallis *P*-value
K	8.67 ± 0.516*	0.000
P1	6.67 ± 1.033*	
P2	4.50 ± 0.548	
P3	5.00 ± 1.095	

Notes: Mann-Whitney analysis between groups found significance between groups K and P1 in the expression of HSP27. $^{*}P < 0.05$ significant difference between groups.

Table 4. Mann-Whitney analysis between groups revealed a significant difference in HSP27 level between groups K and P1.

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*P*-value
HSP27	K	P1	2.000*	0.474	0.002
		P2	4.000*	0.474	0.000
		P3	3.500*	0.474	0.000
	P1	K	-2.000*	0.474	0.002
		P2	2.000*	0.474	0.002
		P3	1.500*	0.474	0.023
	P2	K	-4.000*	0.474	0.000
		P1	-2.000*	0.474	0.002
		P3	-0.500	0.474	0.720
	P3	K	-3.500*	0.474	0.000
		P1	-1.500*	0.474	0.023
		P2	0.500	0.474	0.720

Notes: *$P < 0.05$ significant difference between groups.

widely known PAH prototype is benzo[a]pyrene, a carcinogenic material, environmental contaminants formed due to incomplete combustion processes or pyrolysis of organic materials. Benzo[a]pyrene is a carcinogen because it can enter DNA, bind to the DNA double helix molecule so that it can disrupt the transcription process and cause mutations. The benzo[a]pyrene molecule bound to DNA is the initiator in transformed cells so that it can continue through the promotion and mutation stages. The accumulation of benzo[a]pyrene can cause the conversion and growth of normal cells into malignant or malignant cells, where in this study benzo[a]pyrene was used as the main ingredient that causes cancer [11][14].

Chemotherapy is one of the essential treatments for cancer patients. Although the nature of chemotherapy aims to destroy cancer cells, it also damages normal cells, leading to side effects for cancer patients. These side effects, such as nausea and vomiting, xerostomia, dysphagia, oral mucositis, and fatigue, can affect patients' quality of life, especially their emotional condition, making it difficult to continue treatment [15][16]. Consequently, there are several alternative treatments used by cancer patients. One alternative treatment uses herbal plants, particularly *M. oleifera* or Moringa plants. All parts of the Moringa plant have traditionally been used for medicinal purposes, but the leaves are the most widely utilised, especially as an alternative treatment for cancer. Moringa leaves have anticancer benefits because they contain bioactive flavonoids and isothiocyanates. Flavonoids in Moringa leaves have benefits as antioxidants, anti-inflammatories, and, in this case, especially anti-cancer. Isothiocyanate has benefits as an anti-cancer substance because it can induce apoptosis of cancer cells [17]. Cancer cells are unstable because mutant p53 continues to replicate and transcribe to survive, but the host cells' microenvironment can interfere with survival. Therefore, cancer cells have a mechanism to

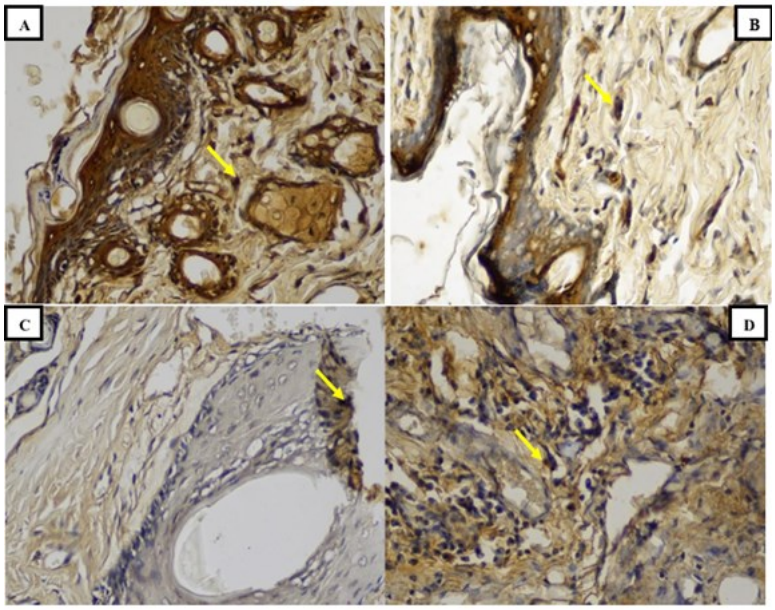

Figure 2. HSP27 expressions by immunohistochemistry between the control (K) and three treatment groups (P1, P2, and P3). Both the control group (K), shown in A, and the P1 treatment group, shown in B, demonstrated the strongest expression compared to the other groups, P2 (C) and P3 (D). All the HSP27 expression was measured by immunohistochemistry staining with a 400x objective lens and was indicated by yellow arrows.

maintain oncoprotein stabilisation so that cancer cells can continue to live and develop. Cancer cells involve several cytokines and chaperones in this mechanism to protect their survival.

IL-10 is an anti-inflammatory cytokine that prevents the formation of adhesions through several mechanisms that inhibit the production of pro-inflammatory cytokines produced by T-helper type 1. Conversely, IL-10 can also spur the proliferation and differentiation of B lymphocytes and T helper lymphocytes type 2. In cancer cells, IL-10 is produced in large quantities so that they can avoid the body's immune cell, that is according to P3 (treatment group).

TNF-α is a pro-inflammatory cytokine that is highly expressed in cancer. The secretion of this cytokine is necessary to support cancer cell death [18][19]. HSP27 acts as a chaperone that repairs oxidative stress-induced protein damage and reduces reactive oxygen species levels by increasing intracellular glutathione levels and decreasing intracellular iron levels [18]. The strong protective effect of HSP27 is mainly due to its vital function in apoptosis regulation. HSP27 can block apoptosis at different stages due to its interaction with several molecules involved in the apoptotic pathway. Numerous studies indicate that HSP27 inactivates the caspase cascade by binding to

Table 5. Immunohistochemistry expression of IL-10 with *p*-value 0.000.

