Euphorbia honey and garlic: Biological activity and burn wound recovery

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ARTICLE INFO
Article history:
Accepted 6 May 2019
Available online xxx

Keywords:
Antimicrobial activity
Antioxidant activity
Wound healing potency
Euphorbia honey
Allium sativum L.
Synergism

ABSTRACT
Currently, chronic wounds and microbial resistance to antibiotics have led to search new healing agents. Combinations of natural products are widely practiced in traditional medicine and exhibited synergistic activity with increased efficacy in treating several pathologies. This study assays the antioxidant, synergistic antimicrobial and burn wound healing activities of Euphorbia honey and Allium sativum (garlic). The minimal inhibitory concentration (MIC) of each natural product was determined against microorganisms commonly found in wound infections. The synergistic antimicrobial effect was assessed by mixing different concentrations of honey and garlic extract below their relative MICs. Subsequently, the antioxidant activity, total phenolic (TPC) and flavonoid (TFC) contents of both natural products and a selected mixture of them were evaluated. Efficacy of that mixture was also evaluated as topical application on male and female Wistar rats skin burn wound, compared to Euphorbia honey and two conventional treatments. Results showed that the mixture honey-A. sativum has synergistic antimicrobial effect against all tested strains. Besides, A. sativum presented higher antioxidant activity along with higher TPC and TFC compared to honey and their mixture. However, the mixture showed higher wound healing activity reflected by shorter epithelialization and wound contraction time, as well as, better histological recovery of the treated tissues. Our results also showed that burn wound healing is not affected by gender. Our findings support the idea of combining natural products as an effective therapy.

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1. Introduction
For many years, raw products isolated from plants and animals have been used in traditional medicine to treat human ailments. Though, the use of natural medicines decreased with the progress of the synthetic drug industry, many developing countries still rely on traditional medicine as the main source of health care [1].

Chronic wounds represent a considerable burden to patients all over the world and especially in poor countries were the health care is deplorable [2]. Besides, burns are one of the most common and devastating forms of wounds where minor burns are generally treated with topical ointment and dressing while severe burns require immediate specialized care. High rates of morbidity and mortality are due to burn wound complications especially to microbial infections [3].
addition, many of the conventional antimicrobial and wound healing drugs are not accessible or affordable for all populations and are only partially effective [4]. Another fact is that the indiscriminate use of these drugs results in several side effects among which toxic effects, allergy and microbial resistance to drugs. Consequently, this led to the search for new healing agents derived from natural sources. Here, traditional medicine holds a great potential as basis of effective alternative therapy [5,6].

Honey and Allium sativum L. (garlic) are natural products that are widely studied due to bioactive compounds they contain and their remarkable therapeutic properties. Honey is an ancient remedy that is slowly re-introduced into modern medicine [7]. To date, unfortunately, studies on local Algerian honeys mainly those belonging to the steppic regions such as euphorbia (spurge) honey are not fully addressed.

Euphorbia honey is largely used in traditional medicine of many Mediterranean countries and is particularly effective to treat asthma, sore throat, cardiovascular diseases, hypertension and favors fertility in women. Due to its antimicrobial, anti-inflammatory and antioxidant activities, boosting effect on immune system, debridement action and stimulating role in wound regeneration, Euphorbia honey contributes significantly in wound healing processes [8]. Biological activities of honeys are due to many factors including acidity, hydrogen peroxide content, osmosality and phytochemical components. Escurodo et al. [9] reported about 200 substances in honey which consists mainly of sugars, water, and other substances such as proteins (enzymes), organic acids, vitamins (especially vitamin B6, thiamine, niacin, riboflavin and pantothenic acid), minerals (potassium, calcium, sodium, magnesium, phosphorus, copper, iron, zinc and manganese), pigments, phenolic compounds, a large variety of volatile compounds, and solid particles derived from honey harvesting.

