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# PEROXIDE AND NON-PEROXIDE ANTIBACTERIAL ACTIVITY IN SOME IRANIAN HONEYS

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## ABSTRACT

A range of some Iranian monofloral honeys were assayed for antibacterial activity with and without hydrogen peroxide which is inactivated by the addition of catalase. It was found that the high amount of antibacterial activity in honeys was due to a factor other than hydrogen peroxide. The test microorganism *Staphylococcus aureus*, was not inhibited by the acidity or the osmolarity of the honey. The association of high antibacterial activity with particular floral sources suggest that the non-peroxide antibacterial activity is of floral origin. The activity of some Iranian honeys were tested and found to be heat stable.
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## INTRODUCTION

The antibacterial activity of honey from different floral sources can vary significantly, but this is not generally accepted.[1] To measure the variation of the inhibine of the antibacterial activity of honey, some investigators devised a five point scale based on dilutions of honey to concentrations ranging from 5% to 25% in the test medium.[2] The inhibine measured in diluted honey is distinct from the bacteriostatic effects of the high osmolarity and low pH of undiluted honey. The inhibition activity is attributed to the hydrogen peroxide generated by the action of glucose oxidase and has been suggested that the variation in heat-sensitivity of the inhibine in honey of different floral sources could result from the presence of peroxide-destroying components from some flowers.[3,4] The inhibine values and hydrogen peroxide concentrations of a range of honeys were compared and it was concluded that hydrogen peroxide was responsible for the major portion of the non-osmotic antibacterial activity in diluted honeys.[4] It has been found that after removing hydrogen peroxide by catalase the antibacterial activity remains.[5] The existence of non-peroxide activity is reported by Dustman (1979), but he claimed that it was only a minor portion of the maximum activity.[6] All of the antibacterial activity in honey could be accounted for by the lysozyme content.[7]

## MATERIAL AND METHODS

### Assay of Antibacterial Activity

An agar well diffusion technique was used to assess antibacterial activity. Agar plates were prepared from nutrient agar (Difco, 23 g/L) to which 10 mL of a culture of bacteria was added at 45°C (*Staphylococcus aureus*, 10 per mL) in 10 mL nutrient broth (Difco, 8 g/L) immediately before pouring. Wells, 6 mm in diameter and 8 mm in depth, were cut in agar; these were completely filled with the solution under test and the plates were incubated at 37°C for 24 h.

The antibacterial activity was recorded as the radial extent of the area cleared of bacterial growth around the well. The antibacterial activity of 20 honey samples from different areas of Iran was tested in original form and diluted with water to 1/4 and 1/8 of their original strength (Table I).

### Removal of Hydrogen Peroxide

An equal volume of a solution of catalase (Sigma, Chem. Co. from bovine liver, Cat. No. E 10: 6000 unit/mL) was added to honey solutions to neutralize hydrogen peroxide and thus allowed to assay the other antibacterial factors. The efficacy of this enzyme solution was tested with solutions of hydrogen peroxide using the agar well

**Table I. Extent of zone of inhibition due to the antibacterial activity of some Iranian honeys, comparing the original form with 1/4 and 1/8 strength diluted form.**

| NO | Area | Original EOZI* | 1/4 diluted EOZI | 1/8 diluted EOZI |
|---|---|---|---|---|
| 1 | Ardabil | 13 | 9 | 7.5 |
| 2 | Urmia | 11 | 8 | 7 |
| 3 | Amoul | 10 | 9 | 7 |
| 4 | Anzally | 9 | 8 | 7 |
| 5 | Ahvaz | 15 | 9 | 8 |
| 6 | Tabriz | 14 | 11 | 8 |
| 7 | Khansar | 14 | 8 | 7 |
| 8 | Doroud | 12 | 9 | 8 |
| 9 | Damavand | 19 | 12 | 9 |
| 10 | Rasht | 12 | 9 | 7 |
| 11 | Zandjan | 15 | 13 | 9 |
| 12 | Sarab | 13 | 8 | Trace |
| 13 | Shahroud | 12 | 9 | 8 |
| 14 | Shiraz | 8 | 7 | Trace |
| 15 | Karaj | 15 | 12 | 9 |
| 16 | Mashhad | 13 | 10 | 7 |
| 17 | Malayer | 12 | 8 | Trace |
| 18 | Mahabad | 14 | 7 | Trace |
| 19 | Neishabour | 14 | 9 | 7 |
| 20 | Hamadan | 13 | 9 | 7 |
| MEAN | | 12.9 | 9.2 | 6.1 |
| SD | | 2.4 | 1.6 | 0.32 |

*Extent of Zone of Inhibition (mm).

