Antimicrobial Activity of Limonene

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ABSTRACT

Essential oil was extracted with steam distillation from the peels of orange, grape-fruit and lemon. The limonene was quantitatively analysed by thin layer chromatography. The extract from the citrus fruits contained from 90 to 95% limonene. The effect of limonene on growth and metabolism of *Staphylococcus aureus* and *Aspergillus oryzae* were studied. Limonene suppressed growth and oxygen uptake of *Staphylococcus aureus* but had no effect on *Aspergillus oryzae*. If these compounds are present as natural constituents of citrus plants, then they may be a good source of both preservative and flavoring agents.

INTRODUCTION

Citrus oil is a major by-product of the citrus processing industry. Commercially important peel oils include lemon, orange, grape-fruit, bergamot etc. These oils are used as flavoring ingredients in a variety of foods such as soft drinks, ice cream, frozen desserts, confectionary and baked foods. They are also used in the perfumery industry. The oil consists of chiefly terpenes hydrocarbons (80–95%) (4). Research has mainly been centered on the methods of isolation, structure, and determination of inidividual terpenes. The toxicity of these components to insects has been reported (1, 2). The inhibitory effect of essential oils of orange and lemon were compared with antibiotic tylosin and nicin. Yeast was more sensetive than bacteria and mold (7).

Most antimicrobial food additives now in use are lipophilic acids which inhibit the growth of unwanted micro-organisms. The mechanism of inhibition remains unknown. The compound used in this report was limonene which was extracted from the peels of citrus fruits.

The purpose of this study was to obtain some general information on the possibility of finding antimicrobial activity in the limonoids from the peels of citrus fruits.

MATERIALS AND METHODS

Extraction and Analysis of Limonoids

The fruits were obtained from the Faculty of Agriculture Experiment Station, Tripoli. The peels were cut into pieces and converted into mash with 300 ml distilled

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water in a blender. The mash was subjected to steam distillation and after collecting about 80 ml of distillate, it was cooled to room temperature. The contents were transferred with the help of 20 ml CH_2 Cl_2 to a separatory funnel. The organic phase was drawn off into a 50 ml flask and the water phase was re-extracted with 20 ml more CH_2 Cl_2. The two extractions were combined and evaporated on the steam bath in the hood until about 1.5 g of pleasant smelling oil was left. This was referred to as extract. The combined CH_2 Cl_2 extracts were analysed by thin layer chromatography with silica gel plates. The chromatographs were developed with cyclo-hexane-ETOAC (6:4) or CH_2 Cl_2–MEOH (96:4) and were sprayed with Ehrlich's reagent.

The relative amount of limonene present was estimated visually by comparison with the standard (5). The limonene was recovered according to Stahl method (6) for further tests.

Culture Propagation and Growth Inhibition Studies

The enterotoxogenic strain of *Staphylococcus aureus* 472 used in this study was obtained from Washington State University, Food Science Department and preserved in the dried form on porcelain beads, according to the method of Hunt *et al.* (3). Stock cultures were prepared weekly by inoculating the organism into Brain Heart Infusion Broth (BHI) and incubated for 18 hours at 37°C. Appropriate concentrations of sweet orange extract and its lemonene were added to BHI after sterilization at 121°C for 15 minutes. Following steralization and cooling, 1 ml of fresh culture of *Staphylococcus aureus* was added to each flask and the flasks incubated in a continuous shaker at 37°C. A control was also inoculated and maintained at the same temperature. Culture growth was measured after 24 hours using a NEPHO Colorimeter Model 9. Percentage inhibitory was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Control-treatment}}{\text{Control}} \times 100$$

Aspergillus oryzae used in this study was obtained from Food Science Department, University of Al-Fateh. The effect of sweet orange extract as well as its limonene on the growth of *Staphylococcus aureus* and *Asperigallus oryzae* were also studied by the standard plate count method agar (pH 7) for *Staphylococcus aureus* and on potato dextrose agar (pH 4.5) for Asperigallus oryzae.

Carbohydrate-starved cells for monometric experiments were prepared by growing cultures for 12 hrs. Oxygen uptake was measured at 37°C by use of the Warburg apparatus. Each reaction flask contained 2 ml bacterial suspensions, 6 mole glucose.

RESULTS AND DISCUSSION

The extract obtained from the sweet orange, lemone and grape-fruit contained about 94%, 95% and 93% limonene, respectively. The other components were not identified. The effect of sweet orange extract on the growth of the gram-positive bacterium *Staphylococcus aureus* is shown in Table 1. With the addition of 0.4% extract in the normal medium, there was about 96% inhibition after 48 hrs. Complete inhibition was observed with 0.5%. The effect of pure limonene is also shown in Table 1. The results appear to indicate that there is more inhibition when using extract than using limonene in BHI.