Furthermore, A. sativum L. has been used for centuries worldwide to treat infectious diseases and it has been known to have antioxidant and antimicrobial activities [10]. Pasteur was the first to demonstrate the antibacterial activity of raw A. sativum extract [11]. It contains carbohydrates, dietary fibers, several enzymes, vitamins, minerals (higher concentrations of potassium and calcium and other minerals such as sodium, zinc, copper, iron, selenium, germanium, magnesium and manganese), polyphenols, carotenoids and at least 33 sulfur compounds (e.g. ajoene, allicin, allin, allyl sulfides, allyl disulfides, allyl trisulfides, cysteine, cyacalilin, cysteine sulfoxides, cystine, diallyl sulfides, dimethyl sulfides, glutathione, disulfides, methionine, methyl sulfides, sulfanes, pseudocordine, thiosulfinates, scordine, trisulfides and tetrathial) that are responsible for garlic pungent odor and its antioxidant, therapeutic, antibacterial and anti-carcinogenic effect [12,13]. Huzafa et al. [14] demonstrated the presence of alkaloids, flavonoids, saponin, tannins and cardiac glycosides in A. sativum bulbs. These phytochemicals are known to have several therapeutic properties. Besides, studies showed that allicin is the active component of A. sativum and has been shown to have many potential targets [15].

For many years, phytobotanical and ethnobotanical research have focused on a single active compound they can extract, purify and apply from plants. Nevertheless, traditional medicines believe that a synergy of all ingredients of the plants yields the maximum of therapeutic efficacy [16]. In fact, the use of combinations of drugs and several plant extracts is widely applied in therapy and had exhibited enhanced antimicrobial efficacy with a reduction in the amount of each product used. This can reduce the risk of possible side effects and treatment costs [17]. As well, the combination of several plants’ extracts can enhance the antioxidant activity [18].

In this context, this study aims to assay the antimicrobial activity of Euphorbia honey and A. sativum separately then to determine the synergistic effect of their combination against selected common pathogenic microbial strains often involved in wound infections. The antioxidant activity along with the total phenolic and flavonoid contents of both natural products as well as of their mixture will be determined. The burn wound healing potency of the identified synergistic mixture will also be evaluated at the macroscopic and histological levels and compared between males and females Wistar rats to determine whether animal gender affect the process of wound healing or not. Our results are expected to give a scientific dimension to the traditional use of such combinations widely used by the local population in Algeria.

2. Material and methods

2.1. Honey

Euphorbia honey used in this study was purchased from a local known apiculture in the steppe region of Tiaret; an upland area in the Tell Atlas located about 100 miles inland from the Mediterranean seacoast of Algeria. Euphorbia honey was identified by specialists in the department of Agronomy and Food Technologies at the University of Tiaret (Algeria).

2.2. A. sativum L

A. sativum L. purchased from the local farmers (Tiaret, Algeria) was identified and authenticated by botanists. The bulbs were peeled and pressed with a garlic press then squeezed using sterile cheesecloth to retrieve the extract.

2.3. Microbial strains and inoculum preparation

Four microbial strains were used in this study and were obtained from the University Hospital Centre, Mustapha Pacha, Algiers (Algeria). These are Staphylococcus aureus (ATCC 33862), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853) and Candida albicans (clinical isolate). The inoculum suspensions were prepared in sterile saline from 24-h-old cultures with a density adjusted to 0.5 McFarland turbidity standards.

2.4. Experimental animals

Adults Wistar rats weighing between 170-200 g, were used in this study to investigate the effect of the assayed natural products on burn wound healing. They were obtained from the Pasteur Institute of Algiers (Algeria) and were kept in standard stainless-steel cages and maintained in the animal house under controlled laboratory conditions with free access to

Please cite this article in press as: L. Ait Abderrahim, et al., Euphorbia honey and garlic: Biological activity and burn wound recovery, Burns (2019), https://doi.org/10.1016/j.burns.2019.05.002
water and food ad libitum (Principles of Laboratory Animal Care NIH publication no, 85-23, revised 1985).

2.5. Antimicrobial activity

2.5.1. Minimal inhibitory concentrations (MIC) of Euphorbia honey and A. sativum

Agar dilution method was used to determine the MIC of the two natural products following the method described by Ait Abderrahim et al. [19]. Briefly, concentrations of honey and A. sativum raw extract were incorporated separately into Mueller-Hinton agar media. The plates were inoculated with the standardized inoculum and incubated at 37 °C for 24 h. The MIC is described as the lowest concentration of the antimicrobial agent that inhibits the visible growth of the tested strains. Experiment was replicated five times to confirm the reached MIC value.

2.5.2. Synergistic antimicrobial activity of the mixture honey-A. sativum

To demonstrate whether there is synergy between the two ingredients, the isobole method of Berenbaum [20] was used. An isobole is a line or curve between points representing the same effect (MIC values of the product). The x and y axes reflect the concentrations of the single individual components. Different concentrations combinations are investigated for the same antimicrobial effect. According to Berenbaum [20], the zero or additive interaction means that the effect of two substances is a pure summation effect and is reflected by a line. The antagonistic interaction is a reduced effect of two substances and is represented by a convex curve. However, synergism refers to the increased effect of two substances when they are combined compared to the separate effects, the result is then a concave curve (Fig. 1) [20].

