Chemical composition, safety and efficacy of Pistacia vera L. *oleoresin essential oils in experimental wounds*

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Abstract

The aims of the study were to evaluate the wound-healing properties in an *in-vivo* model of the essential oils from Algerian and Italian *Pistacia vera* L. oleoresins. The essential oils (EOs) were obtained by hydrodistillation of oleoresins from the trunk of plants. They were analyzed by GC-FID and GC-MS, α-pinene was the main constituent of both EOs. The wound-healing potential of *P. vera* EOs was investigated using an excision wound model in rabbits. The EOs were mixed with petroleum jelly to obtain a topical ointment with a final concentration of 5%. The percentage of the evolution of wound contraction was calculated and histological sections of tissues were examined. Both preparations possess wound-healing activities comparable to that of the reference drug Cicatryl-Bio. Some EOs exhibit a wound-healing potential suggesting that they could find a place in modern therapy.

Keywords: *Pistacia vera* L.; essential oil; α-pinene; toxicity; wound healing activity.

Introduction

The genus *Pistacia,* belonging to the Anacardiaceae family, is widely distributed in the Mediterranean area with numerous species and varieties of wild-growing plants. Some species have an economic value because they produce oleoresins with traditional medicinal uses (1,2). They include *P. lentiscus,* the source of an oleoresin called Chios mastic gum (3), and *P. vera*, which is also the source of true pistachio, widely used as dried fruits or as a food ingredient in Algeria and Italy.

The use of oleoresins as dietary supplement and herbal remedy dates back to ancient Greece, when physicians such as Hippocrates and Galen recommended their use for gastrointestinal diseases (4). Recent studies have confirmed the biological properties of *Pistacia* oleoresins, including antimicrobial effects and their potential for the treatment of various skin, respiratory, gastrointestinal disorders and wound healing (5-9). These activities are probably due to the presence of terpenes and phenolic compounds including acids and alcohols (10). In particular, a wound-healing activity has been ascribed to the whole oleoresin present in *P. lentiscus* and *P. atlantica* (11-13), in which essential oils represent a non-secondary portion. The aim of the present study was to determine the safety and the efficacy of topical ointments prepared with the essential oils (EOs) of oleoresin from *P. vera* trees growing in Algeria and Italy on wounds using an *in vivo* model, as well as to investigate their chemical composition.

Materials and methods

Plant material

The *P. vera* L. oleoresins were obtained in 2014 by making incisions from the base of the trunk of trees growing in M'sila (Algeria) and Bronte (Italy), respectively, as previously reported.[7]

Essential oil extraction and chemical analysis

The EOs were obtained from the whole oleoresin (21 g) by hydrodistillation in a Clevenger-type apparatus for 3 h. The oils (yields of 12% and 16% from Algerian and Italian oleoresins, respectively) were dried on anhydrous sodium sulphate and stored under nitrogen in a sealed vial at -20°C until use.

Gas chromatography (GC) analyses were carried out on a GC-17A gas chromatograph, equipped with a flame ionization detector (FID) and with an operating

