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Evaluation of anti-inflammatory and wound healing properties of Squalene: An important phytochemical component of amaranth oil

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Abstract

The anti-inflammatory effect of crude amaranth (Amaranthaceae; *Amaranthus species*) oil and its wound healing effect are described in this article. One of the main secondary compounds found in amaranth oil is squalene, which also prevents skin damage and has anti-inflammatory properties. It suppresses inflammation, participates in wound healing process and controls the entire process with the help of macrophages. Macrophages are innate body cells that play a vital role in stimulating tissue repair and reducing inflammation. Thus, the aim of this work is to describe the role of squalene in the immune regulation of pro-inflammatory macrophages, which are the first cells to start acting after injury. An experimental cell model was used to analyze pro-inflammatory and anti-inflammatory cytokines. Squalene increases the synthesis of anti-inflammatory cytokines in pro-inflammatory macrophages. In addition, squalene improves the remodeling and repair signal and the recruitment signal of eosinophils and neutrophils responsible for phagocytosis. According to the results, squalene had no toxic effect on the HEK293 cell line in the concentration range from 0.1 to 0.4 mg/ml. This indicates that squalene can be safely used for biomedical, pharmaceutical, and nutritional purposes. The antimicrobial activity of squalene was also studied. The results indicated that squalene can promote wound healing by triggering a macrophage response during inflammation. Hence, squalene can be beneficial during the recovery phase of wound healing, with antimicrobial and anti-inflammatory effects.

Keywords: Amaranth oil, squalene, macrophage, anti-inflammatory effect, wound healing

Full length article **Corresponding Author*, e-mail: elen.ulrich@mail.ru

1. Introduction

Amaranth (Amaranthaceae; *Amaranthus species*) is ornamental plant with high nutritional value [1]. This plant has a varieties of components that are useful and practical for human and animal consumption [2, 3], whereas it has not been used due to its ornamental and wild nature, also lack of scientific evidences [5, 6].

Consumption of amaranth oil is recommended due to the high prevalence rate of certain diseases [7, 8]. Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracos hexane) is the main ingredient in the unsaponifiable fraction of amaranth oil [9]. In addition, the compound is found in cells of human and animal bodies, plants and microbial organisms as a precursor to sterols and many other biologically active terpenoids [10, 11]. The consumption of amaranth oil is common in many European countries, as well as the United States. Recently squalene has been used in medicine, including chemoprophylaxis of certain types of cancer [12, 13].

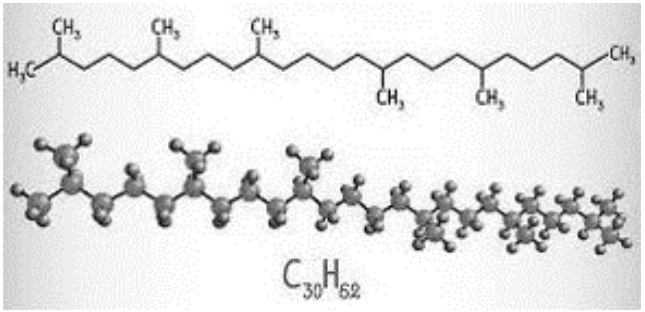

Fig. 1: Squalene structure

Squalene is the main minor compound found in virgin amaranth oil (0.6 to 16 g/kg) and has been described as a substance with prophylactic properties against cancer and atherosclerotic lesions [14].

Now-a-days, attempts for wound healing by natural herbal products are a subject of wide range of researches [15-17]. Sánchez-Quesada et al., [18] had reported about wound-healing and tissue-repairing potential of Squalene. Also, Shanmugarajan et al., [19] reported potential of Squenlene in full-thickness burn healing.

However, Squalene and Squalene-included drugs had notable effect in wound healing and tissue repairing [18,-20]. There are not any reported studies in literature to describe its role in macrophages [21].