Briefly, after determining the MICs of honey and garlic extract separately, an isobole representing a concave curve simulating synergy between the two products was drawn by taking, as points, different values of concentration below the MICs of Euphorbia honey (x axis) and garlic extract (y axis). Those values were used to assay the synergistic antimicrobial activity of the mixtures honey-A. sativum. Different concentrations of the mixture were incorporated into Mueller Hinton media. Plates are inoculated and incubated at 37 °C for 24 h. The mixtures were tested against the same pathogens as described above. Synergism was identified when the mixtures show inhibition of microbial growth.

2.6. Antioxidant activity

The antioxidant activity was assayed on a selected mixture honey-A. sativum that inhibits all the tested strains with minimal ratio. Hence, a ratio 2.8 (v/v) of the mixture honey-A. sativum was selected. DPPH radicals scavenging activity, total phenolic and total flavonoid contents of the mixture were determined as described below and compared to those of Euphorbia honey and A. sativum extract apart:

2.6.1. DPPH radicals scavenging activity

DPPH radicals scavenging activity was assayed as described by Kumar et al. [21]. Briefly; to 1 ml sample, 5 ml of methanol solution of DPPH (33 mg/l) is added. Absorbance is read at 517 nm after 30 min. DPPH radicals scavenging activity is expressed as mg ascorbic acid equivalent (AAEq/g extract). The percentage DPPH radicals’ inhibition of the test samples is calculated following the equation:

$$\text{DPPH scavenging effect} = \frac{(A0-A1)}{A0} \times 100$$

where, A0 is the absorbance of the blank and A1 is the absorbance of the test.

The actual decrease in absorption induced by the test is compared with the positive control (ascorbic acid). The IC50 (concentration inhibiting 50% of DPPH radicals) values is used to compare between the natural products and ascorbic acid.

2.6.2. Total phenolic content

The method described by Nazari Formagio et al. [22] was used to measure total phenolic content (TPC). Briefly, 750 µl Folin Ciocalteu-water (1:14) solution is added to 50 µl of sample and incubated 3 min. 200 µl sodium carbonate (20%) are added. The absorbance is read at 760 nm after 30 min. The total polyphenol content is calculated from the calibration curve and the results expressed as mg gallic acid equivalent (GAEq/g extract).

2.6.3. Total flavonoid content

To assay total flavonoid content (TFC) the method described by Acharya et al. [23] was followed. Briefly, 3 ml of methanolic AlCl3 is added to 3 ml of sample. The absorbance is read at 430 nm after 10 min, using distilled water as a blank. Total flavonoid content is expressed as mg quercetine equivalent (QEeq/g extract).

2.7. Burn wound healing study

The experimental protocol used in this study complies with the ARRIVE (animals in research: reporting in vivo experiments) guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All care was taken to minimize the suffering of the animals. The experimental protocol was approved by the National Ethics Committee.

The wound healing activity was tested on males and females Wistar rats, to determine whether or not the gender

Fig. 1 – Isoboles of the different types of interaction according to Berenbaum [20].
affects the burn wound healing process, according to Pirbalouti et al. [24]. Animals from each sex were divided into four groups in which the healing effect of the mixture was compared to that of Euphorbia honey and two conventional treatments (betadine solution and silver sulfadiazine ointment); the first group received Euphorbia honey, the second group betadine solution, the third one silver sulfadiazine while the fourth receives the mixture Euphorbia honey-A. sativum extract. The rats were anesthetized with an intramuscular injection of ketamine (85 mg/kg) and xylazine (10 mg/kg) [25] and then shaved on the dorsum. Square metals of 1 cm² were used to induce burn wounds. The metals were heated in boiling water during 10 min and then applied 20 s on the dorsal part of the rats. Each piece of the induced burn wound received a specific treatment that was renewed daily the first week then every other day until complete healing of the wound.

2.7.1. Wound epithelialization and contraction
Period of wound epithelialization was calculated as the number of days required for the scar to fall off leaving no raw wound [26]. The mean number of days required for wound epithelialization for each group was calculated. Besides, wound contraction was measured daily by reporting the surface area of each wound on transparent acetate [27].

