In vivo wound healing effect of Italian and Algerian *Pistacia vera* L. resins

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Abstract

Pistacia vera oleoresin is one of the natural products used traditionally for the management of wounds. However, there were no scientific reports documented so far on the wound healing activities to substantiate the claim. This study assesses the potential of the oleoresin of *P. vera* collected in Italy and Algeria for wound healing efficacy via *in vivo* circular wound excision model. Italian and Algerian oleoresins were subjected to purification and successive fractionation to obtain three matrices. The fractions have been characterized using GC-FID and GC-MS analyses. Oleoresins mixed with vaseline (5% w/w) were topically applied on wound excision induced on the dorsum of rabbits. Wound healing effects were evaluated by percent of wound contraction. Biopsies performed after healing were histologically assessed. Phytochemical results showed a high content of terpenoids components inducing an efficient wound healing effect determined by an *in vivo* study. Italian and Algerian oleoresins ointments showed significant wound contraction from day 8 to day 16 as compared to the negative control. The two ointments have not showed statistically difference as compared to Cicatryl, reference drug. These results have also been confirmed by the histological evaluation of the tissues involved. The absence of signs of toxicity on the skin of rabbits indicated the safety of the ointments. The study showed that both oleoresins have a very high effectiveness as wound healing agents and appear to justify their traditional use in wound healing in several countries and offer a scientific support to the treatment of traditional healers.

Keywords: *Pistacia vera* L.; oleoresin; GC-FID /GC-MS; toxicity; *in vivo* wound healing.

Abbreviations:

GC-FID gas chromatography – flame ionization detector

GC-MS gas chromatography – mass spectrometry

1 Introduction

Herbal medicines have been used to treat and prevent various illnesses in many countries throughout the world for centuries. These medicinal herbs typically contain mixtures of different chemical compounds that may act individually, additively, or in synergy to improve health [1,2].

The recent success of drug development from medicinal plants inspires and encourages many researchers to investigate and validate the uses of traditional medicinal plants [1].

The genus *Pistacia* (Anacardiaceae) includes almost twenty species and most of them are considered medicinal plants according to the popular traditions of several countries, in particular, *Pistacia vera*, *P. khinjuk*, *P. atlantica*, *P. terebinthus*, and *P. lentiscus* have been classified and characterized as significant and economically important [3].

Pistacia has an economic value as it is the source of traditional medicinal agent "gum" mastic, an oleoresin obtained by incisions of the trunk of trees, considered as a traditional natural remedy [4,5]. Actual researches have upheld several biological properties of *Pistacia* oleoresins, such as antimicrobial effects, respiratory diseases and gastrointestinal disorders [6-10].

In the Mediterranean area, much attention has been focused on *P. lentiscus* and *P. atlantica* oleoresins for their pharmacological effects such as reducing blood pressure, anti-inflammatory, antimicrobial, hypoglycemic, antioxidant, wound healing and analgesic [11-13]. The effectiveness of oleoresins is probably due to their complex composition, characterized by monoterpenes, together with not volatile components such as triterpenes and phenols [14,15].

In folk medicine, *P. vera* L. has been used as analgesic, carminative, astringent, stomachic, antitussive, diuretic, healing agent and expectorant [6]. Due to economical value and the ethnobotanical importance of *P. vera* L. oleoresisn, and the lack of studies that validated the wound-healing potential of the resins, the aim of the present study has been to evaluate the wound-healing properties of two oleoresins of *P. vera* collected in Italy and Algeria using an *in-vivo* model and to establish its safety by testing the acute dermal toxicity. In addition, the phytochemical study by combinated GC-FID and GC-

MS analyses allowed correlating the detected pharmacological properties with the most abundant bioactive compounds in order to support its traditional use.

2 Materials and methods

2.1 Plant material

The *P. vera* L. oleoresins were obtained by incisions of the base of the trunk of plants in the early autumn of 2019 in Catania, southern Italy at 37° 47' 19.07" N latitude and 14° 50' 1.57" E longitude, and M'sila situated in the central part of Algeria at 35º12'36.97" N latitude and 4º10' 46.08" E longitude.