Group	(x ± SD)	Kruskal-Wallis *P*-value
K	3.00 ± 0.894*	0.000
P1	5.00 ± 0.894*	
P2	7.17 ± 0.753	
P3	7.67 ± 0.516	

Notes: Mann-Whitney analysis between groups found significance between group K and P1 in the expression of IL-10. $^{*}P < 0.05$ significant difference between groups.

Table 6. Mann-Whitney analysis between groups revealed a significant difference in IL-10 level between groups K and P1.

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*P*-value
IL-10	K	P1	-2.000*	0.450	0.001
		P2	-4.167*	0.450	0.000
		P3	-4.667*	0.450	0.000
	P1	K	2.000*	0.450	0.001
		P2	-2.167*	0.450	0.001
		P3	-2.667*	0.450	0.000
	P2	K	4.167*	0.450	0.000
		P1	2.167*	0.450	0.001
		P3	-0.500	0.450	0.687
	P3	K	4.667*	0.450	0.000
		P1	2.667*	0.450	0.000
		P2	0.500	0.450	0.687

Notes: *$P < 0.05$ significant difference between groups.

caspase-3 and cytochrome c released from mitochondria. High intracellular levels of HSP27 can inhibit caspase activation by interfering with upstream mitochondria [20]. In cancer cells, HSP27 protects oncoproteins so cancer cells can continue living. According to P1 (treatment group), the expression of both TNF-α and HSP27 so that it induced apoptosis in cancer cells but they protected the normal cells.

The definition of nanoparticles, according to the National Cancer Institute, is all forms of biological and synthetic materials with dimensions < 1 μm. Nanoparticles are developed and processed so that they can be used as drug-delivery vehicles (carriers), contrast agents (imaging), diagnostic devices, as well as platforms for theranostic agents (agents that function as diagnostic and therapeutic tools), antioxidants (able to react with free radicals in tissues), *in vivo* tumour targeting with high specificity and affinity, and probes in preclinical research for molecular studies of disease. Nanoparticles are often called nanocarriers, nanomaterials, or nanosomes [12].

Some advantages of using nanoparticles as drug delivery materials include their small size compared to microspheres and liposomes. They can easily pass through sinusoidal spaces in the bone marrow and spleen compared to other systems. They also can penetrate intercellular spaces that can only be penetrated by colloidal size, have high stability, are protected from degradation, and possess improved drug/protein stability against enzymatic degradation. They may be employed for controlled release, have excellent tolerability, less liver toxicity, and allow parenteral, oral, subcutaneous, ophthalmic, and rectal routes of administration; they are safe and meet dosing requirements [21] [22].

4. CONCLUSIONS

Moringa oleifera leaf extract with isothiocyanate

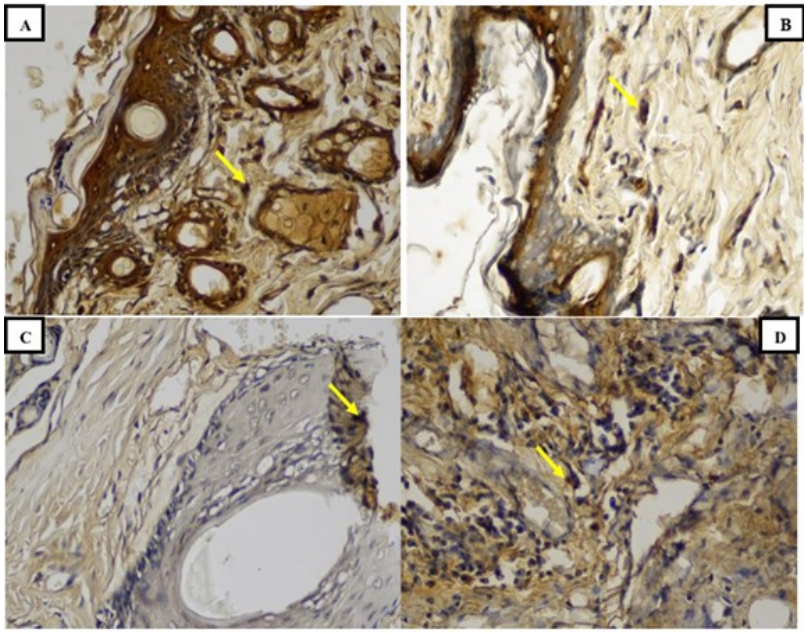

Figure 3. IL-10 expression by immunohistochemistry between the control (K) and three treatment groups (P1, P2, and P3). Both the control group (K), shown by A, and the P1 treatment group, shown by B, displayed the strongest expression compared to groups P2 (C) and P3 (D). All IL-10 expression was measured by immunohistochemistry staining with a 400x objective lens and was indicated by yellow arrows.

content at concentrations of 125 µg/mL (P1), 250 µg/mL (P2), and 500 µg/mL (P3) proved to threat Oral Squamous Cell Carcinoma. It was based on immunohistochemical examination was analysed through TNF-α, HSP27, and IL-10's expression. The expression of TNF-α and HSP27 between control and treatment groups was significantly different which the highest expression in the P1 treatment group. The expression of IL-10 between control and treatment groups was also significantly different which highest in the P3 treatment group. The research, it can be concluded that Moringa oleifera leaf extract could be used as a herbal ingredient for the treatment of oral cancer.

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Conflicts of Interest

The author(s) declare no conflict of interest.

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