**Table II. The difference of antibacterial activity with original and diluted solutions of hydrogen peroxide (1/4 and 1/8) in 10 samples**

| NO | AREA | ONC EOZI* | OWC EOZI | 1/4 diluted nc EOZI | 1/4 diluted wc EOZI | 1/8 diluted nc EOZI | 1/8 diluted wc EOZI |
|---|---|---|---|---|---|---|---|
| 1 | Urmia | 13 | 11.5 | 8 | 7.5 | 6.3 | 6 |
| 2 | Ahvaz | 11.5 | 9 | 8 | 7 | 6.2 | 6 |
| 3 | Tabriz | 10.5 | 9.5 | 7.5 | 7.1 | 6.5 | 6 |
| 4 | Khansar | 19 | 15 | 11 | 8 | 6.6 | 6 |
| 5 | Damavand | 12.5 | 10 | 8 | 7.5 | 6 | 6 |
| 6 | Rasht | 14 | 10.5 | 9 | 8 | 6.4 | 6 |
| 7 | Zandjan | 11 | 9 | 8.5 | 7 | 6 | 6 |
| 8 | Karaj | 14.5 | 13 | 8 | 7.5 | 6.3 | 6 |
| 9 | Mashhad | 18.5 | 14 | 9 | 8 | 6.5 | 6 |
| 10 | Neishabour | 14 | 12 | 8 | 6.5 | 6 | 6 |
| MEAN | | 13.85 | 11.35 | 8.5 | 7.41 | 6.28 | 6 |
| SD | | 2.9 | 2.12 | 1 | 05 | 022 | 0 |

ONC= Original No Catalase
OWC= original with catalase
nc= no catalase
wc= with catalase
*EOZI= Extent of Zone of Inhibition