2.2 Purification of oleoresin

The oleoresin was dissolved at room temperature in diethyl ether to obtain a limpid solution, which was filtered using filtering paper; finally, the solvent was removed under a vacuum with a rotary evaporator.

2.3 Oleoresin purification, fractioning and analyses

The raw Italian and Algerian oleoresins were subjected to purification and successive fractionation as previously reported [16], to obtain three fractions, namely, essential oils, neutral and acidic fractions, the last one was submitted to methylation. The three fractions have been analysed by gas chromatography (GC) combined with flame ionization detector (FID) and mass spectrometry (MS) as previously reported [16]. The characterization of oleoresin components was carried out by comparison of their retention index, computer matching with NIST libraries and fragmentation patterns with those reported in literature [17-20].

2.4 Formulation of the ointment

The animals were randomly divided into 5 groups (Table 1) of 4 rabbits as follows: first group was untreated (UT), second group (RD) treated with the reference drug Cicatryl-Bio (Pierre Fabre, Paris, France), the third group treated with Ointment Resin Italy 5% (ORI 5%), the fourth group treated with Ointment Resin Algeria 5% (ORA 5%), and fifth group with vaseline (VAS).

2.5 Experimental animals

Twenty New Zealand albino rabbits weighing between (2.0 - 2.7 kg) were obtained from Pasteur Institute of Algeria. All the animals were properly caged and maintained under standard pellet diet and water ad libitum, with 12 hours light and dark cycles. Experiments were carried out in accordance with the European Community Council Directive (86/609/EEC). All protocols used in this study were approved by the Ethical Committee of Directorate General for Scientific Research and Technological Development at Algerian Ministry of Higher Education and Scientific Research (CNEPRU DO1N01UN280120150001).

Approximately 24 h before the experimentations, the dorsum of the rabbits was shaved. Care should be taken to avoid abrading the skin, and only animals with healthy, intact skin should be used.

2.6 Dermal toxicity test

The acute dermal toxicity assay was conducted according to the Organization for Economic Co-operation and Development guidelines [21]. ORI 5% and ORA 5% were applied topically on the back of the animals at an amount of 0.5 g per rabbit. 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 14 days following the topical application. At the application sites, the skin was observed for signs of erythema, edema and local injury.

2.7 Healing activity

The rabbits were locally anaesthetized with xylocaine 2% and a circular piece of full thickness of 2.5 cm in diameter was excised carefully. According to the procedure reported in literature [22] excisional wounds were immediately treated. The different preparations (RD, ORI, ORA and VAS) were applied locally at an amount of 0.5 g per rabbit of the different groups once per day during 16 days [23]. The dimensions of excision wounds were measured by tracing the wounds on transparent paper and measuring the following through the graph paper. The percentage of the evolution of wound contraction was calculated using the following formula [24]:

$$\%\ \text{wound contraction} = [(\text{Initial wound size} - \text{specific day wound size})/\text{Initial wound size}] \times 100$$

2.8 Histological sections

At the end of the experimentation, the rabbits were sacrificed and samples from healed skin tissue were taken from the rabbits of controls and treated groups for histological study [25]. Excised tissues were fixed in formalin (10%) for 72 h. All tissues were processed by using conventional histochemical techniques, embedded in paraffin wax, sectioned, mounted on glass slides, deparaffinized and stained with hematoxylin-eosin [26]. The analysis was performed to confirm the experimental results in terms of concentration of inflammatory cells, collagen and angiogenesis in tissue at 16th post wounding days.

2.9 Statistical analysis of results

The results are represented in the form of means ± SEM for each group. All data processing was done using GRAPH PAD analysis and statistical data processing software. All the grouped data were statistically evaluated and the significance of various treatments was calculated using one-way ANOVA followed by Tukey‘s post hoc test. The results were considered statistically significant at 95% confidence level and P-value <0.05.

3 Results

3.1 Chemical composition

The chemical complexity of *Pistacia* resin has imposed an articulated procedure to determine the most complete chemical composition. A first step has been represented by the characterization of the volatile components, which has been carried out by hydrodistillation, the resulting essential oils have been analysed by a combination of GC-FID e GC-MS allowing the determination of the chemical composition of the volatile components of the two resins as reported in Table 2.

