Journal of Essential Oil Bearing Plants

ISSN: 0972-060X (Print) 0976-5026 (Online) Journal homepage: http://www.tandfonline.com/loi/teop20

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To cite this article: Hamid Nazari, Masoud Yavarmanesh & Mohammad Hossein Haddad Khodaparast (2016) In vitro Study to Evaluate the Antibacterial Effect of Pistacia khinjuk Stocks Oil as Compared with Olive Oil on Food Borne Pathogenic Bacteria (Staphylococcus aureus, Escherichia coli, Listeria monocytogenes), Journal of Essential Oil Bearing Plants, 19:1, 125-133, DOI: 10.1080/0972060X.2014.971067

To link to this article: http://dx.doi.org/10.1080/0972060X.2014.971067

Published online: 07 Mar 2016.

Taylor & Francis
Taylor & Francis Group

ISSN Print: 0972-060X
ISSN Online: 0976-5026

In vitro Study to Evaluate the Antibacterial Effect of *Pistacia khinjuk* Stocks Oil as Compared with Olive Oil on Food Borne Pathogenic Bacteria (*Staphylococcus aureus, Escherichia coli, Listeria monocytogenes)*

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Received 31 May 2014; accepted in revised form 24 September 2014

Abstract: The aim of this study was to evaluate antibacterial activity of *Pistachia khinjuk* fruit oil and extra virgin olive oil. The *Pistacia khinjuk* fruit oil was extracted using n-hexane by cold maceration and Soxhlet method. Antibacterial activity was evaluated using disk diffusion and micro broth dilution methods on food pathogenic bacteria (*Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes*). The results showed that the oil from *P. khinjuk* fruit has more inhibitory effect on pathogenic bacteria than olive oil. The best zone of minimum inhibitory concentrations were observed for olive oil and *P. khinjuk* extracted using n-hexane by cold maceration and Soxhlet method on *Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes* so that these ranges were 3.125-6.25 %, 1.156-3.125 %and 3.125-6.25 % respectively. No bactericidal effect was observed for *P. khinjuk* oil whereas this property was only observed in 100 % concentration from extra virgin olive oil. Also, according to disk diffusion method both of *P. khinjuk* oils had only inhibition zone on *Listeria monocytogenes* whereas no inhibition zone was observed for extra virgin olive oil.

Key words: Antibacterial effect, Foodborne pathogenic bacteria, Olive oil, *Pistacia khinjuk* oil.

Introduction

Oils possessing antimicrobial activities are useful in treatment of wound, formulations of antimicrobial creams and lotions for treating skin diseases, as well as in the area of food preservation. For instance, it was reported the efficacy of the seed oil from mahogany (*Khaya ivorensis*) against dermatitis associated with mange and dermatophilosis [1]. In addition, the agourólado (green olive oil produced by crushing the olives without the use of hot water) was, and still is, one of the most important natural medicines for various illnesses among the inhabitants of the Mediterranean [2].

Pistacia khinjuk and *Pistacia atlantica* are the most important species of pistachio in Iran which are spread in places with the attitude around 700-2000 m [3]. Some species of Pistacia have been used in traditional medicine and these plants are used as anti-inflammatory, antipyretic, antibacterial, antiviral, treatment of diarrhea, throat infection, indigestion, tonic, toothache and astringent [4,5,6,7].

There is no report regarding antimicrobial activity of *Pistacia khinjuk* fruit oil. The aim of this study was to evaluate the antibacterial effect of oil from *Pistacia khinjuk Stocks* on food borne

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pathogenic bacteria (*Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes)* in comparison to olive oil.

Material and methods

Sampling and oil extraction

The fruits of *P. khinjuk* were collected from mountains of Jiroft, Iran (a city in southeast Iran). Then the oil was extracted using Soxhlet extraction and cold maceration by n-hexane methods [8,9]. The crude oils extracted were stored at 4°C for further experiments. Also, extra virgin olive oil was prepared from Fadak Company, Qum, Iran.

Preparation of bacteria and culture media

The antimicrobial activity of oil was individually tested against three microorganisms, including *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Listeria monocytogenes* (PTCC 1298). An overnight culture of each bacterial suspension was prepared. Each organism in the stock was sub-cultured into freshly prepared nutrient broth aseptically using a flamed wire loop and then incubated at 37°C for 24 hours. Also, nutrient agar and muller Hinton agar and broth (Merck, Germany) were prepared by using Agar disc diffusion and Micro-broth dilution methods according to manufacturer's instructions.

