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# A novel method for squalene extraction from pumpkin seed oil using magnetic nanoparticles and exploring the inhibition effect of extracted squalene on angiogenesis property

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ABSTRACT

In this study, a new method for extracting and isolating squalene from pumpkin seed oil was developed using magnetic nanoparticles (MNPs). The inhibition effect of extracted squalene on angiogenesis was investigated in chick chorioallantoic membrane (CAM). Squalene extraction from pumpkin seed oil was performed in two steps: fatty acids (FAs) separation *via* hydrothermal adsorption on the magnetic iron oxide nanoparticles and squalene extraction using solvent extraction method at different temperatures. In the first step, the neural network modeling was applied to predict the optimum range of operating parameters for achieving squalene with the highest purity. Results of transmission electron microscopy (TEM) analysis showed that under optimum conditions, the average diameter of the MNPs coated with FAs was about 20 nm. In the second step, the purity of squalene was estimated at 93% with a separation recovery percentage of 83.24% at the optimum temperature of 25 °C to prevent squalene oxidation. The inhibition effect of extracted squalene on angiogenesis in CAM suggested that squalene extracted from pumpkin seed oil had a significant effect on the inhibition of angiogenesis in the chicken embryo chorioallantoic membrane. Finally, this process yielded two valuable products, *i.e.* squalene with high-purity and MNPs coated with FAs.



## 1. Introduction

Squalene is one of the unsaturated hydrocarbons of triterpene. It plays an important role in the synthesis of cholesterol, steroid hormones and vitamin D and E in the human body [1]. Squalene has a variety of applications in food and pharmaceutical industries. It is effective for treating diseases such as dry skin, improving sebum on the skin [2] and treating various cancers [3,4]. One of the main features of squalene is its antioxidant, anti-bacteria and anti-fungal quality [5]. The compounds of squalene in nutrients have a positive effect on cholesterol and heart diseases [6]. Furthermore, the emulsion containing this material is usually used to deliver drugs and adjuvant in vaccines [7,8].

Squalene is derived from various resources including shark liver oil [9], which is one of the main sources of this compound. However, there are general concerns regarding the possibility of source animal mortality caused by contamination biochemical synthesis rout can produce coiled and sterol-like squalene, but this process is expensive due to the utilization of various enzymes and the difficulty of controlling conditions [10,11]. The last resource is squalene extraction from natural oil, which gives a linear structure to this component. The main problem with natural oil source is the low content of squalene. To overcome this problem, the waste of chemical oil refining process called deodorized distillate (DD) can be used. However, the waste oil obtained from these methods affect the quality of extracted squalene.

Various methods have been proposed to extract squalene from vegetable oils. In 2010, Sugihara et al. [12] extracted tocopherols and squalene from DD of rice husk oil by $CO_2$ supercritical fluid (CSF) chromatography. They obtained squalene content with a separation recovery efficiency of 80% after two operations of supercritical fluid and extraction with a purity of 97%. In another study, squalene was separated from DD of palm oil by CSF extraction in the counter-current packed column. Squalene concentration was increased from 2% to 16% with recovery efficiency of more than 95% [13]. Wejnerowska et al. [14] extracted squalene from the grain amaranth using CSF technology with a purity of 60% and separation efficiency of 99%. Squalene was extracted from DD of olive oil phase through distillation in vacuum after esterification

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reaction. Squalene purity was about 78% [15]. Squalene was isolated in a rapid and innovative way using hexane extraction over SPE column from pistachio [16] and other oils [17], with high recovery yield. Quantification by UPLC was later verified and compared to the straight forward NMR analysis [18]. Extraction from olive oil DD was undertaken using centrifugal chromatography. Heptane, acetonitrile and butanol solvents were used for this purpose. Separation was conducted in a silica column of 3 mbar and purity was increased from 23.4% to 95.5% [19].

As mentioned above, in most studies, squalene extraction from natural oils has been undertaken using CSF, chromatography and distillation in the vacuum pressure. In these methods, squalene was extracted directly from natural oil, seed oil or DD. The operation pressure of the equipment used in above methods is greater than the atmospheric pressure. The extreme pressure condition established in these devices suggests that these methods are not economically justifiable. In addition, applying these methods for natural oil or seed oil leads to the dissipation of a large share of oil without nutritious materials such as antioxidants. After extracting squalene from oil, it is soon oxidized, which makes its saving more affordable. Moreover, it is difficult to achieve high purity and high separation efficiency by these methods at the same time. In 2016, Xiao et al. [20] used isolation of squalene with $AgNO_3$ from Camellia oil. At first, oil was saponified under the reaction and then centrifuged. The supernatant containing unsaponifiable phase was placed in methanol and $AgNO_3$. Silver ions tend to conjugate to form unsaturated bond and squalene is separated from other substances with a purity of 37.8%.

In 2013, a combination of solvent extraction, saponification and thin-layer chromatography was used for squalene extraction from Purslane seed. The extracted phase contained squalene, phytosterols and tocopherols from Purslane oil [21], which were used as angiogenesis inhibitors.