The second step concerned the determination of the not volatile components of resins, in this case appliyng the procedure reported in the experimental section two fractions have been obtained, a first one has been called neutral fraction, while the second one has been called acidic fraction. The last fraction has been submitted to methylation to facilitate the successive analyses. In fact, the neutral and the acidic methylated fractions have been analysed by the same procedure adopted for the essential oil analyses, namely

GC-FID e GC-MS, to obtain the chemical compositions reported in Tables 3 and 4, respectively.

The Table 2 lists the chemical composition of both essential oils, which are characterized by the exclusive presence of monoterpenes, hydrocarbons and oxygenated, as previously reported [27]. The hydrocarbons are the predominant components in the two oils, being α-pinene the main component reaching in the two oils ca. 91 and 93%, respectively, β-pinene is the second component but its amount is only slightly above 1% in both oils (Fig. 1). The oxygenated monoterpenes amount to ca. 5% and 2% in Italian and Algerian resins, respectively. The essential oil from Italian resin shows 19 components, whereas the oil from Algerian resin shows only 12 compounds; this is the more consistent difference between the two resins, being all the components quantitatively below 1%. However, both essential oils show that *trans*-sabinol, α-campholenal and *trans*-pinocarveol (Fig. 1) are the main components of the oxygenated monoterpenes.

The Table 3 shows the chemical composition of the neutral fraction of both resins. The first 18 components belonging to monoterpene hydrocarbons and oxygenated monoterpenes and amounting to *ca.* 44 and 31%, respectively, represent a residue of volatile components which are still present in this fraction. The remaining compounds characterized in the fraction are represented by triterpenes amounting to *ca.* 45 and 49% in the Italian and Algerian resins, respectively. Both resins show a similar composition with 13 components, being hydroxydammarenone, tirucallol, olean-18-en-3-one, and 20,24-epoxy-25-hydroxy-dammaran-3-one the main components (Fig. 1).

The Table 4 reports the composition of the acidic fractions from the two resins. The compositions of the two samples are similar but not identical, in fact, Italian resin shows 7 components for a total amount of *ca.* 79%, Algerian resin shows 8 components amounting to *ca.* 50%. The methylisomasticadienonate and the methylmasticadienononate (Fig. 1) are the main components in both resins, whereas the amounts of the other components present some differences between the two resin samples as reported in Table 4.

3.2 Dermal toxicity test

The animals were observed frequently following the topical application of ORI and ORA during the 14 days. No mortality or signs of toxicity were seen. There were no signs of cutaneous irritation no erythema, eschar, edema or any other reactions on the skin of all animals after topical application.

3.3 Healing activity

The assessment of the evolution of the surface of each wound excision is performed on the treated and untreated animals. The results of wound contraction of all groups are reported in Table 5. The wound contraction surface was highly significant ($P < 0.001$) in all treated groups (RD, ORI 5% and ORA 5%) when compared to untreated and vaseline group. No significant difference was found between the group treated by vaseline and the untreated groups. It is remarkable that there was no significant difference between groups treated with the both oleoresin ointments and the reference drug Cicatryl-Bio. The percentage of contraction for the groups treated by ORA and ORI, showed a comparable wound contraction effectiveness than RD. Cicatryl-Bio showed the highest percentage of wound contraction with 82.54%. This value is very close to that obtained with ORA and ORI (79.80% and 72.58% respectively).

3.4 Histological sections

The granulation tissue section of the UT animals showed lower epithelialization, fewer collagen fibers and more inflammatory cells, and thus showed delayed wound healing processes (Fig. 2.1). The same observations were made for the cuts of the batch treated with VAS, showing lesser collagen formation with a greater macrophage aggregation indicating the incomplete wound healing (Fig. 2.2).

The sections of granulation tissue obtained from the RD treated animals showed a significant rise in collagen deposition and proliferating blood capillaries (angiogenesis) with the presence of a thick mature epithelium with well differentiated cells layers (Fig. 2.3).

