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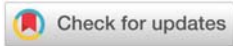
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Research Article

Antibacterial activity of honey and *Nigella sativa* L. seed extracts against animal wound bacteria

Abstract

This study was designed to evaluate the antibacterial activity of Algerian honey and some extracts of *Nigella sativa* L. seeds against animal wound bacteria. To do this, the preparation of *Nigella sativa* L. seed extracts was carried out by macerating the seed powder in increasingly polar solvents (ethyl acetate, ethanol and methanol). The antibacterial activity of honey and *Nigella sativa* L. seed extracts against the bacterial isolates was studied by Agar Well Diffusion Assay. The results showed that the different honey concentrations (20%, 50%, 70% and 100% v/v) were found to be active against all the bacterial isolates. The different *Nigella sativa* L. seed extracts showed a variable activity at the concentration 300 mg/mL against the bacterial isolates. Ethanolic extract of *Nigella sativa* L. seeds was found to be active against all the bacterial isolates. Methanolic extract of *Nigella sativa* L. seeds was found to be active against all the bacterial isolates except *Escherichia coli*. Also, ethyl acetate extract of *Nigella sativa* L. seeds was found to be active against all the bacterial isolates except *Enterococcus faecalis*. In conclusion; Algerian honey (of the region of El-Eulma) and ethanolic extract of *Nigella sativa* L. seeds can be used as an alternative remedy for the treatment of different animal wounds.

Introduction

Although honey is known as a food, there is growing interest in the medicinal properties of honey and its role in the treatment of many different health problems. In traditional medicine, honey has been recognized all over the world for its healing properties for the skin. The ancient Greeks and Egyptians, for example, used the topical application of honey to treat wounds and burns of the skin, and traditional Persian medicine has documented that honey is effective in treating wounds, eczema and inflammation [1,2].

Nigella sativa L. (Black-seed) is an annual herb of the Ranunculaceae family and has been used traditionally for centuries in the Middle East, North Africa, Far East and Asia for the treatment of various diseases [3]. In Saudi Arabia, its oil is used externally for joint stiffness and pain, as well as for asthma and eczema [4]. In Pakistan, Black-seed is used to treat fungal and bacterial infections [5].

The cutaneous wounds are lesions of mechanical origin which are characterized by a solution of continuity of the skin. Accidental wounds result from the action of an animate or inanimate physical agent of the external environment. There are several of accidental causes.

The degree of bacterial contamination and the type of flora conditions the management of the wound. Complications of septic nature are among the various pathological changes of wounds. The treatment of wounds is very varied, the knowledge of their positive or detrimental influences on wound healing makes it possible to choose a therapeutic scheme adapted to each situation. Also, any alteration of a phase of wound healing results in delayed healing. Evolutions of septic nature involve a more or less important infection of the wound. The purpose of the detersion phase is to remove bacteria, dead tissues and other contaminants that may interfere with wound healing. During evolution of septic nature, it is this phase that is concerned. As long as an infection persists, this phase is prolonged, thus delaying the placement of the granulation tissue.

In wounds, the bacteria of the cutaneous flora are found naturally at the level of the wound and will multiply. These bacteria are generally not pathogenic and their proliferation is normally controlled by the detersion phase. However, particular conditions may lead to significant proliferation and wound infection even with originally non-pathogenic bacteria. Transient species are occasionally encountered over a short period and in limited quantities on the surface of the epidermis. When it multiplies, this transient flora may present a pathogenic aspect like secondary invader or superinfecting agent.

Contamination and bacterial colonization of the wound are therefore part of the physiological evolution of wound. When this colonization becomes pathological, it is the clinical infection. Bacterial colonization becomes critical when the host's defenses are outdated or ineffective, then the bacteria invade the cutaneous and subcutaneous tissues adjacent to the wound. The occurrence of infection depends on the number of bacteria, their virulence and pathogenicity factors, and host resistance. All factors influencing these three parameters may contribute to the development of an infection [6]. Many local factors may favor the development of an infection either by promoting bacterial proliferation or by decreasing the effectiveness of host defenses. If the organism can not control the bacterial multiplication, a bacteremia can appear and then eventually a septicemia depending on the general state of the animal. The complication becomes general and can lead to the death of the animal.

Repeated treatments (antibiotherapies, antiseptics) of cutaneous infections can modify the cutaneous flora and select resistant bacteria. The most frequent case is the selection of resistant staphylococci which secrete β -lactamases. Also, there is an increased development of resistance to each antibiotic introduced into clinical practice [7]. Wound infections caused by drug resistant organisms are common and result in increased costs, morbidity and mortality. Therefore, scientific efforts have been made to study and develop new compounds that can be used beyond traditional antibiotic therapy.