Micro-broth dilution method

The zone of minimum inhibitory concentration (MIC) was determined using micro-broth dilution assay [10]. All oils were initially tested at 100 % concentration; then serial dilutions were prepared to 0.195 %. Each well was inoculated with 5 Ml of microbial suspension at a density of 1.5×10^8 CFU/ml and incubated overnight at 37°C. The growth of microorganisms was observed as turbidity determined by measuring optical density at 625 nm (Sunrise Elisa Reader, TECAN, Switzerland).

Tween 80 was used for stability of the suspension (medium-oil) during the incubation period. Also, *ultra turrax* (IKA, Germany) was applied for completely homogenization of the suspension. Due to natural absorbance of the suspension (medium-oil) the experiment was conducted as a blank without bacteria for elimination of background absorbance. Also, gentamicin, erythromycin and chloramphenicol antibiotics were used as positive controls for *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli* respectively.

Agar disc diffusion method

In vitro antibacterial activity of *Pistacia khinjuk* fruit oil was determined using agar disc diffusion method according to EUCAST [11] with some modifications. Briefly, sterile paper discs (6 mm diameter) were thoroughly moistened with 20 µl of different oil concentration (The oils were dissolved in dimethyl sulfoxide 3 %) and placed on the inoculated agar plates, then incubated at 37°C for 18 h. Eventually, the diameters of the inhibition zones were measured.

Results

Extra virgin olive oil: the zone of minimum inhibitory concentration

The zone of MIC according to broth micro dilution method is presented in figure 1. The zones of MIC were 12.5-25 %, 25-50 % and 3.125-6.25 % for *L. monocytogenes*, *E.coli* and *S. aureus* respectively. The highest MIC was observed for *E.coli* so that the zone of MIC was occurred in further dilution.

Pistacia khinjuk oil: the zone of minimum inhibitory concentration

The zone of MIC according to extraction method (soxhlet and cold maceration by n-hexane) is presented in figures 2 and 3. The zones of MIC regarding Soxhlet method were 3.125-6.25, 6.25-12.5 and 6.25-12.5 % for *L. monocytogenes*, *E. coli* and *S. aureus* respectively (Fig. 2) whereas these zones regarding cold maceration by n-hexane method were 1.156-3.125, 3.125-6.25 and 3.125-6.25 % for *L. monocytogenes*, *E. coli* and *S. aureus* respectively (Fig. 3). As a consequence, the highest antibacterial activity was observed for the *P. Khinjuk* oil extracted by cold maceration using n-hexane so that the zone of MIC was occurred in further dilution. Also, regardless of extraction method, *E. coli* and *S. aureus* had the highest MIC so that the zones of MIC were identical in every extraction oil method (Fig. 2 and 3).