Granulation tissue of healed wound ORA 5% and ORI 5% showed very few inflammatory cells, and more collagen fiber, fibroblasts and proliferating blood capillaries (angiogenesis) (Figs. 2.4 and 2.5, respectively). The complete re-

epithelialization provides evidence for the wound healing efficacy of the *P. vera* L. oleoresin

4 Discussion

A wound can lead to serious complications, both in terms of morbidity and mortality. Proper healing of wounds is essential for the restoration of disrupted anatomical stability and functional status of the skin. Rapid wound healing requires fast wound contraction, a shorter epitheliazation period, and adequate gain of tensile strength [28].

In the present study, excisional wound model was used to assess the wound healing effect of Italian and Algerian *P. vera* L. oleoresins. So far, no data have been reported on this plant resin product for wound healing property, knowing that this plant is traditionally claimed in worldwide for its effectiveness in wound healing.

The chemical complexity of the total *Pistacia* oleoresins has been clarified applying the separation procedure previously described, which allowed to define three fractions: volatile components, neutral and acidic fraction. From a chemical point of view all the aforesaid fractions were characterized by the almost total presence of terpenoidic compounds. Monoterpenes hydrocarbons were the main volatile components, tetracyclic and pentacyclic terpenes from the not volatile fractions.

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from a short-term exposure by the dermal route [21]. Assessment of single dermal dose toxicity is an important part of any toxicology program for new pharmaceutical or cosmetic products to be applied on the skin [29]. In our investigation, acute dermal toxicity test showed no signs of toxicity when the resin-based ointments have been locally applied to the backs of rabbits, proving the non-irritant nature of the test samples. Hence, *P. vera* L. oleoresins formulation can be used safely as a topical preparation to treat open wounds. The ointments were formulated by mixing the oleoresins with vaseline for topical application. Vaseline, which is a hydrating and hypoallergenic product, is frequently used as a base ointment for healing wounds by preventing dry skin due to evaporative losses [30].

The results showed that the topical treatment of animals with excisional wounds using *P. vera* resins based-ointment 5% leads to a more rapid repair of wounds similar

with the reference drug treated group and without any toxicity signs on animal skins. The histological data reported that animal skins treated with *P. vera* resins ointment is accompanied by a denser collagen deposition and complete reepithelialization.

In folk medicine, *Pistacia* oleoresisns has been locally applied for wounds and burns [31]. Previous studies explored the effects of the topical administration of different species in the genus *Pistacia* on the wound healing process [32]. The *Pistacia atlantica* oleoresin and hydroethanolic extract improve the burn wound healing process by increasing the angiogenesis, affecting inflammatory phases, upregulating mast cell infiltration, accelerating the proliferation phase, lowering the RNA damage rate and upregulating the hydroxylproline content [31,33]. Also *Pistacia khinjuk* and *P. lentiscus* have proven their effectiveness on the wound healing process in rats and rabbits [10,34,35].

Topical oil from *P. vera* L. promotes the healing process in rats suffering from second-degree burn wounds by repairing epithelium, improving the collage layer in dermis and reducing the wound size [36], whereas essential oils of *P. vera* from the Mediterranean area showed a very strong wound healing activity confirmed by the histological study [27]. A significant wound repair process has been reported for the total extract (MeOH 80%) of *P. vera* L. hulls, which was performed to evaluate the wound healing activity by scratch assay on NIH/3T3 murine fibroblast cells [37].

Many compounds belonging to different chemical classes such as alkaloids, flavonoids, tannins, terpenoids, saponins, and phenolics are involved in wound healing processes [38]. The resins of *P. vera* shows a large presence of terpene derivatives, namely monoterpenes (hydrocarbons and oxygenated) in their essential oils, which have previously been successfully tested as wound healing products [27], and not volatile triterpenes. All these metabolites have a wide range of pharmacological effects, including anti-inflammatory, cardioprotective, neuroprotective, hepatoprotective, nephroprotective, antibacterial, antiviral and wound healing properties too [39-44].

In particular, recent studies show as ursane, oleanane dammarane and masticadiene derivatives, namely tetra and pentacyclic triterpenes, all components present in Algerian and Italian pistacia resins (Tables 3 and 4), are able to accelerate the healing processes of skin ulcers and wounds promoting the synthesis of collagen [45-48].