The antibacterial properties of honey and *Nigella sativa* L. have aroused great interest among researchers. In this research, the antibacterial activity of Algerian honey (of the region of El-Eulma) and some *Nigella sativa* L. seed extracts against animal wound bacteria, is evaluated.

Materials and Methods

Honey sample and *Nigella sativa* L. seeds

The honey used in this study was purchased from an apiary in El-Eulma (Algeria). The seeds of *Nigella sativa* L. were purchased from an herbal shop in El-Eulma (Algeria).

Bacterial isolates

Five bacteria were previously isolated from chronic wounds of domestic carnivores and were maintained at 4°C on nutrients agar slants. These bacterial isolates are: *Escherichia coli*, *Enterobacter* sp., *Staphylococcus aureus*, *Staphylococcus intermedius* and *Enterococcus faecalis*.

Extraction process

The seeds of *Nigella sativa* L. were cleaned, washed, air dried and ground with a mortar to medium fine powder. The extraction was carried out by macerating the seed powder in increasingly polar solvents (ethyl acetate, ethanol and methanol) following the method described by Shahid et al. (2013) [8].

Each of 50 g seed powder was macerated in 500 mL of a different solvent, for two weeks at room temperature, with

occasional agitation to facilitate extraction. The macerates were filtered on filter papers. The filtrates were evaporated using rotary evaporator (Heidolph®) at 50°C. The extracts were stored in sterile glass vials at 4°C until use.

Calculation of percentage yield

The different extracts were weighed and the percentage yield of each extract was calculated using the following equation:

$$\%Y = (Ew / Pw) \times 100. (\%Y: \text{percentage yield, } Ew: \text{extract weight, } Pw: \text{powder weight}).$$

Inoculum preparation

The inoculum was prepared following the method described by Kamal et al. (2010) [9]. Active cultures for each bacterial species were prepared by transferring a loopful of cells from the stock cultures to test tubes of nutrient broth. The inoculated tubes were incubated without agitation for 24 hours at 37°C. The cultures were diluted with fresh nutrient broth to achieve optical densities corresponding to 10⁶ cfu/mL.

Antibacterial effect of honey

The Agar Well Diffusion Method was used to test the antibacterial activity of honey following the protocol described by Akujobi and Njoku (2010) [10] with some modifications.

All media plates (9 cm in diameter) were prepared with Muller-Hinton agar. After agar solidification, the well (7 mm in diameter) was cut from the agar to produce a total of five wells per each agar plate. For test, four honey concentrations (20%, 50%, 70% and 100% v/v) were prepared using sterile distilled water. 100 μ L (10⁵ cfu) of each diluted bacterial suspension were inoculated on Muller-Hinton agar plates using sterile non-absorbent cotton swab. The inoculums were allowed to dry for 5 min. Then, 100 μ L of each honey concentration and sterile distilled water was added separately to each well of agar plate and allowed to diffuse at room temperature for 15–20 min. After incubation at 37°C for 24 hours, all plates were examined for any zones of growth inhibition and the diameter of these zones was measured.

The assay was repeated three times for each honey concentration. The antibacterial effect was recorded as the mean diameter of the resulting inhibition zones of growth in mm.

Antibacterial effect of *Nigella sativa* L.

The Agar Well Diffusion Method was used to test the antibacterial activity of *Nigella sativa* L. seed extracts following the protocol described by Kamal et al. (2010) [9] with some modifications.

All media plates (9 cm in diameter) were prepared with Muller-Hinton agar. After agar solidification, the well (7 mm in diameter) was cut from the agar to produce a total of four wells per each agar plate. For test, just one dose for each extract (30 mg/well) was prepared using DMSO as an organic

solvent. 100 μ L (10^5 cfu) of each diluted bacterial suspension were inoculated on Muller-Hinton agar plates using sterile non-absorbent cotton swab. The inoculums were allowed to dry for 5 min. Then, 100 μ L of each extract solution and blank (DMSO) was added separately to each well of agar plate and allowed to diffuse at room temperature for 15–20 min. After incubation at 37°C for 24 hours, all plates were examined for any zones of growth inhibition and the diameter of these zones was measured.

The assay was repeated three times for each extract. The antibacterial effect was recorded as the mean diameter of the resulting inhibition zones of growth in mm.

Statistical analysis

The in-vitro antibacterial activity was carried in triplicate. The data were then subjected to Microsoft Office Excel 2010 software for statistical analysis. Analysis of variance (ANOVA) to a single factor is established followed by t-Test. All the data were given mean \pm standard deviation (SD). A probability value $P < 0.05$ was taken as significant.

Results and Discussion

Percentage yield

The results correspond to the percentage yield of *Nigella sativa* L. seed extracts are summarized in table 1.