2.7.2. Histological and morphometrical analyses
In order to reduce as much as possible animal testing, histological analyze was limited to the study of samples that have already demonstrated enough interest in this experiment. The tissue specimens of scarred skin were collected at the end of the epithelialization process and fixed in a 5% formaldehyde solution and underwent paraffin inclusion. Histological sections of 5 µm width were performed with the microtome. The paraffin sections were stained by Hematoxylin-Eosin stain for morphological changes and Masson’s trichrome protocol for demonstration of skin collagen. The microphotographs were taken from the generated set of slides with a microscope Leica DMI 3000 (Leica Microsystems, Wetzlar, Germany) and subsequently analyzed using Image-Pro Plus 7.0 (Media Cybernetics, Silver Spring, MD, USA).

Quantitative morphometric studies were performed on the obtained microphotographs in order to evaluate epidermis and dermis thickness, interdigititation index and collagen organization using Fourier transform. At least 5 captions were taken for each case and each parameter, following a method of semi-randomization which excluded altered zones. The magnification of the microphotographs was adjusted individually for each parameter. The mean for each image was calculated and, later, the mean of the case from the values of the 5 images as described by Marcos-Garcés et al. [28].

The interdigititation index was investigated with the above-mentioned computerized image analyzing system. The mouse cursor was led along the interdigititation border, the computer measured its length and calculated the distance between the starting point and end point. From these data the interdigititation index was calculated as described by Timár et al. [29]. Briefly, the length of the line following the interdigititation between two points on the border between the epidermis and the dermis was divided by the length of a straight line between the same two points. Results demonstrating an elevation in interdigititation index might indicate some rejuvenation effect the treatment.

Collagen orientation in the dermis was measured in 20× magnification micrographs following the methodology validated by van Zuijlen et al. [30]. This technique applies the Fourier transform to the image and, over the resulting 2D power plot the length and the width of the central figure are measured. The relation between both data is representative of the orientation of the fibres: values which tend to 0 indicate higher orientation, whilst values tending to 1 define disorientation of the collagen fibres.

2.8. Statistical analysis
All the experiments were replicated at least five times and quantitative data were subjected to analysis of variance. Comparison between groups was carried out using the test of Duncan. The significant differences between means were determined at p < 0.05 level. In all cases, data were examined for normality and homogeneity of variances and identified for any violations of assumptions. The correlation between the measured variables was performed on the basis of the coefficient of Pearson. Synergistic antimicrobial effect of Euphorbia honey-A. sativum extract mixtures on the tested microorganisms was fitted using the polynomial regression model. Results were graphically presented for each variable as subjected means ± standard of deviation.

3. Results

3.1. Antimicrobial activity

3.1.1. Euphorbia honey and A. sativum MICs
The MICs of Euphorbia honey varied from 6 to 22% on the tested strains. However, they ranged from 2 to 4% for A. sativum extract. S. aureus, the Gram positive bacteria, was more sensitive to honey when compared to the tested Gram negative bacteria (E. coli and P. aeruginosa). The yeast C. albicans appeared to be more resistant to Euphorbia honey. On the other hand, S. aureus, E. coli and C. albicans had the same sensitivity to garlic extract while P. aeruginosa seemed, relatively, a bit more resistant to the extract (Table 1).

3.1.2. Synergistic antimicrobial activity of the mixture Euphorbia honey-A. sativum
The different concentration combinations of Euphorbia honey and garlic extract, tested to assay the synergistic antimicrobial

<table>
<thead>
<tr>
<th>Strains</th>
<th>Euphorbia honey (µg/ml)</th>
<th>A. sativum extract (µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>E. coli</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>C. albicans</td>
<td>22</td>
<td>2</td>
</tr>
</tbody>
</table>
activity, inhibited all the tested microorganisms (Table 2). The graphic illustration of the tested concentrations of the mixtures exhibits concave isoboles which demonstrates the synergy between these two natural products as defined by Berenbaum [20] (Fig. 2).

3.2. **Antioxidant activity**

3.2.1. **DPPH scavenging activity**

Analysis of variance demonstrated a significant statistical difference between the tested natural products and their mixture in term of DPPH free radical scavenging activity (p-value < 0.001***). *A. sativum* extract presented the higher free radical scavenging activity expressed by a high equivalent amount of ascorbic acid (0.34 ± 0.03 mg AAEq/g extract) and low IC50 (0.013 mg/ml) comparing to Euphorbia honey (0.24 ± 0.04 mg AAEq/g extract and IC50 = 0.027 mg/ml) and to the mixture of both products (0.13 ± 0.01 mg AAEq/g extract and IC50 = 0.076 mg/ml) (Fig. 3A and B).

