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Investigation of antioxidant effect of *Urtica dioica* (L.) leaf and *Alcea setosa* (L.) flower extracts on fatty acids photooxidation

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ABSTRACT

Light energy, especially in combination with oxygen by producing singlet oxygen ($^1O_2$), can react with the double bonds of unsaturated fatty acids and reduce the quality of food and fats. In this study, the effect of singlet oxygen on photooxidation of fatty acids was investigated. The generation of singlet oxygen and peroxide products in the presence of meso-tetraphenylporphyrin (H_2TPP) as a photocatalyst and light was proved by nuclear magnetic resonance spectroscopy (^1H NMR), visible-ultraviolet spectroscopy (UV-Vis) and iodometric titration. The rate of fatty acid peroxidation determined immediately after photooxidation using meq/kg unit. Effect of hydroalcoholic extracts of *Urtica dioica* (L.) leaf and *Alcea setosa* (L.) flower was compared with synthetic antioxidants and well-known singlet oxygen scavengers. The antioxidant activities of these plants showed that hydroethanolic extracts of *Urtica dioica* (L.) leaf and *Alcea setosa* (L.) flower, respectively diminished conversion of oleic acid to peroxide products 79.45 and 81.05% after 120 minutes photooxidation. While these value for vitamin E (as a fat-soluble chemical antioxidant), sodium azide (as a very strong inhibitor of singlet oxygen) and dimethyl sulfoxide (as a strong solvent in reducing the lifetime of singlet oxygen) were 83.83%, 91.65% and 93.25%, respectively. Also, the hydroalcoholic extracts of *Urtica dioica* (L) leaf, *Alcea setosa* (L) reduced the conversion of linoleic acid (as a oxidizable fatty acid with high degree of unsaturation) to peroxide products by 56.43 and 59.06%, respectively. These results declare high antioxidant efficiency of *Urtica dioica* (L) leaf and *Alcea setosa* (L), in preventing of photooxidation of fatty acids. In this study, the effects of solvent, photocatalyst, light and oxygen in fatty acid photooxidation were also investigated.

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1. Introduction

Reactive oxygen species (ROS) are included to superoxide anion radical, singlet oxygen, hydroxyl radical, and hydrogen peroxide [1, 2]. Biologically, ROS are formed as a natural byproduct of oxygen metabolism. Under certain conditions, such as heat or exposure to ultraviolet rays, their level increases, which causes damage to the cell structure and causes various diseases [3-5]. Also, factors such as tobacco, drugs and radiation are also effective in the production of ROS [6]. The result of the activity of ROS in the body is degeneration, cancer, diabetes, heart failure, brain damage, muscle problems, premature aging, eye damage and overall weakness of the immune system [3-5]. One of the ROS is singlet oxygen, which produce from the excitation of oxygen by light [7]. Reactive singlet oxygen species can react with the double bonds of unsaturated fatty acids and lead to the production of peroxides, which make the quality of the food or fat undesirable. lipid peroxidation reactions, which are fundamental factors in many biological processes such as cancer and cell death, are can be initiated by singlet oxygen [8-11]. In fact, peroxidation is the initiator of chain reactions that lead to the breakdown of phospholipids, or it is the initiator of addition reactions of oxidation polymerization of lipids [12]. The production of singlet oxygen is possible with different physical and chemical methods, but the use of one method is more common than other methods, and that is the use of photsensitizers or photocatalysts [12, 13]. When a component absorbs energy, especially light, a process called photosensitization occurs and singlet oxygen is generated from triplet oxygen. This energy absorber is called photocatalyst or photosensitizer [13]. Porphyrins and metalloporphyrins can act as photsensitizers (photocatalysts) for the photooxidation reaction of fatty acids due to having many destabilized electrons in their structure.