65 software Class VP Chromatography Data System version 4.3 (Shimadzu). Analytical
66 conditions: SPB-5 capillary column (15 m × 0.10 mm × 0.15 μm), helium as carrier gas
67 (1 mL/min). Injection in split mode (1:200), injected volume 1 μL (4% essential
68 oil/CH_2Cl_2 v/v), injector and detector temperature 250°C and 280°C, respectively. Linear
69 velocity in column is 19 cm/s. The GC program temperature was set at 60°C for 1 min,
70 from 60°C to 280°C at a rate of 10°C/min and then reprogrammed at 280°C for 1 min.
71 Percentages of compounds were determined from their peak areas in the GC-FID profiles.
72 Gas chromatography – mass spectrometry (GC-MS) analysis was performed on a
73 Shimadzu GCMS-QP5050A equipped with the same column in the GC-FID, with the
74 following operating conditions: injector and detector temperatures 250°C and 280°C,
75 respectively; carrier gas helium 1mL/min, mass spectra were acquired in the electron
76 mode at 70 eV in the scan mode in the *m/z* range 40-400. Oil solution was injected with
77 the split mode (1:96).
78 *Identification of essential oil components*
79 Compounds were identified by the determination of their GC retention indices relative to
80 C_8-C_{20} alkanes, by comparing their fragmentation patterns with those reported in the
81 literature (14), by computer matching with the NIST MS 107, NIST 21 libraries (15),
82 using the GCMS solution version 1.02 software (Lab solution, Shimadzu).
83 *Preparation of the ointment*
84 The ointments were prepared using a traditional method obtained from herbalists by
85 mixing the EOs separately with petroleum jelly (PJ) (Unilever, France) at a final
86 concentration of 5% to obtain Essential Oil Algerian and Italian Ointments (EOAO 5%,
87 EOIO 5%, respectively). Cicatryl-Bio (CIC) (Pierre Fabre, Paris, France) was used as a
88 reference drug.
89 *Animals*
90 New Zealand albino male rabbits obtained from the Pasteur Institute of Algiers, weighing
91 between (2.0 - 2.7 kg) were used for the study. They were fed *ad libitum* with water and
92 kibble diet. All protocols were approved by the Ethical Committee of Directorate General
93 for Scientific Research and Technological Development at Algerian Ministry of Higher
94 Education and Scientific Research (DO1N01UN280120150001) and according to the
95 International Council for Laboratory Animal Science (16).
96 *Acute dermal irritation*

The study was conducted on rabbits one day after the dorsum was shaved and according to the Organization for Economic Co-operation and Development (OECD) guidelines 404 (17,18). EOAO and EOIO 5% were applied topically on the back of the animals at an amount of 0.5 g per rabbit and then the treated site was covered with a patch. The patch was removed after 4 h and signs of erythema and edema, and the responses were scored at 1, 24, 48 and 72 hours (19). The animals were observed for mortality and any toxic or deleterious effects with special attention given to the first 4 h and then once daily for a period of 16 days following the topical application. At the application sites, the evaluation of edema and erythema was performed and the Dermal Irritation Score (DIS) was calculated by the following formula:

$$\mathbf{DIS} = \frac{\mathbf{Value}\ (\mathbf{erythema}\ +\ \mathbf{edema})}{\mathbf{Nr.\ of\ animals}\ \times\ \mathbf{Nr.\ of\ observations}}$$

The skin irritation of the ointments was classified according to Draize et al., 1944 scale (19).

Eye irritation test

The objective of this study was to determine the potential of the ointments to produce irritation from a single instillation via the ocular route according to OECD guideline No 405 for the evaluation of chemicals, adopted April 24, 2002 (20).

The test consists of a single application of 0.1 mL of Algerian and Italian EO separately in the left conjunctival sack of each rabbit. The non-treated right eyes served as controls. The test was conducted in two stages: instillation was first performed on a single animal (Initial test), and was then applied to the rest of rabbits (Confirmatory test). Signs of ocular irritation, including redness, swelling, cloudiness, edema and hemorrhage, were recorded and scored to evaluate the ocular irritancy at 1, 2, 24, 48, and 72 h after exposure (19). The incidence, severity and reversibility of ocular irritation in New Zealand white rabbits after exposure to *P. vera* oil were classified according to Kay and Calandra (21).

Healing activity

The rabbits were randomly divided into five groups of four rabbits as follows: first group was untreated (UT), second group treated with the reference drug (CIC), third group with EOAO 5%, fourth group with EOIO 5%, and fifth group with petroleum jelly (PJ).

The animals were locally anaesthetized using xylocaine 2% and a circle of 2.5 cm in diameter of the lumbar region was excised. Excisional wounds were immediately