3.2.2. **Total phenolic content**

The total phenolic content differed between the tested products (p-value = 0.001***). *A. sativum* extract presented the higher amount of polyphenols (1.12 ± 0.035 mg GAeq/g extract) however, the statistical test of homogeneity between groups did not show significant difference between Euphorbia honey (0.54 ± 0.11 mg GAeq/g extract) and the mixture (0.76 ± 0.16 mg GAeq/g extract) (Fig. 3C).

3.2.3. **Total flavonoid content**

Significant statistical differences were also observed regarding the total flavonoid content between Euphorbia honey, *A. sativum* and the mixture of both products (p-value < 0.001***). *A. sativum* extract presented higher flavonoid content (4.93 ± 0.04 mg Qeq/g extract) than Euphorbia honey (0.77 ± 0.03 mg Qeq/g extract) and the mixture (1.04 ± 0.02 mg Qeq/g extract) (Fig. 3D).

Significant correlations were found between the tested variables. For all products, the DPPH radicals scavenging

<table>
<thead>
<tr>
<th>Table 2 - Concentrations of the mixture Euphorbia honey- <em>A. sativum</em> tested for the synergistic antimicrobial activity (v/v).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
</tr>
<tr>
<td>Euphorbia honey (%)</td>
</tr>
<tr>
<td><em>A. sativum</em> extract (%)</td>
</tr>
<tr>
<td>Microbial inhibition</td>
</tr>
</tbody>
</table>

Fig. 2 - Synergistic antimicrobial effect of Euphorbia honey- *A. sativum* extract mixtures on the tested microorganisms. Asterisks (*) represent the tested combination of both natural products concentrations. Discontinuous black line (---) represents the curve obtained by connecting all the tested concentrations combinations showing antimicrobial effect. Continuous red line (--) represents the curve tendency using polynomial regression model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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activity was positively correlated with the total phenolic ($r=0.69^*$) and flavonoid contents ($r=0.93^*$). In addition, the total flavonoid content was significantly correlated with the total phenolic content ($r=0.89^{**}$, $p<0.01^{**}$).

3.3. **Wound healing activity**

3.3.1. **Wound epithelialization**

Results show that there is no significant difference between males and females in the time period required for epithelialization (p-value $>0.05$). The shorter time needed for full epithelialization was observed on induced burn wounds treated by the mixture. In addition, treatment by silver sulfadiazine reduced epithelialization time significantly compared to Euphorbia honey alone. However, burn wounds treated by the betadine solution were those epithelialized the latest (Table 3, Fig. 4).

3.3.2. **Wound contraction**

The inflammatory phase begins after inducing burn wounds under all treatments. The most important inflammation response is observed on burn wounds treated with honey and betadine solution in males, and in burn wounds treated with honey in females. This response is reflected by larger wound surface. However, the lower inflammation response was observed on burns treated by the mixture and silver sulfadiazine both in males and females. In addition, the contraction begins around the 8th day in wounds treated with silver sulfadiazine and the mixture and around the 14th day in wounds treated with honey and betadine solution for both genders. Total wound healing is achieved at the 24th day in males and females treated by the mixture, silver sulfadiazine and Euphorbia honey while burn wounds treated with betadine solution achieved total healing at the 28th day (Fig. 5).

**Table 3 – Epithelialization period (day) of the induced burn wounds in Wistar rats.**

<table>
<thead>
<tr>
<th></th>
<th>Silver sulfadiazine</th>
<th>Betadine solution</th>
<th>Euphorbia honey</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>23.66 ± 2.31$^{ab}$</td>
<td>28.33 ± 0.71$^c$</td>
<td>25.66 ± 1.41$^b$</td>
<td>22.33 ± 0.71$^a$</td>
</tr>
<tr>
<td>Females</td>
<td>24.33 ± 0.57$^{ab}$</td>
<td>29 ± 1.41$^c$</td>
<td>26.66 ± 2.12$^b$</td>
<td>23.33 ± 0.71$^a$</td>
</tr>
</tbody>
</table>

Alphabetic letters indicate the homogeneous groups of treatments for each sex, where (a) representing the groups that presented the shortest period of epithelialization and (c) representing the longest period.