The treatment with the resins ointments have had a strong impact on the granulation and epithelialization of wounds, accelerating the tissue repair and reducing the duration of this process. This may be due to presence of the triterpenes, which are probably responsible for the modulation of the production of ROS in the wound microenvironment, accelerating the process of tissue repair and inducing cell migration, cell proliferation and collagen deposition.

5 Conclusions

The 5% resin ointments obtained from Italian and Algerian *P. vera* L. showed significant wound healing properties in *in vivo* excisionnal wound model, which were similar to those of the reference drug Cicatryl. Moreover, acute dermal toxicity assessment on albino rabbits indicates that the ointments are potentially safe over a two-week treatment period, which corresponds to a typical application time in the therapy of wounds. Our data confirm the potential of *P. vera* L. oleoresin for the treatment of wounds and support the use of this plant resin in the traditional medicine as a wound healing agent. This healing potential is due to the presence of volatile monoterpenes and triterpenes, which can be considered a group of molecules particularly promising for the development of new drugs to treat skin injury.

Credit author statement

Amel Boudjelal, Giuseppe Ruberto: Conceptualization, Validation, Formal analysis, Writing, Original Draft, Project Supervision; **Edoardo Napoli, Davide Gentile:** Chemical Methodology, Data Collection, Writing Experimental Section; **Abderrahim Benkhaled, Louiza Benazz, Rahina Beja:** Biological Analyses, Data Collection, Writing Experimental Section. All authors have read and agreed to the published version of the manuscript.

Decalaration of Competing Interest

The authors have declared no conflict of interest.

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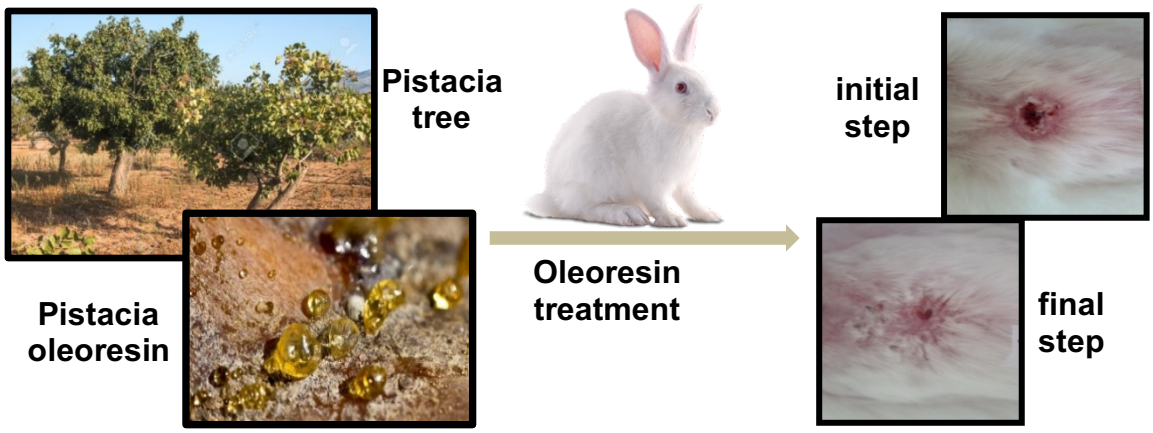
GRAPHICAL ABSTRACT



Highlights

- First report on wound healing activity of *Pistacia vera* L. oleoresin
- The chemical complexity of the total *Pistacia* oleoresins has been established
- Terpenoids are the main components of *Pistacia vera* oleoresins
- *Pistacia vera* oleoresin ointment is a wound healing agent in traditional medicine