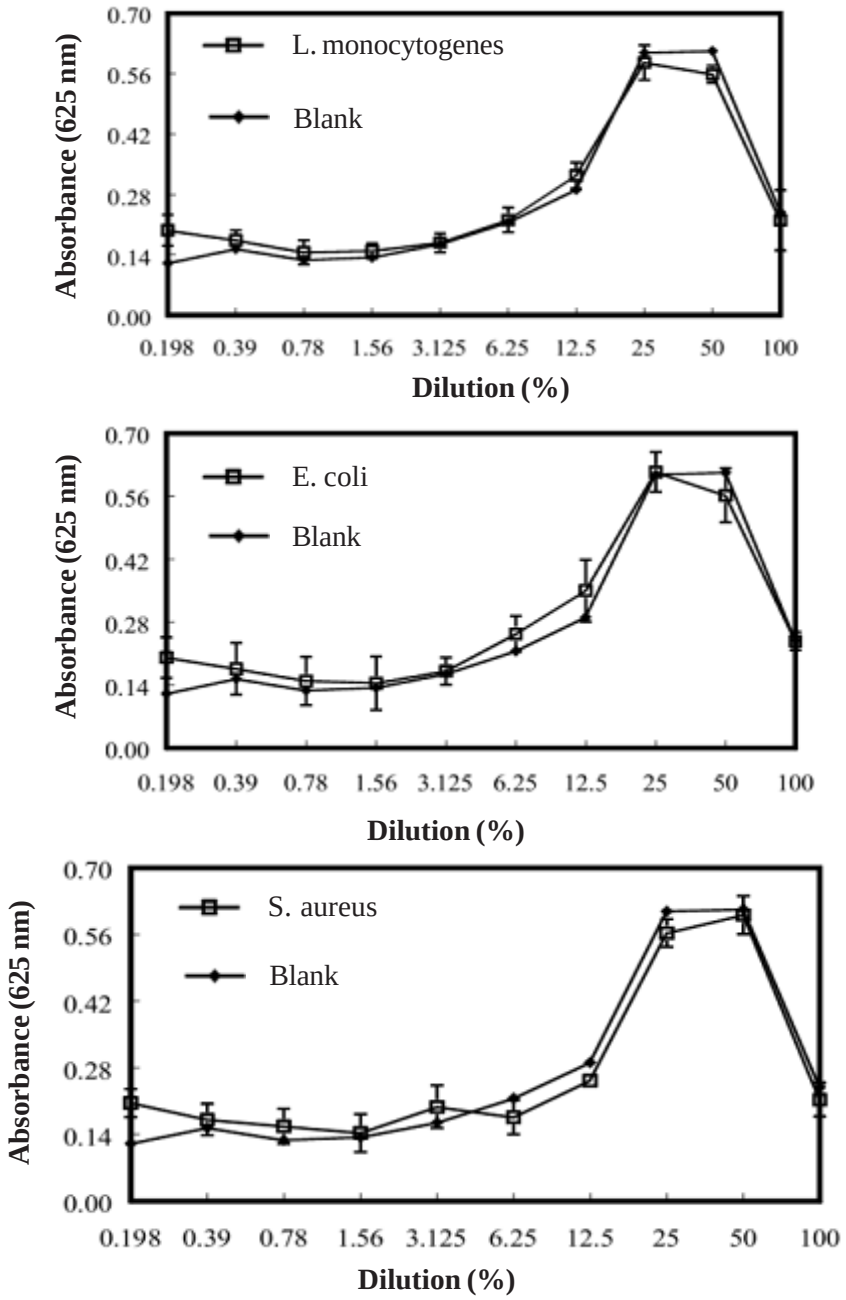

Fig. 1. The zones of MIC for extra virgin olive oil

Agar disc diffusion method: extra virgin olive oil as compared with *Pistacia khinjuk* oil

The results of disk diffusion method are shown in table 1. The inhibition zone was observed only for *L. monocytogenes* by both of *Pistacia khinjuk* oils (soxhlet and cold maceration by n-hexane methods), whereas no inhibition zone was observed for extra virgin olive oil on any of the bacteria.

Discussion

For centuries, antimicrobial activity of fruit oils have been demonstrated so that nowadays, the content of phenolic compounds along with the essential oils and fatty acids profile are the main factors for antibacterial effects [12,13,14,15,16]. Therefore, more inhibitory effect on pathogenic bacteria by *Pistacia khinjuk* fruit oil can essentially be evaluated according to these variables in this study.

Table 1. Disk diffusion method: Antibacterial activity of oils

Oil	Dilution (%)	Zone of inhibition (mm) *E. coli*	*S. aureus*	*L. monocytogenes*
Extra virgin olive oil	100	-	-	-
	50	-	-	-
	25	-	-	-
	12.5	-	-	-
	6.25	-	-	-
	3.125	-	-	-
Soxhlet extraction of	100	-	-	11
P. khinjuk oil	50	-	-	11
	25	-	-	9
	12.5	-	-	8
	6.25	-	-	-
	3.125	-	-	-
Cold extraction of	100	-	-	13
P. khinjuk oil	50	-	-	13
	25	-	-	12
	12.5	-	-	10
	6.25	-	-	7
	3.125	-	-	-

Phenolic compounds

Phenolic compounds extracted from extra virgin Olive oil with antibacterial effect are cinnamic acids, ferulic acids, 4-hydroxybenzoic acid, luteolin, tyrosol, vanillic acid and vanillin, whereas the main phenolic compounds in olive oil are hydroxyltyrosol, tyrosol and oleuropein [15,17]. The quantity of phenolic compounds are very different according to the kind of olive oil or extraction methods. But, the total phenolic compounds in olive oil is nearly 500 mg/kg and 15.27 mg/kg based on gallic acid [18,19,20].