Antioxidants play an important role in the functioning of our immune system by deactivating or inhibiting ROS. These substances remove free radicals from the body caused by energy consumption. If free radicals remain in the body, considering that they play a role in chain reactions, they can damage cell walls and cause cell death [14]. In fact, antioxidants are considered as the most important factor that destroys the chain reactions of free radicals and regenerates the destroyed cells, and chemically or naturally neutralizes the action of free radicals. Ascorbic acid (vitamin C), vitamin E, glutathione, vitamin A, thiols, polyphenols or enzymes such as catalase are some of antioxidants. Also, the complications caused by the use of artificial antioxidants such as BHA, BHT, TBHQ and PG, which have a phenolic structure [15] including carcinogenic properties and coronary diseases, have led to restriction of their usage as food additives in some countries and because of this subject the usage of plant extracts as sources of natural antioxidants are more interested by scientists [16-18].

Nettle with the scientific name *Urtica dioica* (L.) is an herbaceous, annual or perennial plant. These plants grow 1 to 3 meters depending on

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the type of species, soil nutrients and other environmental factors [19-22]. Nettle species have rhizomes. Nettles have many species and subspecies and are found in many parts of the world. For example, common nettle species throughout Asia, Europe and North America, Cannabida Nettle in Siberia, Western Asia and Iran, Incisa Nettle in Australia and New Zealand, Roman Nettle species in Southern Europe, small Nettle in Europe and North America and Nettle gracilenta species or mountain nettle are found in Mexico, Texas and New Mexico. The nettle plant contains compounds such as tannin, lecithin, formic acid, potassium nitrate, calcium, vitamin C and iron. Nettle root can be used in the form of tablets, tea, syrup, extract and capsules [19-22]. Nettle leaves have significant amounts of polyphenolic and flavonoid compounds and are usually used as tea. The oil extract of this plant can be used for local treatments.

Hollyhocks with the scientific name *Alcea setosa* (L.), belongs to the family of Malvaceae. Its flowers, fruits and roots have medicinal uses [23-25]. The two-meter-high Hollyhocks have pink, red, and white flowers that are used to cover medicines. Alcea is a genus of over 80 species of flowering plants in the mallow family Malvaceae, commonly known as the Hollyhocks. It is native to warm, subtropical, tropical, and tropical regions between the north and south of the equator in the world [23-25]. This plant grows well in rich and well-drained soil. Hollyhocks is an ornamental plant that has many therapeutic uses. Some people use Hollyhocks to prevent and treat respiratory disorders and digestive system problems. Also, due to the significant amounts of polyphenolic and flavonoid compounds, Hollyhocks is used to treat scars and painful swelling (inflammation) of the skin [23-25].

Flavonoid compounds have been introduced as singlet oxygen inhibitors, but few studies have been conducted in relation to the antioxidant effect of plant species on the photooxidation of fatty acids [8-10]. In continuation of the studies of this research group on the antioxidant effects of natural and synthetic antioxidants on photooxidation of fatty acids [8-10], the aim of this research is to evaluate the inhibitory property of natural antioxidants of Hollyhocks flower and Nettle leaves, which have significant amounts of flavonoid compounds, against the photooxidation of fatty acids, oleic acid and linoleic acid by singlet oxygen. (figure 1)

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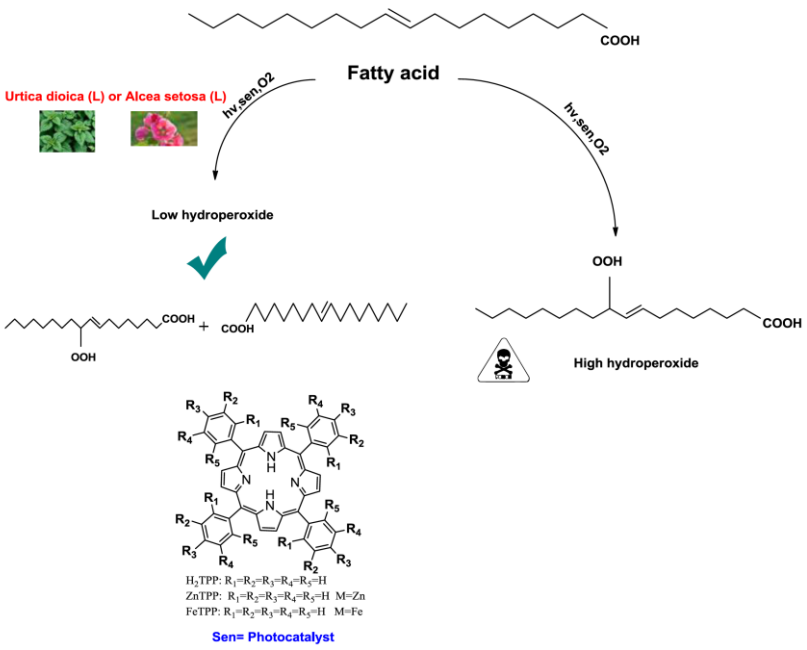