Inflammation process is interactions between external factors and cells that can occur in any tissue in response to injury, infection, ischemia, toxicity, or autoimmune damage [21]. Macrophages are the primary and first cells to appear in the damaged area and can direct and control the inflammatory response [21]. Macrophages of the MPh1 phenotype are usually cytotoxic effectors influencing cytotoxicity and participating in the proinflammatory response, while macrophages of the MPh2 phenotype have anti-inflammatory properties and stimulate the inflammatory response. When the two types of macrophages interact, the healing process usually results in clearing the infection [21]. These macrophages are essential for wound healing; without them, the inflammatory phase can lead to impaired wound closure, granulation tissue formation, severe bleeding, and late wound closure [22]. Some studies have shown that wound healing starts with the response of macrophages MPh1, which occurs at the stage of macrophages MPh2, when the wound is finally closed and inactivated [22].

Numerous studies analyses the effect of squalene on the pro-inflammatory response of MPh1 macrophages [23-25]. The researchers studied cytokines and molecules involved in wound healing after squalene treatment. They observed the recovery potential of herbal components in monocyte cell lines (THP-1) and identified into MPh1 [23-25].

The present study designed with an experimental model of the origin of macrophages and evaluate possible effect of Squalene (herbal component) in wound healing process, *in vitro*.

2. Materials and methods

MPh1-polarized THP1 cells were subjected to the influence of various squalene concentrations. The compound has no toxic effect on macrophage survival rate. The results are calculated as a ratio of cell viability versus control group at 100%. Both drugs have obvious cytotoxicity, but the range from 3.12 to 25 μl appears to reduce cell viability by about 16-22%. On the contrary, cell survival increases at maximum concentration.

A higher inflammatory inhibiting effect was gained after application of a low squalene concentration (1 μM). After treatment with squalene in a concentration of 1 μM, the levels of IL-10, IL-4 and IL-13 increased up to levels unattainable at other concentrations tested. However, as the concentration increased, this effect disappeared. Accordingly, it is clear that squalene affects such inflammation suppressing cytokines as IL-10 and IL-4, regulates their secretion, which exhibited a sharp increase in anti-inflammatory action. In addition, cytokine IL-13 appears to be enforced by the low concentration, but inhibited when the concentration is enriched up to 100 μM. Cytokines IL-4 and IL-13 take part in an antibody response that can antagonize the response of MPh1. It was established that, in comparison with the control, a higher concentration of squalene does not contribute to the development of IL-4 and IL-13; although, at a concentration of 1 μM the compound dramatically increases the content of cytokines (IL-10, IL-4 and IL-13). These three types of major inflammation suppressing cytokines are synthesized by macrophages MPh2; thus, the phenotype MPh1 was induced before squalene was exposed to macrophage.

After treatment with 1 μM of squalene, MPh1 macrophages showed increased formation of eotaxin-2, GCSF, GMCSF, and TIMP-2. High squalene concentration (100 μM) effect body processes non-uniformly, GMCSF and GCSF levels remained unchanged, while the TIMP-2 level increased. All of these structural elements take part in tissue remodeling and produce inflammation-suppressing response to infection. They corresponded to the treating injury reaction caused by MPh2 macrophages after tissue damage. Therefore, in macrophages with an MPh1 distribution, the MPh2 distribution and the squalene response are improved at this concentration.

The yield of IL-1α at all analyzed concentrations increases within a step in 1 μM of squalene, then IL-1α, β and IL-8 increased as well. The yield of IL-1α also increased by 1 and 100 μM comparing to the control data. Except for the concentration of 10 μM, there is no difference between the INF-γ products and the control. This concentration decreases the INF-γ production. INF-γ presents simultaneously both main pro-inflammatory and MPh1 responses signals. This interferon attracts a larger number of macrophages and polarizes along the MPh1 contour, which in turn, supports the pro-inflammatory type of behavior. Such pro-inflammatory signals can be expressed by IL-1β and IL-8, but they can do a recombination function, prompting neutrophil bodies to engulf other presented cells of apoptotic character and then they clear up the space around. Bearing this in mind, the following assumption is proposed: if interferons IL-1β and IL-8 are not pro-inflammatory ones, they can act as recombination stimulants, allowing a suffering immune system to be cleared in a dangerous situation. Thus, even if the macrophages are polarized in the MPh1 state, 1 μM of squalene can still promote the distribution of the MPh2 macrophages to achieve the ultimate goal of wound cleaning and closure.