129 treated according to Draize *et al.* (19). The animals were placed in individual cages with
130 clean litters.
131 Preparations (CIC, EOAO, EOIO and PJ) were applied topically at an amount of
132 0.5 g per rabbit of the different groups once per day during 16 days (22). The initial and
133 successive dimensions of excision wounds were measured by tracing the size of wounds
134 on transparent paper. The percentage of the evolution of wound contraction was
135 calculated using the following formula (23):
136 ***% wound contraction = [(Initial wound size – specific day wound size)/Initial wound size] x 100***
137 *Histological sections*
138 At the end of the 16 days of treatment, the rabbits were sacrificed. The healed skin and
139 0.5 cm of healthy skin were removed for histological study (22). The tissue slices were
140 fixed in formalin (10%) for 72 h. These samples were dehydrated by passage through
141 three successive baths of ethanol. Then they were thinned in two baths of xylene and
142 embedded in paraffin by two successive baths at 60°C for each one. The obtained paraffin
143 blocks were then cut with a microtome, rehydrated and stained with haematoxylin-eosin
144 (24).
145 *Statistical analysis of results*
146 The results are represented in the form of means ± SD and differences were considered
147 significant at $p \leq 0.05$. Calculations were carried out using the GRAPH PAD 7.0 analysis
148 and statistical data processing software. Group comparisons were carried out by ANOVA
149 analysis of variance with two controlled factors (time and treatment). The significance of
150 the difference between the experimental groups was evaluated by the Tukey test.
151
152 **Results and discussion**
153 *Essential oil chemical analysis*
154 Oleoresins are very complex phytochemical mixtures characterized by a large amount of
155 volatile compounds with also a number of non-volatile constituents mainly belonging to
156 the tri and tetraterpenoids substance classes. Other remarkable peculiarities of both *P.*
157 *vera* oleoresin EOs treated in this study were the absence of sesquiterpenes and the very
158 low amount of myrcene, in accordance with Alma *et al.* (25).
159 A total of 39 compounds, accounting for more than 99% of the whole
160 composition, were identified in the *P. vera* EOs, the two essential oils appear very similar

under a chemical point of view. In terms of chemical classes, only two compound groups were represented: the monoterpene hydrocarbons, as the largest group for both oils (96.1% and 91.2%), followed by the oxygenated monoterpenes (3.2% and 7.9%) (Table 1). GC analysis revealed the presence of one main component, α-pinene, in both EOs: >90% for Italian oil and >80% for the Algerian oil. The second main component is in both cases was β-pinene with a relative percentage of 1.6% and 5.3%, respectively. The sum of the content of the two pinene isomers reaches the considerable amount of 93.2% and 87.2%, respectively. In the Italian oil, all other components were present with a percentage <1%, while in the Algerian one, only two additional monoterpene hydrocarbons (camphene and limonene) and two oxygenated monoterpenes (*trans*-3-pinanone and *cis*-sabinol) were present with an amount ≥1% but in any cases below 3%. Previous studies conducted on *Pistacia* EOs from other Mediterranean areas also showed α- and β-pinenes as main components, although the presence of α-pinene was much lower (25,26).

Acute dermal irritation

In the assessment, evaluation and classification of the toxic characteristics of substances, the determination of acute dermal and eye toxicity is a very important step. As an initial step, it is used for establishing a dosage regimen in subchronic and other studies and may provide information on dermal and eye absorption and the mode of toxic action of the substances by this route (27). In the evaluation of acute dermal irritation/corrosion, both ointments were classified as not irritant, according to guideline 404 of the Organization for Economic Cooperation and Development (OECD) and Draize et al. 1944 (18,19). The Algerian and Italian ointments can be considered minimally irritant based on the guideline of the Organization for Economic Cooperation and Development 405 (OECD) and the classification of Kay and Calandra, 1962 (20,21). As to the toxicity, the short-term treatment with a *P. vera* EOs appears safe. A previous study indicated that the vegetable oil extracted from the fruits of *P. lentiscus* is slightly irritating to the skin and the eye of rabbits after a single application (11). The safety of *P. vera* EOs could be ascribed to the very low toxicity of α-pinene and of many other monoterpene hydrocarbons, which have been generally recognized as safe (GRAS) (28).

The animals were continuously observed during the 16 days following the topical application of OEOA 5% or OEOI 5%. No signs of toxicity or mortality were seen. The

rabbits did not show any critical changes in behavior and breathing, or any disability in feeding and water utilization, or postural irregularities and loss of hair. There were no signs of cutaneous irritation, no erythema, eschar, edema, or any other reactions on the skin of all animals after topical application. The DIS for both ointments was equal to “0".