Fig 1 Fatty acid photooxidation in the presence and absence of *Urtica dioica* (L) or *Alcea setosa* (L)

2- Materials & methods

2-1- Materials

All tested antioxidants were obtained from Zardband Company in the hydroethanolic form. The total flavonoid concentration in the hydroalcoholic solution of Hollyhocks flower was 0.015 mg/ml. Also, the total flavonoid concentration in the Nettle leaf hydroalcoholic solution was 0.013 mg/ml. Linoleic acid was purchased from Sigma. Also, oleic acid, dimethyl sulfoxide (DMSO), sodium azide, pyrrole, benzaldehyde, propionic acid, dichloromethane, dimethylformamide (DMF), zinc (II) chloride ($ZnCl_2.4H_2O$), manganese (II) chloride ($MnCl_2.\ 4H_2O$) and solvents were purchased from Fluka, Merck and Kimia Aksir without further purification. Tetraphenylporphyrin (H_2TPP), MnTPPCl and ZnTPP were prepared according to Lindsey's method [26].

2-2- photooxidation reaction tests of oleic acid in the presence of plant antioxidants

For the photooxidation of fatty acids, a solution containing 0.2 ml (6.3×4^{-10} mol) of oleic acid or 0.1 ml of linoleic acid (3.2×4^{-10} mol) along with 1 ml of H_2TPP, MnTPPCl, ZnTPP (1×10^{-4} M) photocatalyst and also, 2 ml of hydroalcoholic antioxidant were added to acetonitrile solvent to reach a final volume of 20 ml. Then, for 120 minutes, irradiation was done by 288 LED lamps (1 watt) with intensity 59660 LUX, and wavelength more than 350 nm. It should be noted that the temperature of the photoreactor was set at 29-30°C by a cooling fan.

2-3- Peroxide measurement by iodometric titration method

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2.5 ml of the sample was placed in an Erlenmeyer flask, and then 18 ml of acetic acid and 12 ml of chloroform were added to the sample in the flask. The flask was shaken until the sample was dissolved in the solution. Next, 0.5 ml of potassium iodide saturated solution was added to the contents of the flask and shaken for 1 minute to dissolve in the mixture, and 30 ml of distilled water was added to the solution. Then, the contents inside the Erlenmeyer flask were titrated against the 0.1 N sodium thiosulfate standard solution until the yellow color almost disappeared. Finally, about 0.5 ml of starch indicator solution was added. The titration continued until the blue color in the solution disappeared [27].

2-4- Instrumental devices for analysis

In order to identify synthetic porphyrins, a Shimadzu-2100 model UV/Vis spectrophotometer equipped with a deuterium-tungsten lamp and an array detector at a wavelength of 300 to 700 nm was used. BRUKER AMX-300 MHz NMR spectrophotometer was used to identify the products obtained from oleic acid oxidation.

2-5- The statistical analysis of the experiments

The statistical analysis of the experiments was carried out in the form of a completely random statistical design with three repetitions. The analysis of the results was done using SAS software version 9.3 and then the average results were compared using Duncan's test and graphs were drawn with Excell software.