3. Results and discussion

To evaluate the results obtained with the matrix protein, TNF-α was estimated by immunoassay method. It demonstrated no existing essential differences; but after treating MPh1 macrophages with squalene, there was established an increase in TNF-α production depending on the applied squalene concentration. This finding may be explained by both anti- and pro-inflammatory cytokines presented in the applied preparation with squalene. The fusion process of this interferon was suppressed to 1 and 10 μM, but increased to 100 μM. TNF-α is one of the basic pro-inflammatory interferon that activate the pro-inflammatory response, including Th1-lymphocytes, B-lymphocytes and MPh1 macrophages.

Researchers have reported that production of NO does not change after squalene treatment, but the effect was reduced to the amount of from 10 to 100 μM. This fact confirms considerable diversity between all analyzed concentrations [26, 27].

Concerning the safety of squalene, we studied its cytotoxicity in *in vitro* systems in relation to HEK293 cell cultures (a cell line obtained from human embryonic kidneys). Figure 2 shows the results of determining the dose-dependent effect of squalene on the HEK293 cell line after 24 hours of exposure and Figure 3 – after 48 hours.

According to the results (Fig. 3 and 4), squalene had no toxic effect on the HEK293 cell line in the concentration range from 0.1 to 0.4 mg/ml. This indicates that squalene can be safely used for biomedical, pharmaceutical, and nutritional purposes. The antimicrobial activity of squalene was also studied.

The study of antimicrobial properties was carried out in relation to pathogenic and opportunistic micro-organisms that cause food poisoning and various human diseases and determined the minimum inhibitory concentrations. The findings are presented in Table 1.

Analysis of the data allowed us to conclude that squalene exhibits antimicrobial activity in relation to all investigated strains of pathogenic and opportunistic microorganisms (table 1). Table 1 show diameters of zones for pathogens were not considerable as microbial contamination. The present finding is in agreement with Bindu et al., [28] who showed anti-virulent effects of squalene for treating *S. aureus* infections. Also, data of table 1 is in agreement with Fan et al., [29] who reported antibacterial effect of squalling-included formulated drug for control of *E. coli*, and *S. aureus* infections (anti-biofilm activity).

Although limited studies revealed that squalene is a prominent agent in preventing inflammation, the study detailed the effect of squalene on pro-inflammatory macrophages, the first cell to control inflammation in trauma, and its relationship to regeneration and remodeling of tissues [18, 30].

With the aim to promote inflammation (caused by infections, tumors or tissue damage), the researchers boosted activity of human monocytes to modify into pro-inflammatory macrophages MPh1, which are the first bodies of immune system to give response to any inflammation. Thereafter, monocytes were processed with squalene to analyze the response behavior of macrophages. The data obtained along the experiment show that squalene has no cytotoxic effect on macrophages, and its polarization is close to that of MPh1, but it acts in two even opposite directions, depending on its solution concentration. Lower squalene concentration (1 μM), even though MPh1 polarization has a pro-inflammatory effect, has an anti-inflammatory response.

First, squalene appears to increase synthesis of IL-10. It presents a cytokine with anti-inflammatory properties, that can inhibit the production of antigen and pro-inflammatory cytokines, and pro-inflammatory cytokines are a key to treatment of many diseases such as inflammatory bowel syndrome. In addition, squalene (1 μM) potentiates such macrophages as IL-4 and IL-13. All the macrophages (except IL-10) are all major cytokines and contribute to the anti-inflammatory polarization of the MPh2 macrophage. IL-13 and IL-4 are variants of cytokine Th2, they both act in the following ways: 1) take part in the anti-inflammatory process and 2) balance the MPh1 response activity by regulating repair of wound.

One more point, squalene increased GMCSF, GCSF, and eotaxin-2. It is known that these chemotactic agents can control the eosinophil related activity, including their differentiation, proliferation and recruitment. Eosinophils are establishes to impose a beneficial effect in suppressing allergies, stimulating tissue repair and host defense against parasites. Nevertheless, they are also engaged into regeneration and remodeling of tissues. Squalene not only generates production of anti-inflammatory bodies, it accompanies the tissue remodeling and regeneration process by translating signals of anti-inflammatory type and gathering immune system's cells in a target area. In addition, production of TIMP-2 gene and protein is increasing. It is a metalloproteinase-2 (MMP-2) inhibitor of natural origin. The latter is connected with the destruction of pathological biological tissues states when there are chronic ailments such as inflammatory arthropathia or cancer. Thereby, the observed increase in expression of TIMP-2 after exposure to squalene will inhibit the tumor and pro-inflammatory activity of MMP-2 associated with tissue destruction, which this process is described by Watari et al., [31] and Jang and Lee [32].