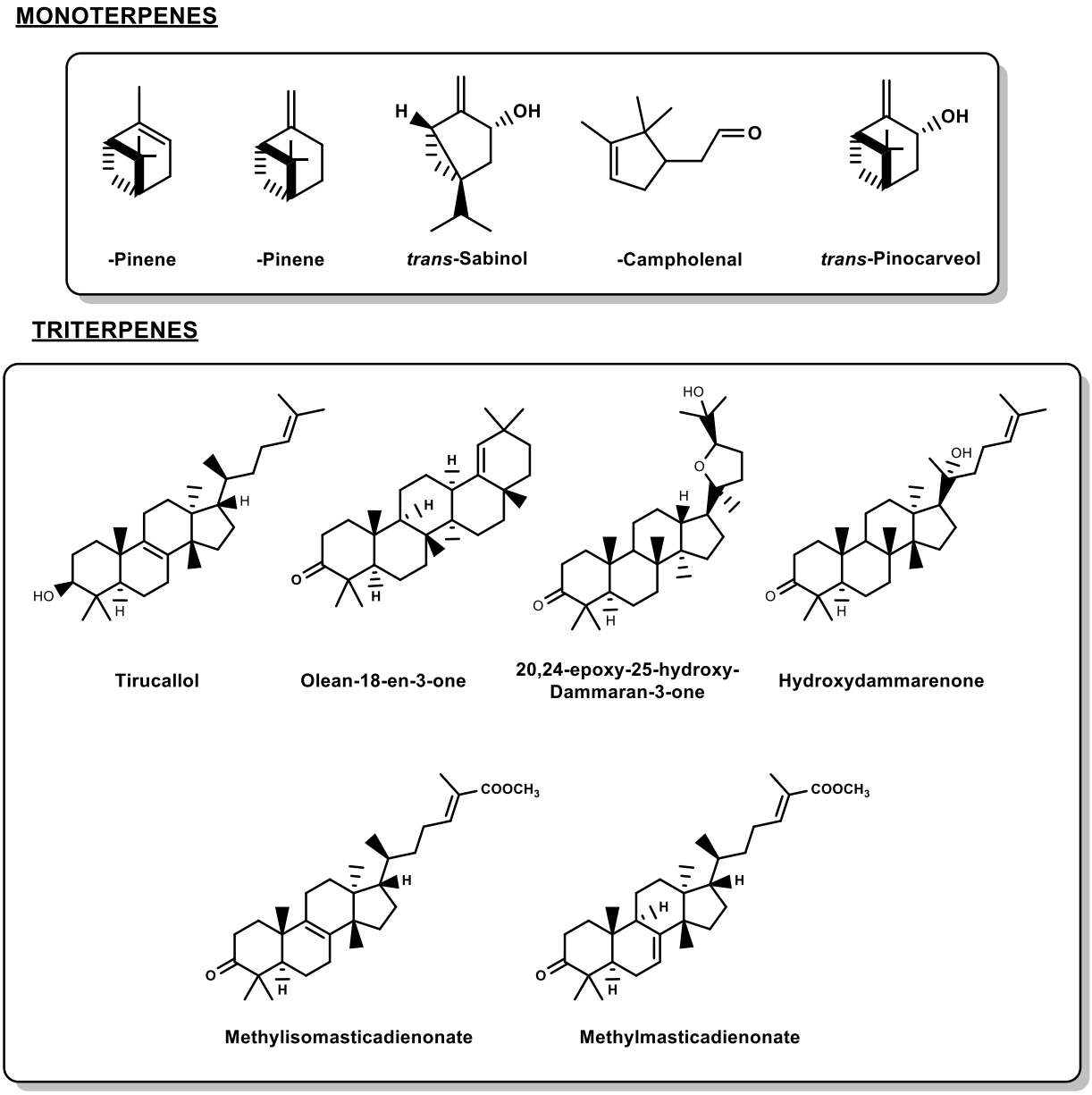


Figure 1. Main terpenoidic components from essential oils, neutral and methylated acidic fractions of Italian and Algerian Pistacia resins.