3- Results and discussion

3-1- Photooxidation of oleic acid in the absence of plant antioxidants

The photooxidation of oleic acid to peroxide products showed that the presence of light, air and the H_2TPP photocatalyst play a key role in the reactions (Table 1, entry 1) and the absence of any of them means that the reaction does not progress (Table 1, entry 2, 3 and 4). It should be noted that sodium azide, as a strong scavenger of singlet oxygen [28], stopped the production of peroxide (Table 1, entry 5). Also, the peaks of the ^1H NMR spectrum with chemical shift values of 4.25 ppm and 9.4 ppm confirmed the photooxidation reaction of oleic acid by the photocatalyst and showed that the production of peroxide from oleic acid was done by singlet oxygen. (figure 2)

Table 1 Oleic acid oxidation by singlet oxygen in different condition[a]

Entry	Photocatalyst	Light	Oxidant	PV(meq/kg)
1	H_2TPP	LED Lamp	O_2	623
2	H_2TPP	LED Lamp	-	Trace
3	-	LED Lamp	O_2	Trace
4	H_2TPP	-	O_2	Trace

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5[b]	H_2TPP	LED Lamp	O_2	Trace

[a] 6.3×10^{-4} mol oleic acid, 19ml acetonitrile (solvent), 1 ml (0.0001 M) H_2TPP, air (1atm) and 288 power LED lamps, 1 W, 2.3 V (59660 LUX). [b] 0.5gr NaN_3 was added as a singlet oxygen scavenger.

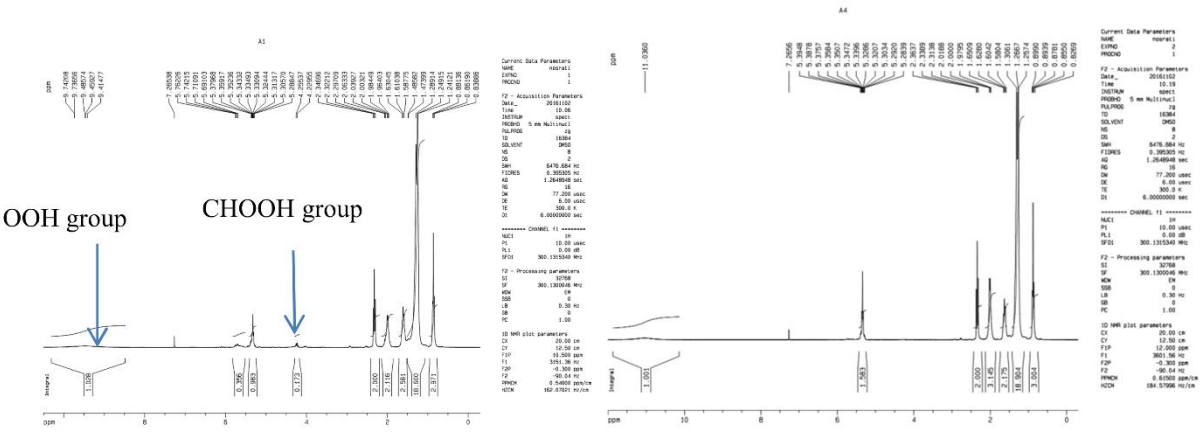

Fig 2 ^{1}H NMR spectra of oleic acid after photooxidation in the absence (right) and in the presence (left) of H_2TPP as a photocatalyst (photosensitizer)

The results of Table 2 show that H_2TPP as a metal-free photocatalyst was able to convert oleic acid to peroxide with a higher efficiency than metal-containing porphyrins such as ZnTPP and MnTPPCl. This result confirms that the main rout of reactions is generation of singlet oxygen because according to the literature, metal-containing porphyrins do not have a good ability to produce singlet oxygen [29]. In fact, paramagnetic metal complexes have a shorter triplet state lifetime than diamagnetic metal complexes or metal-free porphyrins, and this leads to the production of singlet oxygen with a lower quantum efficiency, and finally, the amount of peroxide produced decreases. Also, the comparison of photooxidation of oleic acid at different times revealed that the highest conversion of oleic acid into peroxide is occurred after 120 minutes. In another study, as a result of changing the solvent from acetonitrile to ethanol and methanol, the amount of peroxide production in the photooxidation reaction of oleic acid decreased by 37.54% and 81.41%, respectively, which was a confirmation of the photooxidation reaction by means of singlet oxygen, because in polar solvents such as methanol and ethanol, due to the formation of hydrogen bonds, the lifetime of singlet oxygen is decreased [30].