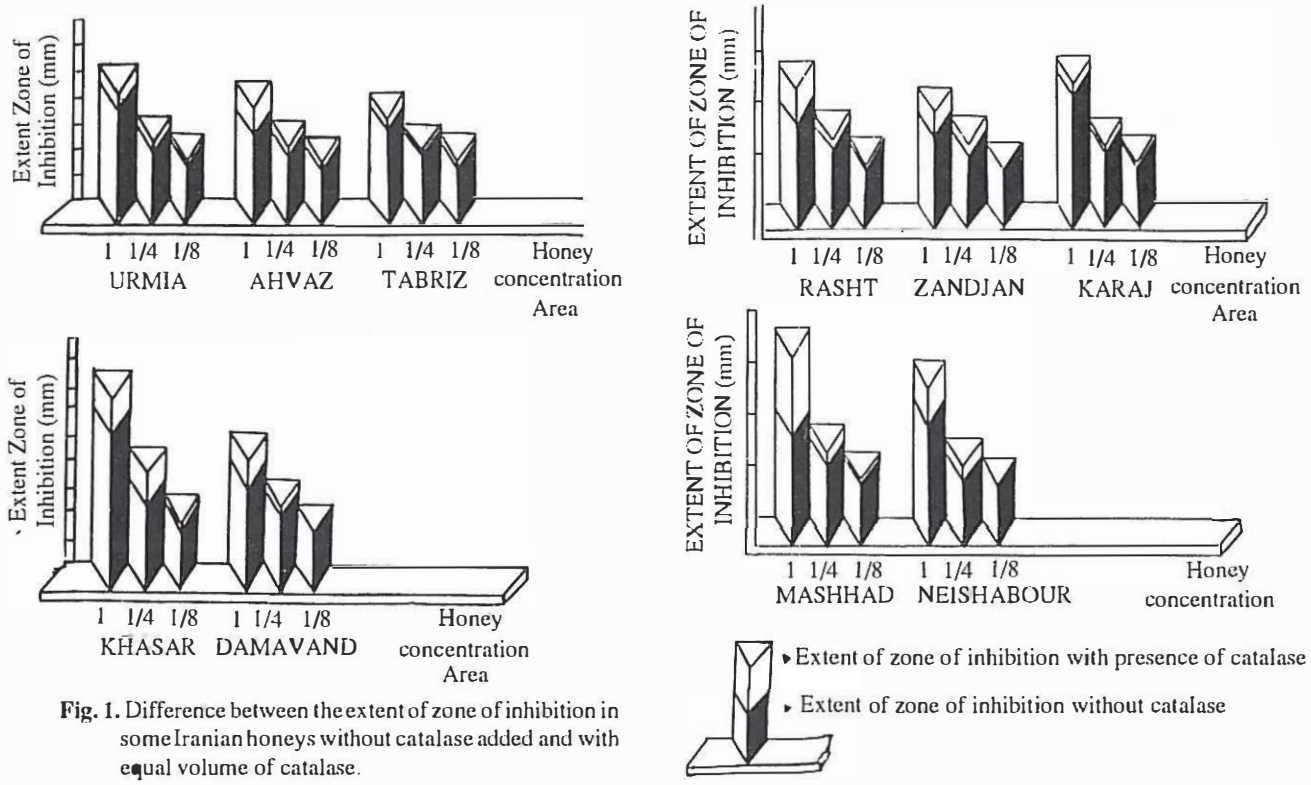


**Fig. 1.** Difference between the extent of zone of inhibition in some Iranian honeys without catalase added and with equal volume of catalase.

diffusion technique with *Staphylococcus aureus* in the plates. The solutions were prepared by mixing aqueous hydrogen peroxide solutions with equal volume of clover honey with no detectable antibacterial activity. The maximum concentration of hydrogen peroxide present in Khansar honey was estimated by comparing antibacterial activity of a sample of Khansar honey with that of a range of concentrations of hydrogen peroxide.

10 samples from different areas of Iran were compared with each other for antibacterial activity both undiluted and diluted to 1/4 and 1/8 (Table II).

Hydrogen peroxide solutions were mixed with an equal volume of catalase. There was no detectable inhibition of bacterial growth on the plates, showing the amount of catalase concentration less than sufficient to eliminate all of antibacterial activity in the honey due to the presence of hydrogen peroxide (Fig. 1).

### Selection of Bacterial Culture

A variety of species of bacteria (*Staphylococcus, E. coli, Salmonella*) were tested for their resistance to the high osmolarity of honey. In the wells of plates prepared with cultures of each strain were put a series of dilutions of the composition and the average of 20 samples of Iranian honeys were found (Jamali, 1973): 40.5% glucose, 43.9% fructose, 7.2% sucrose, 8.4% water.

The strains were tested at the same time for their sensitivity to the antibacterial activity of honey. A series of dilutions of Iranian honeys were put in the wells alongside with one containing sugar solution (this type of honey has particulary high antibacterial activity). A series of dilutions of Iranian honeys with catalase added to remove hydrogen peroxide were also included in order to compare the bacteria with respect to their sensitivity to any other type of antibacterial factors. The strain selected from the results of these tests, *Staphylococcus aureus*, was checked for its growth inhibition by the acidity of the honey under the test system used. The pH of one of the most antibacterial honeys was approximately 4, and was assessed by titration of a solution of this honey at 1/4 strength with 9.8 mM $Na^+$ gluconate.

The pH of this solution was measured also. A solution of gluconic acid of equivalent concentration was prepared by dissolving the appropriate quantity of the D-gluconate in water and after 30 minutes adding $Na^+$ gluconate to adjust this solution to the same pH as at that of the honey solution. The antibacterial activity of the gluconic acid solution and the honey solution were then assessed by the agar well diffusion technique in a plate containing *Staphylococcus aureus*.

### Heat Stability of the Honeys

The effect of heat on the antibacterial activity of some Iranian honeys was tested. The honeys (10 samples, 1 g each sample) diluted with an equal volume of water were heated

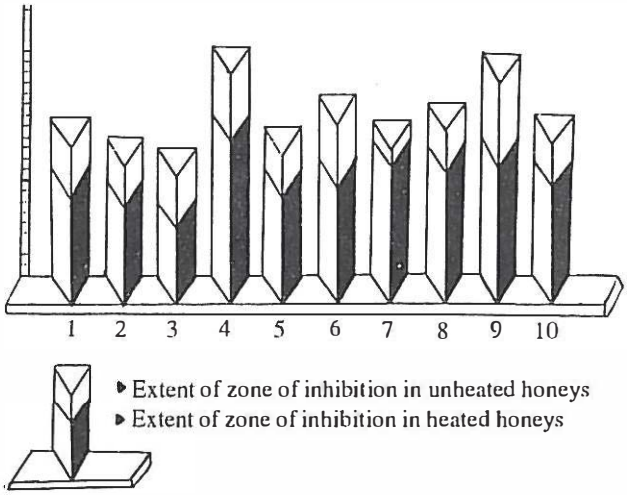


**Fig. 2.** The effect of heat on antibacterial activity of some Iranian honeys.

for 30 minutes at 60°C. After cooling, the antibacterial activity was assessed on plates containing *Staphylococcus aureus*. Reduction in inhibition of growth was observed in the samples (Table III, Fig. 2).