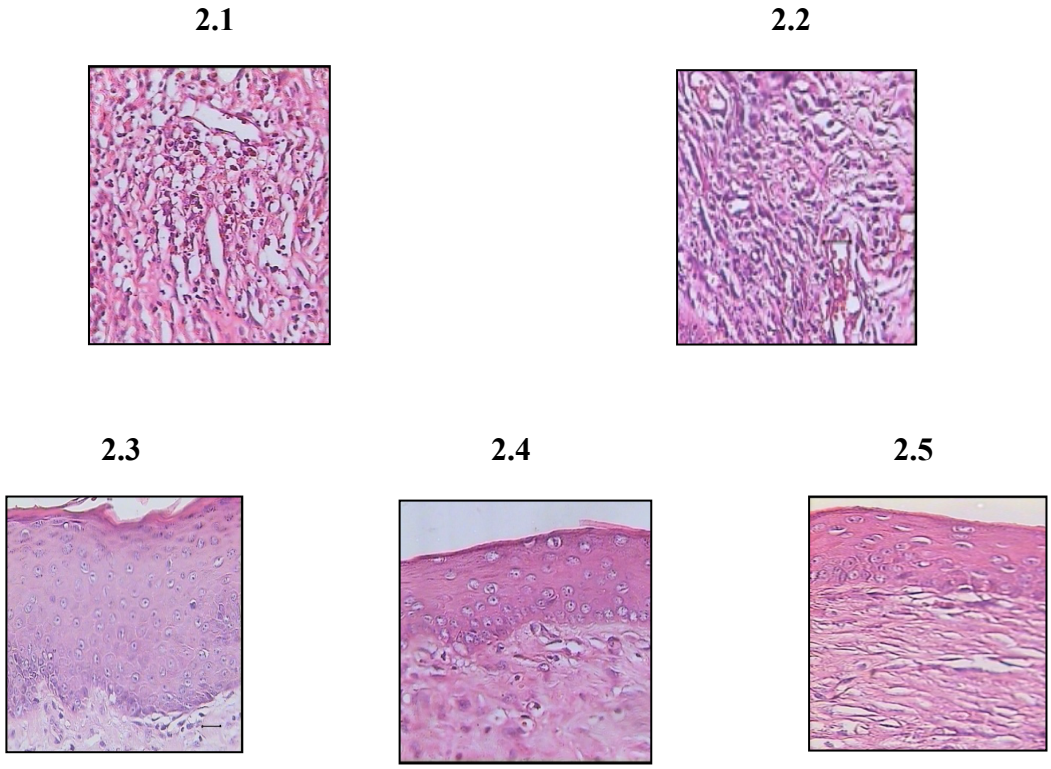


Figure 2. Histological examination of healed excised wound after hematoxylin and eosin staining (40x).

Table 1. Different groups and treatments.

Groups	Treatments
1 UT	Untreated
2 RD	Reference Drug (Cicatryl)
3 ORI 5%	Ointment Resin Italy
4 ORA 5%	Ointment Resin Algeria
5 VAS	Vaseline

Table 2. Volatile components of *P. vera* L. oleoresin.

#[a]	Class/Compound	*% of Italian Essential oil*	*% of Algerian Essential oil*
	Monoterpene hydrocarbons[b]	**94.63**	**96.41**
1	α-Pinene	91.25	93.34
2	Camphene	0.43	0.43
3	Thuja-2,4(10)-diene	0.38	0.29
4	Sabinene	0.06	0.05
5	β-Pinene	1.12	1.26
6	Myrcene	0.05	0.10
7	Δ-3-Carene	0.10	0.13
8	Menthene	0.15	0.15
9	*p*-Cymene	0.46	0.12
10	Limonene	0.31	0.42
11	*cis*-Ocimene	0.05	0.06
12	*trans*-Ocimene	0.07	0.06
14	Menthatriene	0.20	-
	Oxygenated monoterpenes[b]	**4.41**	**1.77**
13	dehydro-Linalool	0.07	-
15	α-Campholenal	0.62	0.36
16	*trans*-Pinocarveol	0.65	0.37
17	*trans*-Sabinol	0.97	0.44
18	*trans*-Pinocamphone	0.08	0.06
19	Pinocarvone	0.08	0.03
20	Mentha-1,5-dien-8-ol	0.50	-
21	Isopinocamphone	0.04	-
22	Terpinen-4-ol	0.09	0.06
23	*p*-Cymen-8-ol	0.02	-
24	*cis*-Pinocarveol	0.01	-
25	α-Terpineol	0.08	0.05
26	Myrtenal	0.33	0.07
27	Borneol	0.04	-
28	Verbenone	0.41	0.04
29	*trans*-Carveol	0.26	0.19
30	Carvone	0.05	0.03
31	Bornyl acetate	0.11	0.07

a) The numbering refers to elution order, and values (relative peak area percent) represent averages of 3 determinations

b) Identification based on retention index (RI) relative to standard mixture of *n*-alkanes on SPBTM-5 column, comparison with mass spectra reported in literature.