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Consequently, these results show that Wistar rat gender did not affect the process of burn wound healing. Besides, wounds treated with the mixture and silver sulfadiazine showed mild inflammatory response and rapid wound healing compared to those treated by honey alone or betadine solution.

3.3.3. **Histological and morphometrical study**

Comparison of the histological morphology between normal and scarred skin treated by the different tested substances was performed on the basis of epidermis and dermis thickness, the interdigitation index and the orientation of collagen fibers by the Fourier transform (Table 4 and Fig. 6). The thickness of the epidermis was determined through 15 representative measurements performed on each of the micrographs taken at 10× magnification. However, the thickness of the dermis was determined in each of the micrographs taken at 5× magnification. The thickness of both epidermis and dermis varied depending on the treatment.

The obtained results demonstrated that samples treated with betadine and honey in male and female did not differ significantly in terms of dermis thickness. However, samples treated with the mixture honey-garlic extract presented thinner dermis.

Moreover, epidermis thickness did not differ significantly in males between samples treated with betadine and the mixture while wounds treated with honey showed significantly thinner epidermis. In females, however, the wounds treated with honey and the mixture presented significantly thinner epidermis in comparison with samples treated by betadine.

Regarding the interdigitation index, no significant difference was noted between the three treatments applied on male and female. Furthermore, there was no significant difference
in Fourier transform values between males and females which indicates that there is no difference among the different treatments regarding the orientation of the collagen fibers (Fig. 6).

4. Discussion

The side effects engendered by the irrational use of chemical and synthetic drugs led the balance to incline toward the use of natural ingredients and medicinal plants [31].

This study investigates the biological activity of the mixture of two natural products widely used in traditional medicine Euphorbia honey and A. sativum in term of synergistic antimicrobial effect, antioxidant activity and burn wound healing capacity in comparison to conventional treatments (sulfadiazine ointment and betadine solution).

Regarding the antimicrobial activity, A. sativum, when tested alone, exhibited better MIC values against the tested microbial strains compared to Euphorbia honey. All the MIC values of A. sativum were comparable for S. aureus, E. coli and C. albicans except for P. aeruginosa that exhibited a bit higher MIC. Al Masaudi and Albureikan [32] demonstrated the antimicrobial activity of garlic extract against the same strains tested as well on methicillin-resistant S. aureus among others. Feldberg et al. [33] showed that allilcin is responsible for the antimicrobial activity of A. sativum extracts by interrupting and partially inhibiting DNA and protein synthesis as well as by inhibiting RNA synthesis. Other sulfur compounds such as diallyl disulfide, S-allylcysteine and diallyl trisulfide also have been shown to have antimicrobial activity [34]. The variability in the structure of microbial strains may also play a role in the vulnerability to garlic components. Garlic may inhibit cell wall synthesis by inhibiting transpeptidation enzymes involved in the cross-linking of the polysaccharide chains of bacterial cell wall and also activates lytic enzymes [32]. Joe et al. [35] also reported that the antimicrobial properties of garlic are due to the inhibition of succinic dehydrogenase.

Furthermore, our results revealed that Euphorbia honey had an antimicrobial activity against all the tested microorganisms. In fact, several studies have demonstrated the antimicrobial activity of different honeys on numerous bacteria including, anaerobes, Gram-positive and Gram-negative bacteria [36] and fungi such as C. albicans [37,38]. Wasihun and Kasa [39] also found that honey inhibited the growth of multidrug resistant S. aureus, E. coli and P. aeruginosa. Our study revealed that the bacteria tested were more sensitive to Euphorbia honey, at lower MIC, than the yeast C. albicans. Possible reason for these variations in response to honey might be due to difference in the cellular organization of the microorganisms [39]. Resistance can be due to low permeability of the cell wall, genetic capacity to express resistance and/or mutation in chromosomal genes that regulate resistance genes [39]. S. aureus showed the lowest MIC value compared to the Gram-negative bacteria E. coli and P. aeruginosa. This result agrees with that of Molan [36] who showed that S. aureus is one of the most bacterial species sensitive to the activity of honey. The enzymes found in honey play an important role in its antibiotic properties. Some of these convert the complex sugars to glucose forming a super-saturated solution with high osmolality that inhibits bacterial growth [8]. Glucose oxidase converts glucose to gluconic acid, which gives honey its low pH and hydrogen peroxide that has antimicrobial action [40,41]. As well, methylglyoxal was found to be responsible for the antibacterial action of honey. The botanical origin also plays an important role in the antimicrobial activity. Honey acquires characteristics from plants that bees have visited and fed on. The main sources of honey phenolic compounds are plants. Depending on the floral origin honey may possess higher or lower antioxidant, antibacterial, or radical-scavenging activity [42]. Researchers isolated several phytochemical compounds with therapeutic properties from plants belonging to the Euphorbiaceae family, mainly alkaloids, cyanogenic glycosides, phenolics, diterpenes, glucosinolates, coumarins, seed and other lipids, tannins, and triterpenes. In addition, the genus Euphorbia was shown to be extremely rich in polyphenolics [43]. Phenolics are responsible of the antimicrobial action; it has been related to their ability to denature proteins [44]. Polyphenols also act against microorganisms either by chelating metal ions needed for microbial growth, or by non-specific interactions with cell wall proteins or microbial extracellular enzymes [45].