Table 2 Effect of Photocatalyst on oleic acid photooxidation[a]

Entry	Photocatalyst	60 min	120min	240 min

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1	TPP	311	623	419
2	ZnTPP	138	207	103
3	MnTPPCl	69	124	96

[a] 6.3×10^{-4} mol oleic acid, 19ml acetonitrile (solvent), 1 ml (0.0001 M) Photocatalyst, air (1atm) and 288 power LED lamps, 1 W, 2.3 V (59660 LUX).

It should be noted that the degradation of H_2TPP photocatalyst, which was recorded by UV-Vis spectroscopy (Figure 3), showed that singlet oxygen had an effective role in the reaction that destroyed the photocatalyst.

Time (min)	Abs	H_2TPP photodegradation (%)
0	1.14	0
30	0.78	31.58
60	0.69	39.48
90	0.66	42.11
120	0.61	46.5

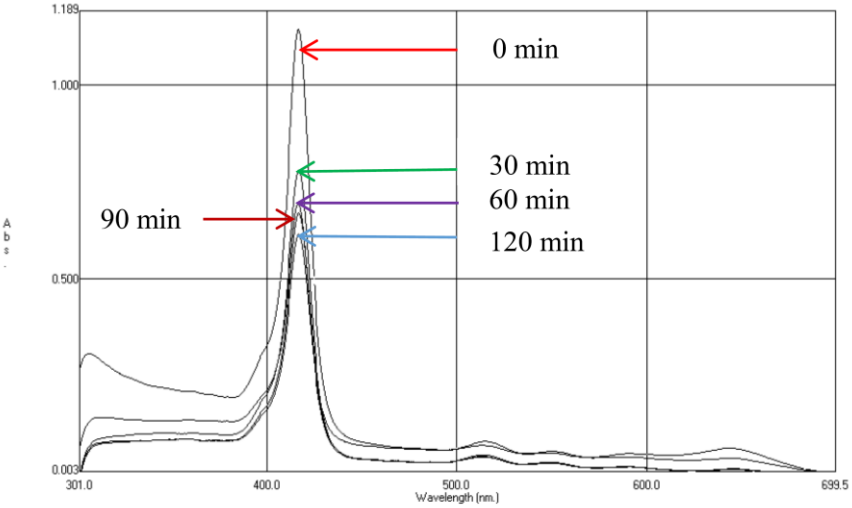

Fig 3 Effect of photooxidation time on H_2TPP degradation

3-2- Antioxidant effect of Nettle leaf extract and Hollyhocks flower extract on oleic acid photooxidation

The effect of antioxidant activity of Nettle leaves and Hollyhocks flowers on oleic acid photooxidation is shown in Figure 4. Based on the results of this table, Nettle leaf and Hollyhocks hydroalcoholic extracts were able to reduce the conversion percentage of oleic acid fatty acid to peroxide procucts by 79.45 and 81.05%, respectively, in the optimal time of 120 minutes, while this amount for vitamin E to as a fat-soluble chemical antioxidant, sodium azide as a very strong inhibitor of singlet oxygen [28], and dimethyl sulfoxide as a strong solvent in reducing the lifetime of singlet oxygen [30], 83.30%, 91.65% and 93.25%, respectively. These results indicate the high antioxidant properties of Nettle leaves and

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Hollyhocks in preventing the photooxidation of fatty acids.