Eye irritation test

No particular behaviour was observed in the rabbits, which appeared active and healthy. No corneal opacity or iritis was observed during the test period (Table 2). All treated eyes exhibited conjunctivitis 1 h after EOs instillation, but the overall incidence of irritation decreased with time and completely disappeared after 24 h. The maximum total mean obtained at 1 h was 24 (n=4) with a corresponding ocular irritation index of 6. No clinical signs or changes in animals associated with the administration of the preparations were evident.

Evaluation of the healing process

The assessment of the evolution of the surface of each wound excision was performed on all the treated and untreated animals. The results showed a significant reduction in wound closure (cm and % contraction) in groups treated with CIC, EOAO 5% and EOIO 5% (*** $p < 0.001$) in comparison with UT and PJ (Table 3). There was no significant difference between groups treated with both ointments and the reference drug Cicatryl-Bio. The EOIO 5% proved to be efficient in the healing process with 87.55 ± 0.14% of contraction of excision wounds in rabbits, which was similar to that obtained with the reference drug Cicatryl-Bio (82.54 ± 0.18%).

Histological study

According to Figure 1, the cuts of the batch treated with the reference drug (CIC) showed a complete re-epithelialisation with the presence of a thick mature epithelium with well-differentiated cell layers and also higher collagen deposition. The inflammatory fleshy bud was clearly visible in the cuts of the untreated group or the treated group with PJ and the re-epithelialization was incomplete with the presence of disorganized cells typical of an immature epithelium and very less collagen deposition. On histological examination, the treated groups (Cicatryl, EOAO 5% and EOIO 5%) showed significant collagen deposition and complete re-epithelialization. The treatment with the ointments has therefore a strong impact on the granulation and epithelialization of wounds, accelerates tissue repairment and reduces the duration of this process. The effect can be

correlated to the composition of the ointments. Thus, α-pinene, the major constituent of *P. vera* EOs, together with β-pinene and limonene are monoterpenic compounds which have shown anti-inflammatory, antibacterial, antioxidant and anti-hypernociceptive properties which support the wound-healing process (6,29-31). Moreover, α-pinene stimulates the inflammatory cell production (macrophage type-2) which is a key regulation step of the wound healing process. The anti-inflammatory effect is essential to shorten the healing period as well as to reduce pain and scarring (29).

In an *in vivo* study, a *P. atlantica* resin extract with an α-pinene content of 46.57% showed a concentration-dependent effect on the healing of burn wounds after 14 days of treatment by increasing the concentration of basic fibroblast growth factor (BFGF) and platelet-derived growth factor and by improving angiogenesis. Indeed, increased concentration of basic fibroblast growth factor is known to greatly enhance wound healing (6).

The batches treated by the EOAO 5% and the EOIO 5% showed a complete re-epithelialization. All treated samples showed a complete maturation of the epidermis and the presence of well-differentiated granular layer and also higher collagen deposition.

Conclusions

The present study revealed that essential oils of *P. vera* from the Mediterranean area possess strong wound-healing activity associated with a lack of side effects such as skin and eye irritation. Wound-healing percentages at different time points during 16 days of observation showed effectiveness of both Algerian and Italian *P. vera* essential oils compared to the untreated and petroleum jelly treated groups.

These results are also confirmed by the examination of histological sections where a complete re-epithelialization of the skin layers, similar to the one obtained with Cicatryl, the reference drug treatment, was observed. These findings indicate that these ointments could be effective protective agents.

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Disclosure of statement

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Table 1. Components of *P. vera* oleoresin essential oils[a].