In the herbal medicine context, synergy can mean that the combined effect of a number of herbal components is greater than the sum of each of the individual components [46]. Throughout our study, the combination honey and garlic extract has proven to be effective antimicrobiol agent against the microbial strains investigated. Our results are in concordance with those of Andualem [12] who demonstrated the synergic antibacterial effect of Tenegh honey and garlic mixture against E. coli, Salmonella typhi, S. aureus and Streptococcus pneumonia among others.

Several studies have tested combinations of honey and various substances, including conventional antibiotics [7,17], plant extracts [47], royal jelly [48] and propolis [49]. Although various combinations of honey and other compounds have been tested, the effect of these combinations on
microorganisms is still not well understood. Wagner and Ulrich-Merzenich [50] and Ulrich-Merzenich et al. [16] explained the synergistic effect by several mechanisms: (a) synergistic multi-target effects; where constituents of a monoeffect or a multi-extract combination affect several targets (enzymes, receptors, proteins, DNA/RNA . . . ). (b) Pharmacokinetic and physiochemical effects; where a particular concomitant compound in an extract, often without particular pharmacological effects alone, may increase the solubility and/or the resorption rate of main components in the extract.

Fig. 6 – Histological structure of Male and Female rat Wistar skin. (a) Thickness of the epidermis and dermis stained with H-E (15 representative measurements performed on each of the micrographs taken at 20 × magnification). (b) Disposition of collagen fibers in the dermis stained with Masson’s trichrome (magnification 20 ×). (c) Scatter plot of the collagen orientation in the dermis with the Fourier transform. The thickness of both epidermis and dermis varied depending on the treatment. There were no significant differences regarding the orientation of the collagen fibers.
and thereby enhance its bio-availability and simultaneously result in a higher efficiency of the extract. (c) Interactions of agents with resistance mechanisms of bacteria; occur when antibiotics are combined with natural products that are capable to partially or completely suppress bacterial resistance machinery.

Regarding the antioxidant activity, garlic extract presented the higher antioxidant activity compared to Euphorbia honey and their mixture. This antioxidant activity was significantly correlated with the amounts of total phenolics and flavonoids. Previous studies have demonstrated the direct correlation between antioxidant activity and phenolic content [51]. Moreover, selenium and organosulfur compounds found in garlic have been shown to be responsible of the antioxidant activity [52]. High levels of alliin, allyl cysteine, allyl disulfide and allicin found in A. sativum are powerful antioxidant agents [53]. Other bioactive compounds such as dietary fibers and other microelements contribute to this activity [54]. Besides, total phenolic content did not differ between Euphorbia honey and the mixture however, the total flavonoid content and the antioxidant activity were significantly higher in Euphorbia honey which leads to conclude that the difference in the antioxidant activity between honey and the mixture was mainly attributed to the amount of total flavonoid. Similar results were reported by Ceksteryte et al. [55]. In addition, the phytochemicals found in honey also serve antioxidant and antimicrobial functions [8].

This study, reported for the first time the burn wound healing activity of Euphorbia honey and the mixture Euphorbia honey-A. sativum L. compared to silver sulfadiazine and betadine solution in terms of wound epithelialization, contraction time and histological recovery. Results showed that the mixture of these natural products and silver sulfadiazine induced a shorter epithelialization and contraction time period in comparison to betadine solution and Euphorbia honey in both males and females. The histological morphometric studies revealed also a comparable histological structure to normal skin with higher interdigitation index indicating some rejuvenation effect of the mixture.