Mean while, the amount of phenolic compounds in oil extracted from the shell of *Pistacia khinjuk* is nearly 99.67±7.07 mg/kg, whereas based on gallic acid this amount is 26.6 mg/kg in the kernel of *Pistacia khinjuk* [21,22]. Also, there is no information on the type of phenolic compounds extracted from the kernel of *Pistacia khinjuk.*

According to the main role of phenolic compounds (antibacterial activity), it seems that higher amount of these compounds in *Pistacia khinjuk* kernel oil causes higher antibacterial activity than olive oil (Fig. 2 and 3).

Antibacterial mechanisms of phenolic compounds due to binding to the cell wall of bacteria are presumably the enzymatic inhibition of oxidized compounds, chemical reaction with the sulfhydryl groups and non specific chemical reaction with the proteins [13,23].

Essential oils profile

Essential oils are one of the main components in the kernel of *Pistacia khinjuk* which are transferred presumably to the oil during extraction processing (Table 2) [7]. Among these components the antibacterial activity of α-pinene and terpinolene has been demonstrated previously [7].

Fatty acids profile

Antibacterial activity of unsaturated fatty acids (18 carbons) along with esterified fatty acids (12 carbons) has been proved on gram positive bacteria and some of yeasts [14]. Antibacterial mechanism of lipophilic glycerides and their derivatives is likely damaging the substrate transition into the bacterial cell due to more transition of protons across the bacterial cell membrane and disruption