The extract contained 94% limonene, and the inhibition found to be function of limonene. However, the extract was a more effective inhibitor than limonene (Tables 1

Table 1. The effect of sweet orange extract and its limonene on the growth of *Staphylococcus aureus*, based on turbidity.

Treatment %	Inhibition after 48 hours (%) Limonene	Extract
0.1	0.11	52
0.2	5	86
0.3	89	91
0.4	94	96
0.5	96	100
Control	0	0

Mean of 3 replicates.

and 2). This shows that there are other compounds in the extract which are also inhibitors of *Staphylococcus aureus*, explaining why the extract caused more inhibition than limonene alone.

The relative effects of the extracts and limonene are also shown in Table 2 by measuring the total count. These results indicate that after 24 hrs., no viable organisms were present in the 1% extract, whereas with 1% limonene the total counts were 44×10^6 and with 2% only 7×10^6. In the presence of 3% limonene, the total counts were zero. After 48 hrs. the total counts gradually increased (Table 2). These results were in agreement with the turbidity measurements (Table 1). There was no effect of extract on the growth of *Aspergillus oryzae*.

Table 2. The effect of extract and limonene on the growth of *Staphylococcus aureus* determined by viable counts.

Treatment %	Total counts $\times 10^6$			
	24 hours		48 hours	
	Extract	Limonene	Extract	Limonene
1	0	44	44	88
2	0	7	10	78
3	0	0	0	20
Control	129	132	135	143

Mean of 3 replicates.

The effect of extract on the oxygen uptake by bacteria is shown by Fig. 1. In the presence of 0.5% extract in the reaction flask, the oxygen uptake is reduced to 50%. When limonene was increased to 1% there was complete inhibition of O_2 uptake.

These results suggest that limonene is a strong inhibitor of *Staphylococcus aureus*. The results also suggest that citrus oil may contain other compounds which could have antibacterial properties in addition to the limonene.

On the basis of these results, the addition of citrus oil to various foods could serve as dual purpose i.e. as a flavoring ingredient and as an antimicrobial agent.

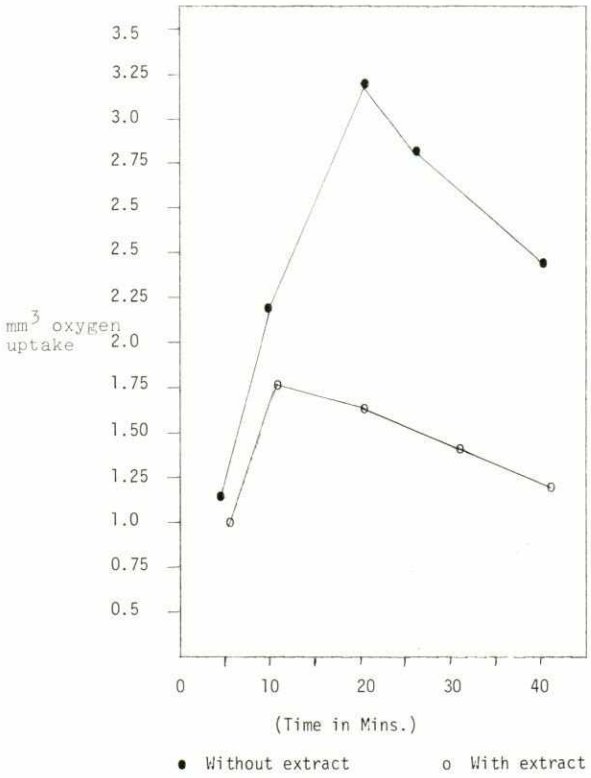

Fig. 1. The effect of 0.5% extract on the oxygen uptake by *Staphylococcus aureus*.

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نشاط الليمونين المضاد للميكروب

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المستخلـــص

بالتقطير بالبخار تحصل الباحثان على مستخلص الزيت العطري من قشور البرتقال والليمون الهندي والليمون ، بالتحليل الكروماتوجرافي بالطبقة الرقيقة تم تقدير الليمونين الذي اتضح أنه يكون ٩٠ — ٩٥ من وزن المستخلص وبدراسة تأثير هذا الليمونين على تكاثر الميكروب المكور العنقودي (*Staphylococcus aureus*) اتضح التأثير المثبط لليمونين على تكاثر البكتريا وعلى استهلاك الأوكسجين (خارج الجسم) ، ولكن لم يظهر أي تأثير على الفطر (*Asperigillus oryzae*).