In this study, the inflammatory response was more pronounced in wounds treated by honey and betadine solution. It is well known that microbial infections as well as oxidative damage extend the inflammatory phase [56]. Studies have reported that many molecules such as vitamins (C, E, and A), glucose, amino acids, antioxidants, alkaloids, fatty acids, proteins, water and zinc found in natural products enhance the process of wound healing [57,58]. In fact, researchers reported that the antioxidant substances play an important role in the healing process. They are believed to protect protease inhibitors from oxidative damage. They counter the excess proteases and reactive oxygen species (frequently formed by neutrophil accumulation in the wound-ed area in the inflammatory phase) that destroy fibroblasts and other cells as well render skin lipsids less supple [5]. The presence of trace elements such as Zn, Fe, Cu, Mg, Co and Mn modulates integrin expression during reepithelialization promoting keratinocyte proliferation [59]. As it was shown previously, the tested mixture and honey demonstrated antioxidant and antimicrobial activities. This can explain why the burn wounds treated with these two natural products showed remarkable results at wound epithelialization and contraction times with good histological structure recovery. As a dressing on wounds, honey provides a moist healing environment, prevents infections, deodorizes, and reduces inflammation, edema, and exudation. Honey can stimulate and modulate the immune system it plays both pro-inflammatory and anti-inflammatory action [59]. It increases the rate of healing by stimulation of proliferate phase [57]. The acidic nature of honey stimulates the formation of granulation tissue by releasing oxygen from the hemoglobin. Development of capillaries can be stimulated by hydrogen peroxide contained in honey as well as the growth and proliferation of fibroblasts and epithelial cells in wound tissue. Besides, A. sativum with its antioxidant and antimicrobial properties could improve the healing process. The mixture and Euphorbia honey may increase wound contraction by enhancing the activity of fibroblasts and eliminating microorganisms in the inflammatory phase allowing wound contraction.

Besides, the wound healing action of the mixture was more effective than honey alone; this may probably be due to the constituents present in both products; a function of either the individual or the synergistic effects of the constituents of both products. These results are in accordance to those of Sidik et al. [60] who demonstrated that wound healing was accelerated when garlic extract is combined with honey and proved to be more effective than honey alone. Furthermore, even though the sulfadiazine showed a good effect, studies reported that the use of some treatment for skin injuries such as betadine solution, saline and antibiotic ointments as sulfadiazine led to side effects such as toxic effects on fibroblasts, lymphocytes and cells, allergy and microbial resistance [5,4]. In fact, studies have shown that even though silver sulfadiazine is an effective antibacterial agent, the topical application of this product on skin wound healing is highly cytotoxic to human dermal fibroblast cells leading to impaired dermal regeneration in addition to a decreased mechanical strength of dermal tissue [61].

Concerning gender influence, Viguié [62] reported a difference between male and female mice in brain damage healing which indicates that animal gender can influence the biological response and can even induce changes in the genes. Researchers attributed this heterogeneity to the sexual hormones mainly oestrogen since it exerts an antioxidant effect. Bellanti et al. [63] reported that oestrogen level is positively correlated with plasma antioxidant capacity and antioxidant enzymes expression. Nevertheless, results obtained throughout our study showed no significant difference in burn wound healing between male and females Wistar rats.

5. Conclusion

This study provides an alternative natural treatment to the commonly used drugs in treating wounds and microbial infections. Euphorbia honey and A. sativum proved to be effective antimicrobial agents separately and in combination assuming a synergistic antimicrobial action. However, the antioxidant activity was higher in A. sativum than honey and their mixture. Besides, significant correlations were found between antioxidant activity, total phenolic and flavonoid
contents and antimicrobial activity. Moreover, the mixture honey-A. sativum showed an enhanced wound healing poten-
cy compared to Euphorbia honey and the conventional treatments. It should be noted that burn wound healing was
not affected by rats gender.

Apart from their availability and the low amount used of each product, our results offer the advantage of an increased
antimicrobial and wound healing efficiency without side effects and at low costs. Combination of natural products
could lead to a new spectrum of therapeutic even preventing microbial drug resistance. Further studies are needed to
determine the underlying mechanism of synergistic action and how they interfere with wound healing process.

Conflict of interest

None.

Acknowledgments

Authors gratefully acknowledge Dr. M. Teresa Sagrado and Pr. Javier José Boix from the department of Pathology, Faculty of
Medicine and Odontology, University of Valencia (Spain) for their help and technical assistance concerning histological studies.

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