Table 3. Composition of neutral fractions of *P. vera* L. oleoresins.

#[a]	Class/Compound	*% Italian neutral fraction*	*% Algerian neutral fraction*
	Monoterpene hydrocarbons[b]	**35.94**	**24.94**
1	α-Pinene	34.87	21.96
2	Camphene	0.17	0.35
3	Thuja-2,4(10)-diene	-	0.20
4	β-Pinene	0.63	1.92
5	*p*-Cymene	-	0.08
6	Limonene	0.27	0.43
	Oxygenated monoterpenes[b]	**8.49**	**5.91**
7	α-Campholenal	0.78	0.32
8	*trans*-Pinocarveol	1.80	1.12
9	*trans*-Sabinol	3.47	2.10
10	*trans*-Pinocamphone	0.07	-
11	Pinocarvone	0.20	-
12	Terpinen-4-ol	0.07	-
13	*cis*-Pinocarveol	-	1.12
14	α-Terpineol	0.08	-
15	Myrtenal	0.45	0.33
16	Verbenone	0.98	0.23
17	*trans*-Carveol	0.44	0.33
18	Bornyl acetate	0.15	0.36
	Triterpenes	**45.26**	**48.85**
19	Unknown 1	1.04	0.87
20	28-Norolean-12-en-3-one	1.15	2.94
21	Tirucallol	7.58	8.52
22	Olean-18-en-3-one	4.21	3.84
23	β-Amyrin	1.86	2.24
24	Unknown 2	1.79	2.12
25	28-Norolean-12,17-dien-3-one	1.88	0.95
26	Olean-18-en-3-ol	1.30	1.18
27	14,17-nor-3,21-dioxo-β-Amyrin-17,18-didehydro-3-dehydroxy	1.40	1.42
28	20,24-epoxy-25-hydroxy-Dammaran-3-one	3.01	3.49
29	Hydroxydammarenone	17.81	17.76
30	Ursolic aldehyde	1.85	1.47
31	Oleanolic aldehyde	0.38	2.05

c) The numbering refers to elution order, and values (relative peak area percent) represent averages of 3 determinations

d) Identification based on retention index (RI) relative to standard mixture of *n*-alkanes on SPBTM-5 column, comparison with mass spectra reported in literature.

Table 4. Composition of acidic fractions of *P. vera* L. oleoresins.

#[a]	Class/Compound	*% of Italian acidic fraction*	*% Algerian acidic Fraction*
	Triterpenes	**78.86**	**50.37**
1	Methyl-2,3,23-trihydroxyolean-12-en-28-oate	-	1.96
2	Moronic acid methyl ester[b]	5.27	3.15
3	Oleanolic acid methyl ester[b]	6.88	6.16
4	Ursolic acid methyl ester[b]	7.35	1.27
5	Methylisomasticadienonate[b]	25.33	16.74
6	Methylmasticadienonate[b]	23.94	13.73
7	Methyl-3-acetoxy-3-epi-isomasticadienolate[b]	2.55	3.47
8	Methyl-3-acetoxy-3-epi-masticadienolate[b]	7.54	3.89

e) The numbering refers to elution order, and values (relative peak area percent) represent averages of 3 determinations

f) Identified with comparison of their retention time and mass fragmentations with those of authentic standard.

Table 5. Effect of different treatments on the evolution of the healing process of excision wounds 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
RD	34.36± 0.42**	59.71± 0.38***	69.39± 0.35***	82.54± 0.17***
ORA 5%	51.18± 0.59**	66.72± 0.33***	76.83± 0.25***	79.80± 0.13***
ORI 5%	23.80± 0.35**	53.34± 0.31***	63.48± 0.37***	72.58± 0.36***
VAS	18.97± 0.18	28.97± 0.17	41.25± 0.31	48.06± 0.13

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

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