In addition, the symptoms of chronic inflammation are usually attenuated in the pro-inflammatory macrophages MPh1, and the macrophages themselves are paradoxical. 1 μM of squalene decreases the production of NF-kB. The factor is defined as an element controlling the pro-inflammatory response and contributing to inflammation in a chronic form. NF-κB is able to regulate both its own activity and inflammatory process, if it is provoked by other molecules (such as TNF-α); thus, contributes to inflammation as well. Notably, that it was observed just statistically insignificant decrease in TNF-α concentration (1 μm), although it is suggesting that squalene is a naturally occurring compound with anti-inflammatory properties that may help in treating inflammatory chronic ailments. NF-κB is inhibited along the releasing of cytokines with anti-inflammatory functions such as IL-4, IL-13 and IL-10. Their number is growing under the squalene influence. Moreover, appearance of NO is also a sign of chronic swelling in MPh1 macrophages, which is not significantly affected by squalene treatment, but appears to decrease after squalene treatment. As a result, squalene can contribute to the resolvent state of macrophages, which are more similar to the MPh2 phenotype comparing to the MPh1 one.

On the other hand, squalene is not a transactor of the resolvent response at low or insignificant concentrations. If its concentration equals from 10 to 100 μM, it lost its healing activity and elicited a pro-inflammatory behavior (at concentration of 100 μM). In fact, it is described the anti-aging effects of squalene consumption, but it emphasizes that "the ratio risk and benefit effect of applying squalene in high concentrations is rather high, consequently it is not recommended for the treatment of skin tissue aging." This research is devoted to the phenomenon of double opposite activity of one and the same compound. The study depends only on using various concentrations of the compound under observation. Although the amount is essential, as the amount of compound increases, the low-level biomolecular processes involved in squalene action can change. The biological mechanism of action of squalene requires careful study, but there is reason to believe that large amounts of this compound can destroy the resolvent process mechanism and cause the opposite behavior, which may be associated with an increased response to high levels of squalene [33, 34].

Whereas anti-inflammatory cytokines are producing by macrophages MPh1 even after treatment with squalene, the studied element can elicit an MPh1 response and promote the conversion of MPh1 macrophages to MPh2 ones. Such transition has previously been pointed out in tumor growth; it includes molecular signals provoking altering the two states of macrophages. Continued research is necessary to prove this supposition; however, squalene can interface the polarization of macrophages at focus of inflammation.

Squalene treatment failed to reduce some pro-inflammatory cytokines such as IL-1α and IL-1β, which are presiding to IL-8 activation. Mentioned cytokines are designated for a group of neutrophils eligible to engulfing other cells of apoptotic character or clearing other lesions formed during acute state of any inflammation. If chemokine s IL-1 and IL-8 contribute to the inflammatory response, it is expected that INF-γ will increase because this is a sign of a pro-inflammatory response that can develop into chronic inflammation and then cancer. Interestingly, IFN-γ did not increase with squalene treatment.

Thus, there are points that squalene promotes tissue remodeling and repair, as well as recruiting neutrophil molecules responsible for clearing dead cells and detritus. This compound affects the MPh1/MPh2 balance to ensure proper wound healing.

As a result, squalene seems to control inflammation with a help of pro- and anti-inflammatory signals, including wound healing, cessation of swelling, and subsequent tissue regeneration and remodeling, but there is no MPh1 response, thus persistence of chronic inflammation is avoided. Squalene may be one of the many compounds in amaranth oil that have anti-inflammatory and wound healing effects, which suggested by Lacatusu et al., [35].