## RESULTS

1. 20 samples of honey from different areas of Iran had the antibacterial activity equivalent to 12.9 ± 2.4 mm (original), 9.2 ± 1.6 mm, and 6.1 ± 0.32 mm (diluted with water to 1/4 and 1/8) zone of inhibition, respectively (Table I).
2. After removing hydrogen peroxide by catalase in some samples the antibacterial activity was compared. The comparison showed 13.85± 2.9 mm zone of inhibition for original, 8.5± 1 mm, and 6.26±0.22 mm for 1/4 and 1/8 diluted honeys, respectively (Table II, Fig. 1).
3. After 30 minutes heating in water bath (60°C), a reduction of inhibition of bacterial growth was observed from 15.58 ± 3.07 mm to 11 ± 2.9 mm (zone of inhibition) in some of the samples (Table III, Fig. 2).
4. Survery of honey samples. The extent of the largest zone of inhibition recorded from the honey samples was 22 mm (Khansar, Table II). All honey controls with hydrogen peroxide alone gave a clear zone of inhibition of approximately 22 mm, those with hydrogen peroxide and catalase together showed no inhibition. So, it can be concluded that where a honey has high total antibacterial activity, it also has a high activity after removal of the hydrogen peroxide.

**Table III. The effect of heat on the samples.**

| NO | AREA | EOZI* (original) | EOZI (reduction by heat) |
|---|---|---|---|
| 1 | Urmia | 14.8 | 10.5 |
| 2 | Ahvaz | 13 | 9 |
| 3 | Tabriz | 12.2 | 7 |
| 4 | Khansar | 21.5 | 15 |
| 5 | Damavand | 14 | 10 |
| 6 | Rasht | 16.8 | 11 |
| 7 | Zandjan | 14.5 | 13 |
| 8 | Karaj | 16 | 12.5 |
| 9 | Mashhad | 20.7 | 13 |
| 10 | Nieshabour | 15 | 13 |
| Mean | | 15.58 | 11 |
| SD | | 3.07 | 2.93 |

*EOZI = Extent of Zone of Inhibition

## DISCUSSION

This investigation provides conclusive evidence that antibacterial activity (antibacterial activity) in some Iranian honeys is due to hydrogen peroxide only. This effect could not be possibly due to honey's osmolarity or acidity. In the microorganism test, *Staphylococcus aureus* resisted this bacteriostatic effects, even at concentrations equivalent to 1/4 strength, yet was inhibited by the non-peroxide activity of a number of honeys diluted to 1/8. The amount of catalase added was sufficient to destroy the antibacterial effect of hydrogen peroxide at concentration equivalent to that of honey at 1/4 strength.

The data presented in the present study show a large variation between different honeys, the high non-peroxide factors occurring in honeys with a high overall activity.

The agar well diffusion technique used is one of the least sensitive techniques, the test solution becomes diluted as it diffuses into the agar. The additional activity is presumably of floral origin, since the high activity generally was associated with honeys from some floral sources and not others.[1]

There are some discrepancies, but as discussed by Molan,[1] it is likely that identification of the sources of these honeys may be in error to some degree.

## REFERENCES

1. Molan PC, Smith IM, Reid GM: A comparision of antibacterial activity of some New Zealand honeys. J Apic Res submitted,

1988.
2. Donald H, Witzenhausen R: Ein verfahran zur beurteilung der ortlichen inhibitorischen keim vermehrungs hemmenden wirkung von honigsorte verschiedener. Herkunft Z Hyg Infetkrannkh 141: 33-7, 1955.
3. Whith JW, Suber MH, Schepartz A: The identification of inhibine. Am Bee J 102: 430-1, 1962.
4. Whith JW, Suber MH: Studies on honey inhibine, 2. A chemical assay. J. Apic Res 2: 93-100, 1964.
5. Adcock D: The effect of catalase on the inhibine and peroxide values of various honeys. J Apic Res 1: 38-40, 1962.
6. Dustman J: Antibacterial effect of honey. Apicta 14: 7-11, 1968.
7. Gonnet M, Larie P: Influence du chauffage surle facteur antibiotique present dans lemiels. Annls Aheill 34: 349-64, 1960.