It is possible to separate the unsaponifiable phase of seed oil for squalene extraction. Natural oils contain unsaponifiable phases such as squalene, tocopherols and sterols [22]. The unsaponifiable phase does not react with alkaline water, refusing to engage in saponification and esterification reaction [5,23]. Previous studies have shown that enhanced squalene purity could be achieved by separation of FAs from oil using saponification reaction. However, this reaction leads to the creation of FA soap that creates micelles and bubbles in the water solution. As a result, squalene is trapped in micelles and the separation efficiency declines at the extraction stage.

One of the main novelties of this study is reducing FAs by adsorption on MNPs before extraction in a two-phase separator. This is the first study to propose a new method for squalene extraction from pumpkin seed oil. It is desirable to achieve high recovery and purity at the atmospheric pressure. Pumpkin seed oil contains FA (98.4%), oleic acid (37–43%) and linoleic acid (37–43%) [24]. These FAs have extensive applications in pharmaceutical industry and the treatment of prostate diseases [25]. As a pharmaceutically important and well-cultivated plant in Iran, this oil was selected for the case study. In the proposed method, magnetic nanoparticles (MNP) have been used to retrieve FAs after saponification reaction. At this stage, magnetic nanoparticles with a coating of natural biological materials were produced. Given the low content of squalene in natural oils, generating a byproduct using other components of oils can be economically justified. In the proposed method, the entire oil contents can be used by coating MNPs with FAs. Another novelty of this study is using neural network modeling based on SA-LOOCV-GRBF method to obtain suitable condition for FA adsorption on MNPs. In the next step, squalene was easily extracted from unsaponifiable phase using the solvent extraction method. This convenience was due to the reduced FA amounts induced by adsorption on MNPs. At this stage, the optimal operating temperature to prevent squalene oxidation during purification steps was determined. This is the first study to explore the optimum temperature for oxidation prevention. In the end, the effect of extracted squalene, as an angiogenesis inhibitor, on the CAM model was investigated, which constitutes another novelty of this paper.

## 2. Materials and methods

### 2.1. Materials

$FeCl_3 \bullet 6H_2O$ (97%), $FeCl_2 \bullet 4H_2O$ (99%), potassium hydroxide, acetone, ethanol, hexane, toluene (99%) and ammonium hydroxide (27–30%) were purchased from Merck Company (Darmstadt Merck, Germany). Bromophenol blue and phenolphthale indicator were purchased from Dr. Mojallali Company, Iran. Pumpkin seed oil was achieved from Zarband Company, Iran. Standard squalene for gas chromatography-flame iodized detector (GC-FID) analysis with a purity of more than 98% was purchased from Sigma Aldrich Company. Fertilized eggs were obtained from Race Ross Company of Tus Mashhad, Iran. Paraffin sterile (Paraffin Pathological) was purchased from Fara Company, Iran.

### 2.2. Iron oxide MNPs synthesis

Co-precipitation reaction was used for the synthesis of 5.2 mmol iron oxide MNPs. First, $FeCl_3$ and $FeCl_2$ with a molar ratio of (2/1) were dissolved in 50 ml distilled water. They were poured in a balloon under atmospheric argon. The temperature was set at 45 °C and almost 4–5 ml ammonia solution was added to the solution to adjust the pH to 11. After stirring the solution at 800 rpm for 30 min, nanoparticles appeared and the solution turned black [26]. Finally, MNPs was washed with distilled water and separated with a magnet until excess ammonia was completely removed from the MNPs surface.

### 2.3. Saponification reaction

Saponification reaction was performed using ASTM standard method [27] to determine the total amount of FAs in the pumpkin seed oil. To do so, the total amount of FAs (in the form of an ester, free FAs and triglycerides) participating in the saponification reaction was calculated.

5 g of the oil sample was poured in two separate containers (one original and one control container) and 50 ml ethanolic potassium hydroxide was added to them. The original sample was heated under reflux condition at 80 °C for 60 min, and it was then titrated with HCL (0.5 N). The consumed volume of HCL was assigned as $V_1$. In addition, the control sample was titrated with HCL (0.5 N) and the volume of HCL at this stage was obtained ($V_2$). The number of FAs moles in the pumpkin seed oil or saponifiable value before adsorption on MNPs ($SV_1$) was computed according to the following equation:

$$\boldsymbol{SV_1 = (V_1 - V_2) \times C} \quad (1)$$

where $C$ is the concentration of HCL.

### 2.4. Saponification reaction to pumpkin seed oil

The saponification reaction was applied to the SV analysis using the method described in the previous stage, except that the amount of oil was determined based on SV and the average molecular weight of fatty acids. The SV amount revealed KOH moles in ethanolic potassium solution, which was added ($SV_1$) to the reaction under reflux condition (temperature of 80 °C for 1 h). As a result, oil was converted into soap and exposed to the reaction. Then, it was retained in the oven at the same temperature for 4 h until

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