The percentage yield of *Nigella sativa* L. seed extracts showed maximum amount of ethyl acetate extract 23.22% and

Table 1: Percentage yield of *Nigella sativa* L. seed extracts.

Extract	% Yield
Ethyl acetate	23,22
Ethanollic	8,37
Methanolic	8,98

minimum of ethanolic extract 8.37%. The percentage yield of methanolic extract was 8.98%.

These extraction yield results are different from those reported by other authors. The quantitative difference between the extraction yields is attributed to the methodological difference of extraction. Also, the extraction product varies in terms of quality, quantity and composition according to climate, soil composition, plant organ, age, etc. [11].

Antibacterial activity of honey

In this section, the antibacterial activity of different concentrations of honey against bacterial isolates from animal wounds, was studied. These bacterial isolates are Gram-positive (*Staphylococcus aureus*, *Staphylococcus intermedius* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Enterobacter sp.*).

The antimicrobial activity of honey can be the result of high sugar concentration, acidity, production of hydrogen peroxide,

flavonoids, phenols or other unidentified components present in honey [12]. Certain types of honey contain other bioactive components with antibacterial activity, including methylglyoxal, lysozyme and defensin-1 [13]. In addition, it is suggested that presence of different strains of *L. acidophilus* in honey obtained from different sources may contribute to the antimicrobial properties of honey [14]. When honey is diluted, an enzyme (glucose oxidase) is activated in honey and catalyses the slow production of hydrogen peroxide which generally is the major antibacterial factor in honey [15]. This antibacterial activity varies significantly from honey to honey [15]. Activity of hydrogen peroxide in undiluted honey is suppressed by the low pH of honey since the glucose oxidase enzyme has an optimum pH of 6.1 with a minimum activity of pH 5.5 and a maximum of pH 8 [16]. The antibacterial activity of honey depends on synergism between all the bioactive components and honey containing more than one active substance has a higher potency as an antimicrobial agent [17].

The results correspond to the antibacterial activity of honey are summarized in table 2.

All the bacterial isolates were found to be sensitive to all the honey concentrations but with varying degrees. The difference

Table 2: Antibacterial effect of honey against bacterial isolates.

Bacteria	Average diameter of zone of inhibition (mm)			
	20%	50%	70%	100%
<i>Escherichia coli</i>	9,66 \pm 1,15	11,66 \pm 2,08	12,33 \pm 1,15	19 \pm 1,73
<i>Enterobacter sp.</i>	13,66 \pm 0,57	13,66 \pm 1,52	14,33 \pm 2,51	15 \pm 2
<i>Staphylococcus aureus</i>	15,66 \pm 4,04	18 \pm 4,35	16,66 \pm 0,57	19 \pm 3,46
<i>Staphylococcus intermedius</i>	10,33 \pm 0,57	11,66 \pm 0,57	12 \pm 1	16,33 \pm 0,57
<i>Enterococcus faecalis</i>	13,66 \pm 0,57	12,33 \pm 0,57	12 \pm 0	14,33 \pm 0,57

Note: The results are represented by the mean \pm SD.

in sensitivity between these bacterial isolates was significant ($P < 0.05$) to each of the honey concentrations.

The highest sensitivity of all these bacterial isolates was observed with undiluted honey. This result is explained by the fact that the antimicrobial activity of undiluted honey depends on its high sugar content lowering the water activity and the dilution of honey will change its osmotic effect [16], its acid pH and its antibacterial effect [16,18]. The antibacterial effectiveness of diluted honey against the bacterial isolates suggesting the presence of antibacterial activity other than simple sugar-dependent hyperosmolarity.

The smallest zone of inhibition was observed with concentration 20% against *Escherichia coli* and its diameter was 9.66 \pm 1.15 mm. The largest zone of inhibition was observed with undiluted honey against *Staphylococcus aureus* and *Escherichia coli*, and the diameters of these zones of inhibition were 19 \pm 3.46 mm and 19 \pm 1.73 mm respectively.

Basualdo et al. (2007) [19], reported that *Staphylococcus aureus* was the most sensitive bacterium to several honey samples marketed in Argentina, but *Escherichia coli* and *Enterococcus faecalis* were resistant. Also, Khalil et al. (2014)

[20], reported that *Staphylococcus aureus* was the most sensitive bacterium to different honey samples marketed in Pakistan and *Enterococcus faecalis* was also sensitive to these honey samples. In other study, Omafuvbe and Akanbi (2009) [21], reported that *Escherichia coli* was the most sensitive bacterium to some commercial Nigerian honey but *Staphylococcus aureus* was resistant.