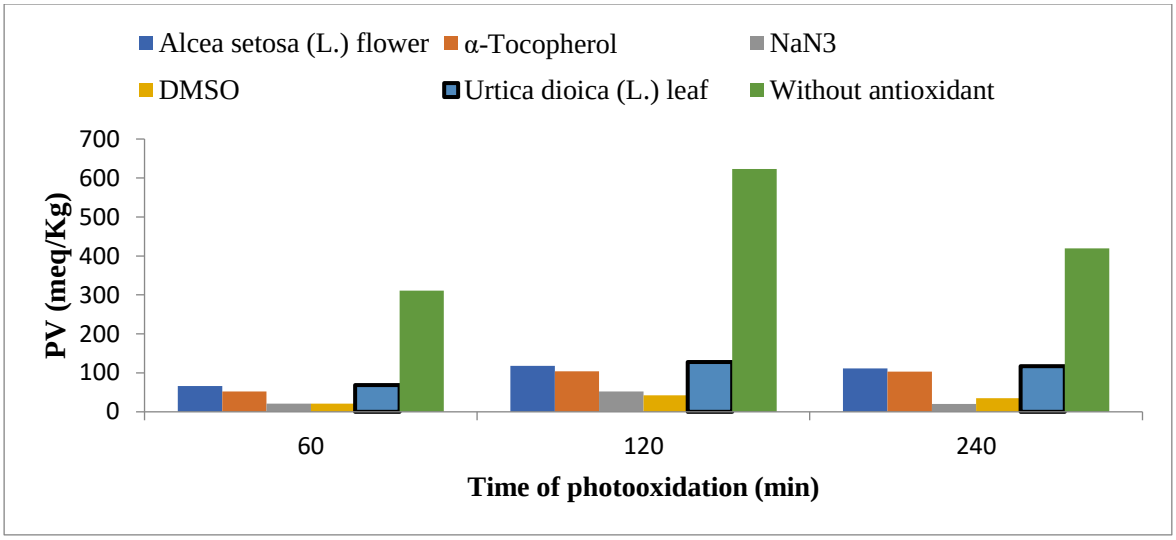

Fig 4 Effect of hydroalcoholic extract of *Urtica dioica* (L.) leaf, *Alcea setosa* (L.) flower and singlet oxygen scavengers on oleic acid photooxidation in acetonitrile as a solvent, 1- Dimethyl sulfoxide (DMSO) was used as a solvent, 2- 15 mg of α-Tocopherol (the daily intake of an adult) was used.

3-3- Antioxidant effect of Nettle leaves and Hollyhocks flowers on linoleic acid photooxidation

Linoleic acid is more reactive to oxidation than oleic acid due to its higher degree of unsaturation. The noteworthy point of this research is the significant antioxidant effect of Nettle leaves and Hollyhocks flowers on linoleic acid photooxidation. Based on Figure 5, Nettle and Hollyhocks extracts were able to reduce the conversion percentage of linoleic acid to toxic peroxide by 56.43 and 59.06%, respectively, in the optimal time of 120 minutes.

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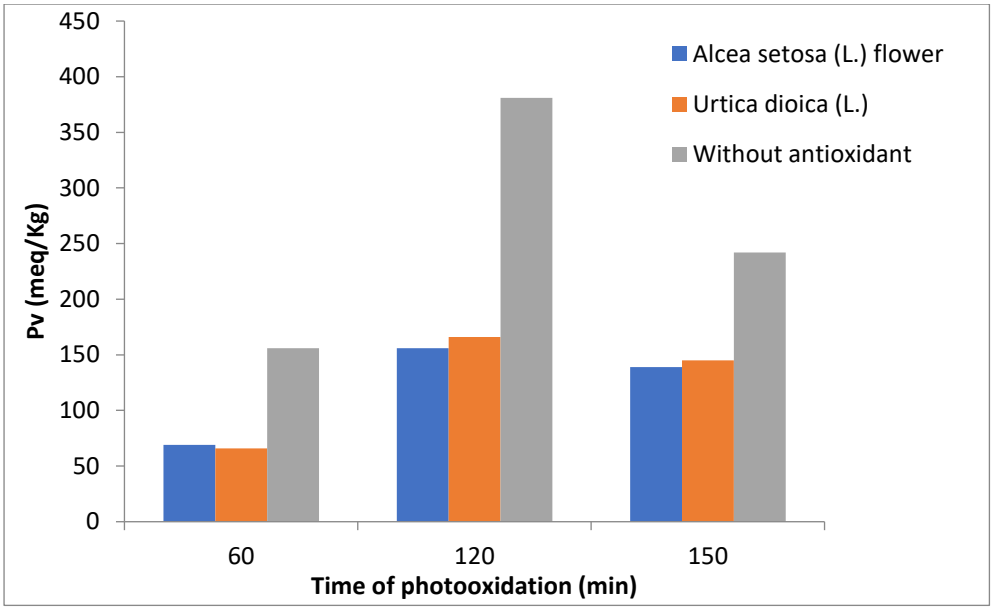

Fig 5 Effect of Effect of hydroalcoholic extract of *Urtica dioica* (L.) leaf, *Alcea setosa* (L.) flower on linoleic acid photooxidation in acetonitrile as a solvent, 1- (3.2×10^{-4} mol) linoleic acid was used.