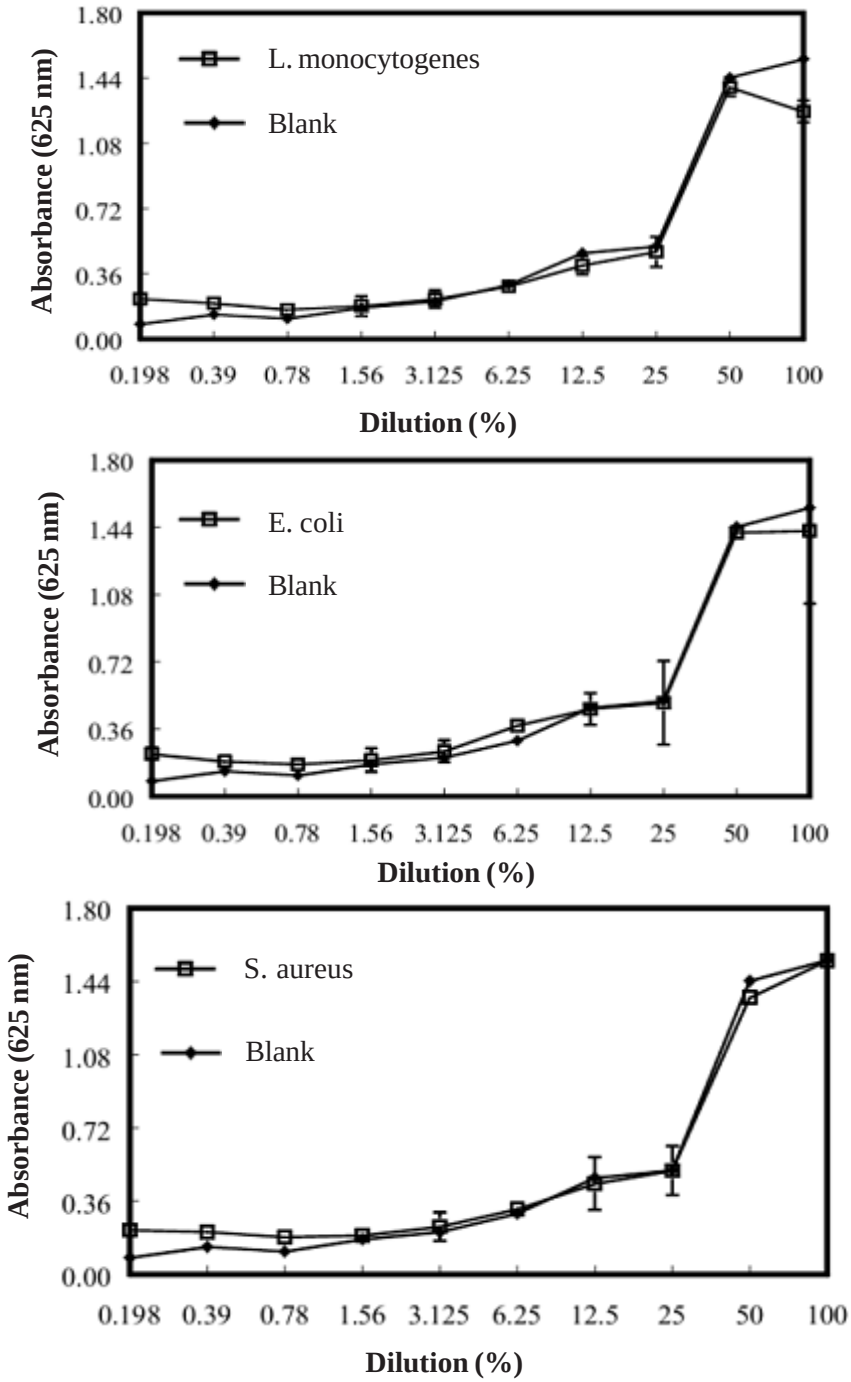

Fig. 2. The zones of MIC for *Pistacia khinjuk* oil extracted by soxhlet method

of the cell membrane proton pump [14].

Comparison between the fatty acids profile in olive and *Pistacia khinjuk* kernel oils to some extent confirms more antibacterial activity of *Pistacia khinjuk* oil, which is due to the higher concentrations of linoleic, myristic and *cis*-11-eicosenoic acids. Also, unlike the olive oil, there are pentadecanoic and heptadecanoic acids in *Pistacia khinjuk* kernel oil (Table 3 and 4) [24,25].

Conclusions

The main reasons for more inhibitory effect of *Pistacia khinjuk* fruit oil on pathogenic bacteria compared to olive oil are as below:

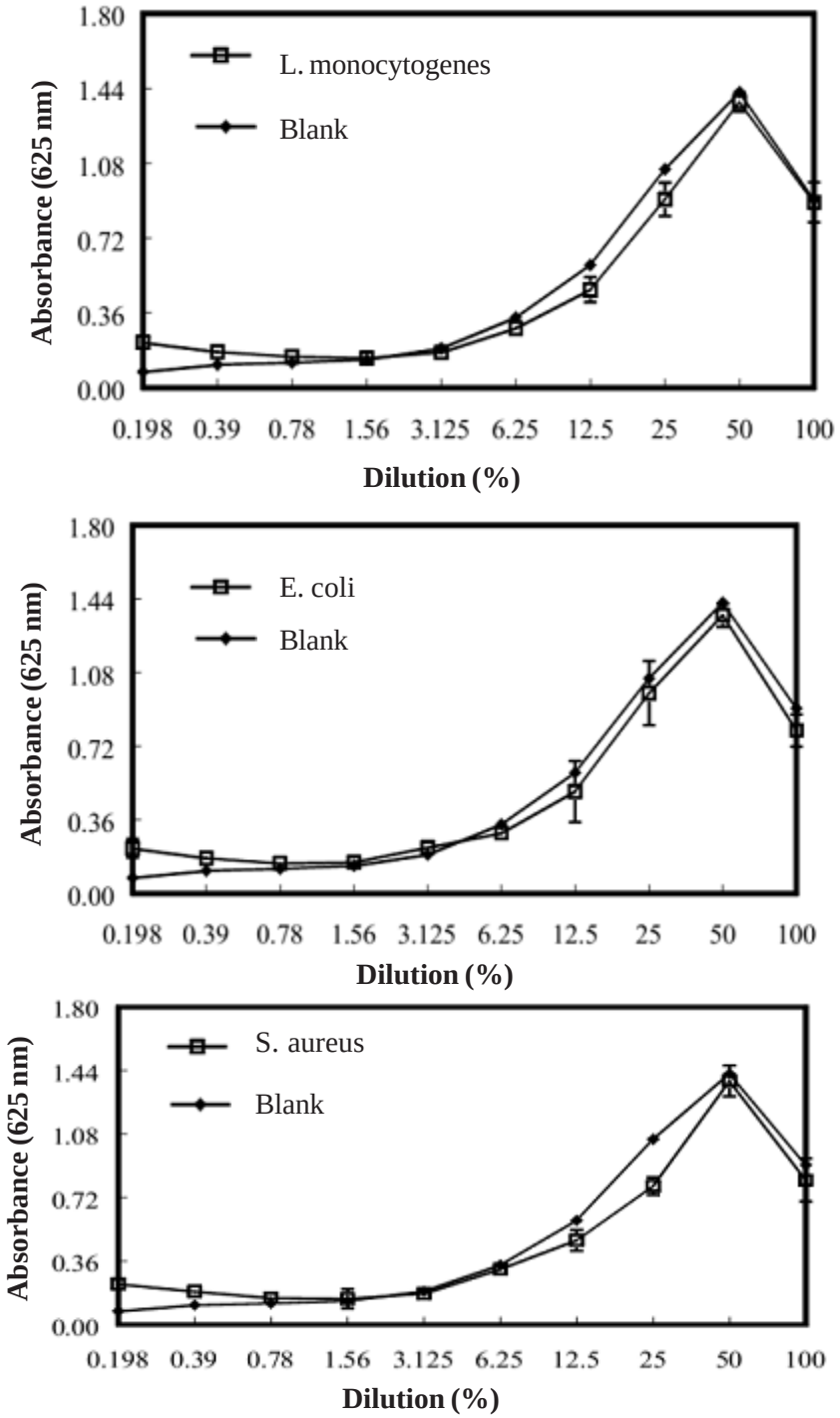

Fig. 3. The zones of MIC for *Pistacia khinjuk* oil extracted cold maceration by n-hexane method

1. Higher amount of phenolic compounds in *Pistacia khinjuk* kernel oil.
2. Presence of α-pinene and terpinolene as the main essential oils with distinct antibacterial activity in the kernel of *Pistacia khinjuk.*
3. Presence of more fatty acids with distinct antibacterial activity in the kernel of *Pistacia khinjuk.*

Acknowledgments

The authors wish to thank Mr. Ali Mohammadi Sani for his scientific support of this project.

Table 2. Essential oils profile in the kernel of *Pistacia khinjuk*

No.	Compound	R.T (min)	(%) (w/w)
1	α-Pinene	11.59	15.28
2	Sabinene	13.09	0.90
3	Phellandrene	15.95	52.33
4	Δ-Limonene	16.78	4.08
5	1,3,6-octatriene	17.33	1.30
6	γ-Terpinene	17.71	1.54
7	α-Terpinolene	19.51	0.58
8	(Z)-4,8-Dimetyl-1,3,7 nonatriene	19.91	1.01
9	L-Linalool	20.64	1.69
10	Thujopsene	35.16	1.47
11	Caryophyllene oxide	41.63	1.65
12	Hexadecanoic acid	57.36	1.08
13	Octadecanoic acid	59.83	6.26
14	9- Octadecanoic acid	63.39	1.21
15	Ethyl oleate	64.20	1.37
	Total		91.75

Table 3. Fatty acids profile in the olive oil

Fatty acid		Codex alimentarius (2003)	IOOC (2003)
Lauric	C 12:0	Not present in discernible amounts	Not specified
Myristic	C 14:0	< 0.1	< 0.05
Palmitic	C 16:0	7.5-20.0	7.5-20.0
palmitoleic	C 16:1	0.3-3.5	0.3-3.5
Heptadecanoic	C 17:0	< 0.5	≤ 0.3
Heptadecanoic	C 17:1	< 0.6	≤ 0.3
Stearic	C 18:0	0.5-5.0	0.5-5.0
Oleic	C 18:1	55.0-83.0	55.0-83.0
Linoleic	C 18:2	3.5-21.0	3.5-21.0
Linolenic	C 18:3	< 1.5	≤ 1.0
Arachidic	C 20:0	0.8	≤ 0.6
Eicosenoic	C 20:1	Not specified	≤ 0.4
Behenic	C 22:0	< 0.3	≤ 0.2
Erucic	C 22:1	Not present in discernible amounts	
Lignoceric	C 24:0	< 1.0	≤ 0.2

Table 4. Fatty acids profile in the kernel of *Pistacia khinjuk*

Fatty acid (%)		*Pistacia khinjuk* oil
Myristic	C 14:0	-
Palmitic	C 16:0	23.4±0.24
Palmitoleic	C 16:1	7.74±0.07

table 4. (continued).

Fatty acid (%)		***Pistacia khinjuk* oil**
Heptadecanoic	C 17:0	0.05±0.08
Heptadecanoic	C 17:1	0.2±0.16
Stearic	C 18:0	2.39±0.05
Oleic	C 18:1	52.03±0.16
Linoleic	C 18:2	12.02±0.2
Linolenic	C 18:3	1.5±0.19
Eicosenoic	C 20:1	0.25±0.05
Behenic	C 22:0	-

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