#	RI[b]	RI[c]	Class/Compound	*Italian*	*Algerian*
				% (±SD)	
			Monoterpene hydrocarbons	**96.2**	**91.1**
1	922	926	tricyclene		0.2 (0.01)
2	931	930	α-thujene	0.1(0.02)	0.2 (0.01)
3	936	939	α-pinene	91.5 (0.30)	81.9 (0.23)
4	951	954	camphene		1.2 (0.04)
5	954	960	thuja-2,4(10)-diene	0.8 (0.15)	0.5 (0.02)
6	969	968	verbenene	0.2 (0.09)	
7	977	975	sabinene	0.2 (0.03)	0.2 (0.01)
8	976	979	β-pinene	1.6 (0.03)	5.3 (0.06)
9	987	990	myrcene	0.6 (0.02)	t
10	1005	1002	Δ-3 carene	0.1 (0.04)	0.1 (0.00)
11	1006	1003	α-phellandrene	0.1 (0.00)	t
13	1015	1017	α-terpinene	t	t
14	1023	1024	*p*-cymene	0.1 (0.02)	0.2 (0.01)
15	1033	1029	limonene	0.5 (0.01)	1.0 (0.03)
17	1041	1037	*cis*-ocimene	0.1 (0.01)	0.1 (0.00)
18	1051	1050	*trans*-ocimene	0.1 (0.01)	0.1 (0.01)
20	1088	1089	terpinolene	0.2 (0.05)	0.1 (0.00)
			Oxygenated monoterpenes	**3.2**	**7.1**
16	1032	1031	1,8-cineole	0.5 (0.02)	
19	1099	1093	6-camphenone	t	t
21	1114	1109	6-camphenol		0.1 (0.01)
22	1117	1126	α-campholenal	0.1 (0.02)	t
23	1124	1139	*trans*-pinocarveol	0.1 (0.00)	0.1 (0.00)
24	1138	1142	*trans*-sabinol	0.3 (0.01)	0.4 (0.03)
25	1140	1144	*cis*-sabinol	0.5 (0.03)	1.3 (0.02)
26	1149	1145	*cis*-verbenol		0.5 (0.03)
27	1156	1160	*trans*-3-pinanone		2.6 (0.04)
28	1163	1165	pinocarvone	0.9 (0.03)	0.3 (0.01)
29	1165	1170	α-phellandren-8-ol	0.1 (0.00)	0.1 (0.01)
30	1175	1171	menthol	t	
31	1180	1177	terpinen-4-ol	0.6 (0.02)	0.1 (0.01)
32	1189	1183	*p*-cymen-8-ol	t	
33	1189	1184	*cis*-pinocarveol	t	
34	1192	1188	α-terpineol	0.1	0.2 (0.02)
35	1197	1195	Myrtenol	t	0.5 (0.02)
36	1211	1196	Myrtenal	0.2 (0.02)	0.1 (0.00)
37	1216	1205	verbenone	t	0.2 (0.01)
38	1227	1220	*trans*-carveol	0.1 (0.01)	0.3 (0.02)
39	1247	1243	Carvone	t	t
40	1291	1285	isobornyl acetate	0.2 (0.00)	0.3 (0.01)
			Others	**0.3**	
12	1008	1005	*p*-cresyl methyl ether	0.3 (0.01)	

[a] The numbering refers to elution order, and values (relative peak area percent ±SD) represent averages of 3 determinations (t=trace, <0.05); [b] Retention Index (RI) relative to standard mixture of *n*-alkanes on SPB-5 column; [c] Literature Retention Index (RI);

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Table 2. Incidence, severity and reversibility of ocular irritation in New Zealand white rabbits after exposure to *P. vera* EOs (n = 4 rabbits/oil).

Time post-instillation (h)	Corneal opacity	Iritis	Conjunctivitis	MMT value*	O.I.I.**
1	0/0/0/0	0/0/0/0	4/6/8/6	24/4	6
24	0/0/0/0	0/0/0/0	0/0/0/0	0	0
48	0/0/0/0	0/0/0/0	0/0/0/0	0	0
72	0/0/0/0	0/0/0/0	0/0/0/0	0	0
96	0/0/0/0	0/0/0/0	0/0/0/0	0	0
168	0/0/0/0	0/0/0/0	0/0/0/0	0	0

*MMT = Maximum Mean Total; **O.I.I. = Ocular Irritation Index

Table 3. Effect of different treatments on the evolution of the percentage of excision wounds contraction in New Zealand albino rabbits.