There are no studies on the antibacterial activity of honey against *Staphylococcus intermedius* and *Enterobacter sp.*, except that of Al-Waili et al. (2013) [17], who reported that *Enterobacter aerogenes* was sensitive to honey from Saudi Arabia.

The variation between our data and earlier reports may be attributed to many factors which may influence the antimicrobial activity of honey. These factors include physico-chemical properties, botanical origin, entomological origin and symbioses with beneficial bacteria of honey [21].

Antibacterial activity of *Nigella sativa* L.

In this section, the antibacterial activity of some *Nigella sativa* L. seed extracts against bacterial isolates from animal wounds, was studied.

Polar solvents (ethanol and methanol) and a moderately polar solvent (ethyl acetate) were used to extract secondary metabolites from *Nigella sativa* L. seeds, that differ in polarity and structure, and thus different solvent extracts showed distinct biological properties.

The antibacterial activity of ethanolic, methanolic and ethyl acetate extracts of *Nigella sativa* L. seeds is largely attributable to TQ since Singh et al. (2014) [22] and Suresh Kumar et al. (2010) [23], reported that these extracts were rich in TQ. Halawani (2009) [24], Shohayeb and Halawani (2012) [25] and Jrah Harzallah et al. (2011) [26], demonstrated that this bioactive component has a very powerful antibacterial effect.

The results correspond to the antibacterial activity of different *Nigella sativa* L. seed extracts are summarized in table 3.

At concentration of 0.3 g/mL, ethanolic extract of *Nigella sativa* L. seeds was found to be active against all the bacterial isolates. Ethyl acetate extract of *Nigella sativa* L. seeds was found to be active against all the bacterial isolates except

Enterococcus faecalis. The difference in sensitivity between the bacterial isolates was significant ($P < 0.05$) to each of these both extracts. Methanolic extract of *Nigella sativa* L. seeds was found to be active against all the bacterial isolates except *Escherichia coli*. The difference in sensitivity between the bacterial isolates was not significant ($P > 0.05$) to this extract.

The largest zone of inhibition was observed with the ethyl acetate extract against *Staphylococcus intermedius* with a diameter of 15.66 ± 0.57 mm. The smallest zone of inhibition was observed with the ethanolic and ethyl acetate extracts against *Escherichia coli* with a diameter of 10 ± 0 mm.

Shahid et al. (2013) [8], reported that *Escherichia coli* was sensitive to methanolic and ethanolic extracts, but resistant to ethyl acetate extract of *Nigella sativa* L. seeds. *Staphylococcus aureus* was sensitive to methanolic, ethanolic and acetate ethyl extracts of *Nigella sativa* L. seeds. In other study, Mishra (2011) [27], reported that *Escherichia coli* was resistant to methanolic and ethanolic extracts, and sensitive to ethyl acetate extract of *Nigella sativa* L. seeds. *Staphylococcus aureus* was sensitive to methanolic, ethanolic and acetate ethyl extracts of *Nigella sativa* L. seeds. In the study of Sellami et al. (2013) [28], ethanolic extract of *Nigella sativa* L. seeds was found to be inactive against *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter cloacae*.

There are no studies on the antibacterial activity of methanolic, ethanolic and ethyl acetate extracts of *Nigella sativa* L. seeds against *Staphylococcus intermedius* and *Enterococcus faecalis*.

Negative results do not mean the absence of bioactive constituents; the active compounds may be insufficient to show activity with the applied concentration [29]. Indeed, the different sources of extracts, agro-climatic factor, manipulation of experiment and phytochemical ingredients in extract also contribute to the differences in obtained results [30].

Conclusion

The results demonstrate that Algerian honey (of the region of El-Eulma) can be used as a natural liquid dressing to treat infected animal wounds. Also, ethanolic extract of *Nigella sativa* L. seeds is an effective antibacterial agent, which can be used as an alternative remedy for the treatment of infected animal wounds.

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Table 3: Antibacterial effect of *Nigella sativa* L. seed extracts against bacterial isolates.

Bacteria	Average diameter of zone of inhibition (mm)		
	Ethyl acetate extract	Ethanolic extract	Methanolic extract
<i>Escherichia coli</i>	10 ± 0	10 ± 0	0
<i>Enterobacter sp.</i>	13,66 ± 0,57	11,66 ± 0,57	11,66 ± 0,57
<i>Staphylococcus aureus</i>	12 ± 1	10,66 ± 1,15	12,66 ± 0,57
<i>Staphylococcus intermedius</i>	15,66 ± 0,57	12 ± 1	13,66 ± 0,57
<i>Enterococcus faecalis</i>	0	12,66 ± 0,57	12,33 ± 1,15

Note: The results are represented by the mean ± SD.

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