3-4- discussion

Many studies have been done in connection with the oxidation of oils and fats in the presence of natural oxidants. In the research conducted by Noor et al., it was observed that the iodine index of palm oil containing turmeric leaf extract shows a higher iodine index compared to the oil containing hydroxybutyl toluene during frying [31]. Also, in the research conducted by Man et al., it was observed that the level of fatty acids in oil samples containing 0.2% turmeric leaf extract is similar to oil containing 0.4% rosemary and sage extract [32]. In the research conducted by Naz et al., it was also observed that vanillic acid, caffeic acid, and ferulic acid (inhibitor phenols) and 0.02% tea extract reduced the peroxide index of the olive, corn, and soybean oils [33]. Auto-oxidation of oils and fats occurs through a self-propagating free radical mechanism. According to the theory of Farmer and Boland, the radical chain reaction of autoxidation of unsaturated fatty acids consists of 4 stages: initiation, diffusion, breakdown of hydroperoxides and the end, which can lead to the production of peroxide [5-11]. Meanwhile, in the photooxidation reaction, a sensitizer such as chlorophyll absorbs light energy and is excited. The excited photosensitizer can become a fundamental or singlet photosensitizer, or it can transfer its energy to the basic molecule of oxygen, which is in triplet form, and produce the active species of singlet oxygen. It is known that singlet oxygen reacts 1000 to 1500 times faster than triplet oxygen [5-11]. In this way, compared to autoxidation, photooxidation is a much faster reaction. Also, the oxidation mechanism is different in terms of the type and amount of hydroperoxides produced [5-11]. According to these materials, in this research, a new method for oleic acid fatty acid oxidation was presented in the presence and absence of Nettle leaf and Hollyhocks flowers

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hydroalcoholic extract. For this purpose, LED light energy was used to activate triplet molecular oxygen ($^3O_2$) to singlet molecular oxygen ($^1O_2$) in the presence of porphyrin and metalloporphyrin photocatalysts in the direction of oleic acid oxidation. At first, to prove that the reactions are carried out through the path of singlet oxygen, investigations were carried out and the results of NMR spectroscopy and UV-Vis spectroscopy proved that the oxidation of oleic acid is carried out by singlet oxygen. Next, in one of the most important analyzes that were conducted, the antioxidant properties of a number of natural and chemical antioxidants, including Nettle leaves, Hollyhocks flowers, and vitamin E, were investigated on the photooxidation of oleic acid. Since, based on scientific findings, Nettle leaves have 65 mg of polyphenolic compounds per 100 grams of leaves [22] and Hollyhocks flowers has 73 mg of polyphenolic compounds per 100 grams of flowers [24], these plants can Show proper antioxidant properties. The results of this research showed that the process of trapping active species of singlet oxygen in these plants is very close to vitamin E. The noteworthy point in this study was that the photooxidation of linoleic acid, which has more reactivity and oxidizing ability than oleic acid, was significantly reduced in the presence of the hydroalcoholic extract of the leaves of the Nettle species and the flowers of the Hollyhocks flowers species. It seems that the high antioxidant performance of Nettle leaves and Hollyhocks flowers against photooxidation reactions is due to phenolic and especially flavonoid groups, which are found in high amounts in the of these plants [34] According to the literature, flavonoid compounds play a very critical role in trapping reactive singlet oxygen species (Figure 6), and the flower of the Hollyhocks and leaf of Nettle are rich in these compounds.