Groups	Wound contraction (%)			
	Days			
	4	8	12	16
UT	19.84± 0.17	23.28± 0.13	32.10± 0.47	36.84± 0.49
CIC	34.36± 0.42*	59.71± 0.38***	69.39± 0.35***	82.54± 0.17***
EOAO 5%	42.59± 0.38**	64.90± 0.29***	81.33± 0.36***	85.30± 0.33***
EOIO 5%	44.76± 0.33**	67.50± 0.42***	79.85± 0.39***	87.55± 0.25***
PJ	18.95± 0.18	28.97± 0.17	41.25± 0.31	48.06± 0.13

Values are expressed as means ± SD (n = 4); * p <0.05, **p<0.01 and ***p<0.001 (when treated groups are compared to the UT group).

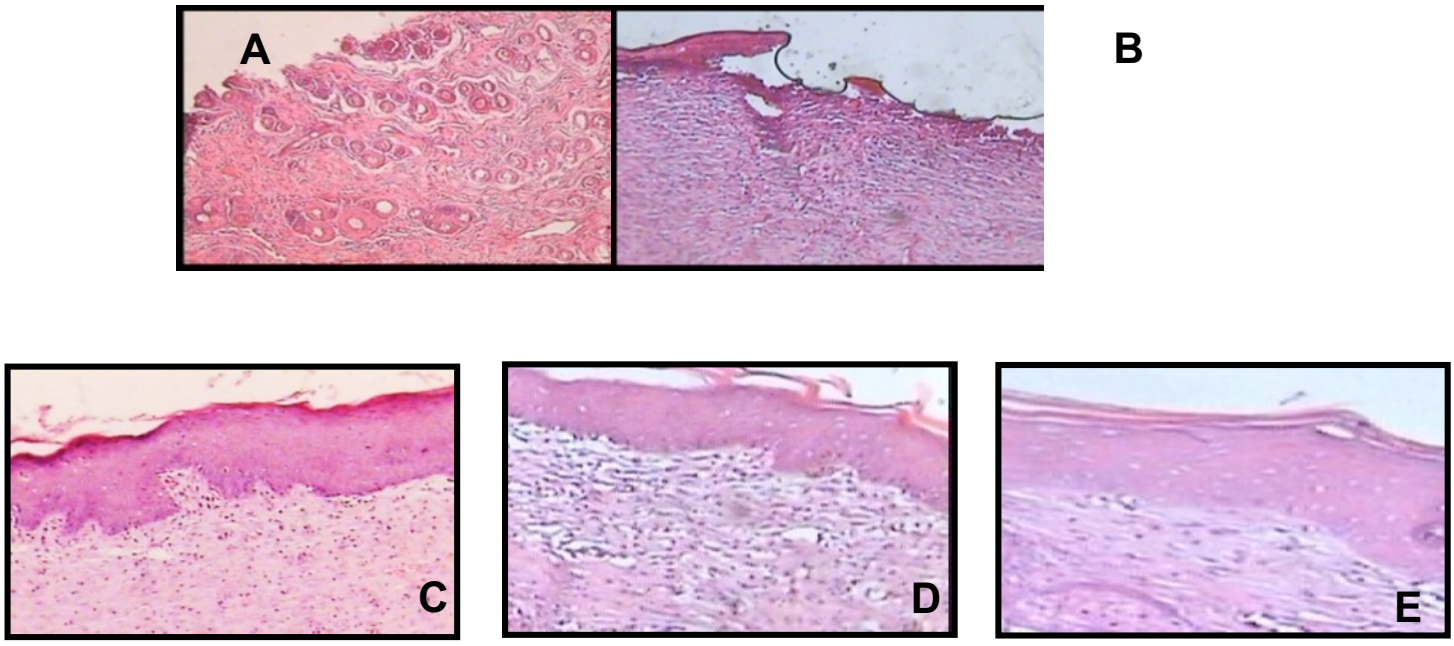

Figure 1. Histological evaluation of wound skin sections stained with hematoxylin-eosin (10 x magnification). **A** and **B** [untreated (UT) and petroleum jelly (PJ) groups, respectively] showing the inflammatory fleshy bud. **C**, **D** and **E** [cicatryl-bio (CIC), 5% essential oil Algerian ointments (EOAO 5%) and 5% essential oil Italian ointments (EOIO 5%) groups, respectively] showing a thick mature epithelium with well differentiated cells layers and higher collagen deposition (complete reepithelisation)

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