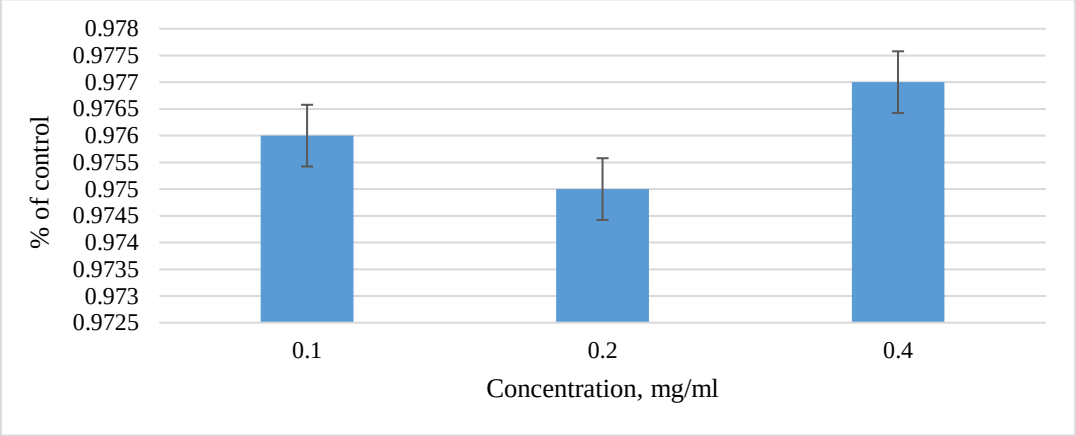

Fig. 2: Effect of squalene concentration on the HEK293 cell line after 24 h of exposure

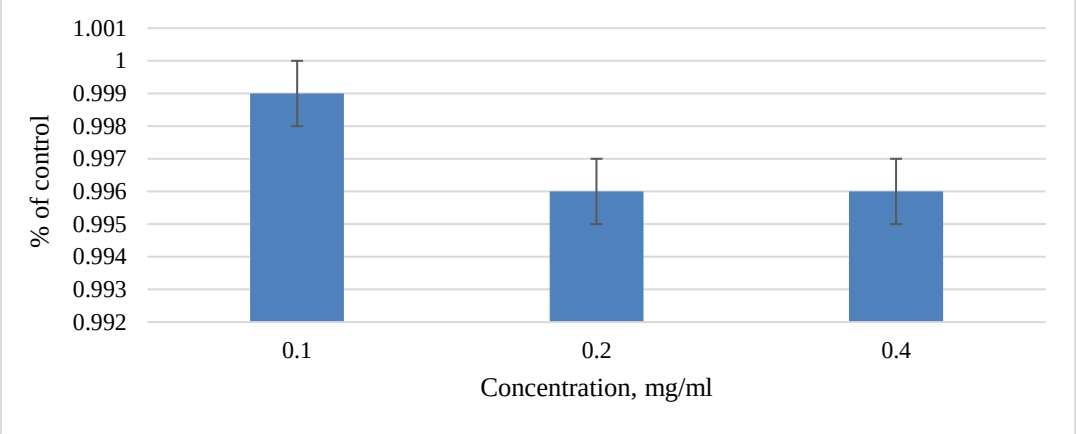

Fig. 3: Dose-dependent effect of squalene on the HEK293 cell line after 48 h of exposure

Table 1: Diameter of the lysis zone of microorganisms in the presence of squalene

Sr. No	Microorganism	Lysis zone diameter, mm
1	*E. coli*	22.00±0.66
2	*S. enterica*	25.00±0.75
3	*S. aureus*	19.00±0.57
4	*P. aeruginosa*	22.00±0.66
5	*B. mycoides*	25.00±0.75
6	*A. faecalis*	23.00±0.69
7	*P. vulgaris*	20.00±0.60
8	*Sh. Flexneri*	22.00±0.66
9	*L. monocytogenes*	24.00±0.72

4. Conclusion

Squalene had no toxic effect on the HEK293 cell line in the concentration range from 0.1 to 0.4 mg/ml. This indicates that squalene can be safely used for biomedical, pharmaceutical and nutritional purposes. Also, this component has antimicrobial effect and may decrease microbial population of wound or damaged tissue. In addition, due to its anti-inflammatory properties and its ability to remodel and close the wound, it may be beneficial in the later or final stages of natural anaplerosis process. It is noted that squalene may be used as a beneficial product of natural origin; it can guide treating for injuries by immune system modulation of macrophages. Essential implicit cells are involved in healing process.

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