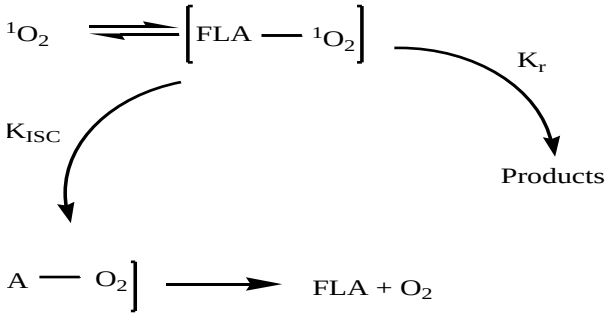

Fig 6 The mechanism of flavonoids barricade against singlet oxygen

The important point of this research is that until now no research has been done on the antioxidant properties of Nettle and Hollyhocks against the active species of singlet oxygen, and from now on these plants can be included among the substances that have antioxidant properties and used to prevent photooxidation of fats.

4 - Conclusion

Nettle leaves and Hollyhocks flower have very good antioxidant properties to prevent the oxidation of fatty acids and can be taken orally to prevent diseases caused by oxidative stress in the biological environment. Also, the extract of these plants can be added as an additive to edible oils to prevent the oxidative deterioration

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of fatty acids. Finally, it is suggested to use this plant in the In-vivo tests for using in pharmaceutical companies.

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بررسی اثر آنتی اکسیدانی عصاره برگ گزنه (L.) *Urtica dioica* وگل ختمی (L.) *Alcea setosa* بر فتواکسیداسیون اسیدهای چرب

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چکیده

انرژی نور، به ویژه در ترکیب با اکسیژن با تولید گونه فعال اکسیژن یکتای ($^1O_2$) می تواند با پیوندهای دوگانه اسید های چرب غیراشباع واکنش دهد و کیفیت غذا و چربی ها را کاهش دهد. در مطالعه حاضر اثر اکسیژن یکتایی بر اکسیداسیون اسیدهای چرب مورد بررسی قرار گرفت. تولید اکسیژن یکتایی و محصولات پراکسیدی در حضور کاتالیزگر نوری H_2TPP (مزو-تترا فنیل پورفیرین) و نور به وسیله روش های طیف سنجی روش های طیف سنجی رزونانس مغناطیسی هسته‌ای ^1H (NMR)، طیف‌سنجی مرئی-فرابنفش (UV-Vis) و تیتراسیون یدومتری اثبات شد و مقدار پراکسید شدن به عنوان پارامتر اکسیداسیون در اولئیک اسید بلافاصله پس از اکسیداسیون به صورت (meq/kg) تعین شد. در این مطالعه به مقایسه بررسی اثر عصاره هیدروالکلی برگ گزنه وگل ختمی به عنوان آنتی اکسیدان گیاهی و طبیعی در مقابل آنتی اکسیدان های سنتزی و مهارکننده های اکسیژن یکتایی پرداختیم. نتایج آنتی اکسیدانی این گیاهان نشان داد که به ترتیب عصاره های هیدرواتانولی برگ گزنه و گل ختمی توانستند به میزان۷۹.٤۵ و ۸۱.۰۵ درصد از تبدیل فتواکسیداسیونی اولئیک اسید به پراکسید در مدت زمان ۱۲۰ دقیقه جلوگیری کنند. در حالی که این مقدار برای ویتامین E (به عنوان یک آنتی اکسیدان شیمیایی محلول در چربی)، سدیم آزید (به عنوان مهارکننده بسیار قوی اکسیژن یکتایی) و دی متیل سولفوکساید (به عنوان حلال قوی در کاهش طول عمر اکسیژن یکتایی) به ترتیب ۸۳.۳۰، ۹۱.٦۵، ۹۳.۲۵ درصد بود. همچنین عصاره های هیدروالکلی برگ گزنه و ختمی توانستند به ترتیب درصد تبدیل اسید چرب لینولئیک اسید (به عنوان یک اسید چرب با درجه غیر اشباع بالا و اکسید پذیر) به گونه سمی پراکسید را تا ٥٦.٤۳ و٥۹.۰٦ درصد کاهش دهند. این نتایج دلالت بر خاصیت آنتی اکسیدانی بالای برگ گزنه و گل ختمی در جلوگیری از فتواکسایش اسیدهای چرب دارد. در این مطالعه اثرات حلال، کاتالیزگر نوری، نور و اکسیژن نیز مورد بررسی قرار گرفتند.

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