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The Effects of Limonene and Orange Peel Extracts on Some Spoilage Fungi

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ABSTRACT

Orange peel extract solution contains mucilage flavor and three types of glycosides. Limonene is one of the terpenoides in some vegetables, fruits and food; that plays as an antioxidant in juice. As it has anti-microorganism effects on some bacteria, we carried out the antifungal effects of limonene versus orange extract. In the present study the effects of orange extract and two types of limonene against *Candida albicans*, *Aspergillus niger*, *Aspergillus* sp. and *Penicillium* sp. has been studied using agar well diffusion and paper disc diffusion. (S)-(-)-Limonene had more inhibitory effect on examined fungi; in both well diffusion, and paper disc diffusion experiments. *Aspergillus niger* was more sensitive related other fungi. Orange peel extract didn't show any antifungal activity on tested fungi. The results showed that orange peel extract does not have any antifungal activity. However Limonene (S)-(-) type has been shown to have inhibitory effects on filamentous fungi.

1. Introduction

Limonene is a kind of mono cyclic Terpene (136.24 KD molecular weight) which found in the various food stuffs such as sour fruits, orange, carrot and coffee (Rangma et al., 1983; Roberto et al., 2009; Pourbafrani et al., 2010). There are two types of this compound R (+), R(-) in plants and have important role in synthesizing of many compounds (Graebin et al., 2010). Orange is one of the citrus fruits and limonene is one of the most abundant compounds in it (Perez-Cacho and Rouseff, 2008a). However its amount is different in various citrus fruits and in sour type of citrous fruits is more than the others. Its amount in some sour fruits is 84% (Mosaddegh et al., 2004; Sharma and Tripathi, 2008).

This substance can be used as healthy flavor in fruit juices , drinks and ice cream and also it has antioxidant effects (Perez-Cacho and Rouseff, 2008b; Roberto et al., 2009; Marostica et al., 2009). It has been proved that limonene is inhibitor factor for prolifrating of lymphomas cell and stimulating factor for normal life of lymphocytes (Manuele et

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al., 2008). Moreover the anti-cancer effect of limonene have suggested due to chemical factors on animal models (Roberto et al., 2009). Studies have shown that limonene has regulatory effects on immune system of mouse by increasing white blood cells and increasing production of total antibody (Raphael and Kuttan, 2003) and also can increase the number of alveolar macrophage and their activities (Hamada et al., 2002).

However, the complete function of this compound has not been identified (Perez-Cacho and Rouseff, 2008a), but the inhibitory effect of it has been shown on some organisms by combination with the other plant extracts (Matsuoka et al., 1990; Raybaudi et al., 2006; Gutierrez et al., 2008). This compound has been identified as a toxic material for microorganisms in citrus fruits (Pourbafrani et al., 2007). It has been shown that materials containing limonene have antifungal effect on various types of filamentous-fungi (*Aspergillus niger*, *Penicillium* sp.) and *Candida albicans*. Furthermore, limonene has anti-leishmania and anti-salmonella effect (Graebin et al., 2010; O'Bryan et al., 2008).

According to wide applications of this extract in drugs and food stuffs as flavor and preservative and anti-microorganism material, various types of tests such as measuring the anti-microorganism effect against fungi needs to be done in order to increase the quality of food stuffs such as decreasing the amount of sugar and amino acids (Montesins, 2003; Fletcher et al., 2006; Raybaudi et al., 2006; Chang et al., 2008; Sharma and Tripathi, 2008). The present study was carried out according to evidences based on antifungal effect of plant by-products to determine the antifungal effect of limonene and orange peel extracts.

2. Material and Methods

2.1. Limonene

Both types of limonene (R-(+) Denorotatory and R-(-) Levorotatory) were purchased from Merck Co, Germany, and Orange peel extract from Ramsar decoction factory, Mazandaran, Iran.

2.2. Fungi

Fungi which used in this study were *Candida* species (isolated from vaginitis), *Aspergillus niger* (isolated form otomycosis) *Aspergillus* sp. and *Penicillium* sp. (isolated from the environment). These fungi were identified to genus and species level by standard method of mycology.

2.3. Antifungal effect in solid media (tube)

A little amount of fungal colony was cultured in the tube containing sabouraud dextrose agar supplemented with chloramphenicol, and left 24 hours at room temperature. Then one drop of limonene or orange peel extract was added onto the colony and after 5 days the growth of fungi in samples was studied compared with positive control (containing fungi without extract or limonene). This method was repeated two times and mean±SEM was calculated and have been added in the results.

2.4. Antifungal effect by well diffusion method

Fungal suspension in 1×10^6 cells/ml has been cultured using the microbiology method on plate containing sabouraud dextrose agar with chloramphenicol. Then limonene and extract (20, 50 and 100 micro liters), were poured in 6.5 mm diameter well. The plates and negative control (physiological serum) were incubated at 25°C for 48 hours. The zone inhibition colony growth was measured and compared with the controls.

2.5. Paper disc diffusion method

In this method, paper disc with 6.4 mm in diameter (purchased from Padtan Teb. Co. Iran) were treated with limonene, orange peel extract (10, 20, 30 micro liters) and physiological serum (negative control). These papers were put on the plates containing selected fungi. Plates were incubated at 25°C for 48 hours and then inhibition of fungal growth around paper discs was measured and compared to controls.

2.6. Fungal suspension and paper disc method

In this method 2 ml of fungal suspension (1×10^6 cells/ml) was added to the plate liquefied culture media (45-50°C). After 2 hours paper discs containing 10, 20, 30 micro liters limonene or orange peel extract or physiological serum (negative control) were put on the media at 25°C for 48 hours.

Inhibition of fungal growth was measured and compared with the controls.

2.7. Statistical analysis

The statistical analysis has been done by SPSS software (version 18). The variance less than 0.05 were considered as significant.

3. Results

Antifungal effect of orange peel extract and limonene in tube showed the growth of all fungi was less than in control group. Although all fungi were inhibited by limonene and orange peel extract, the inhibition of fungi by orange peel was less than limonene. *Aspergillus niger* was the most sensitive fungi to the limonene among the tested fungi, and also the effect of R-(+) limonene was more than R-(-) (Table 1). In addition, by adding of various types of limonene and orange peel extracts immediately after culture, no effects on the fungus growth has been observed.

In well agar diffusion, R-(-) limonene has been shown to have the best antifungal effect which had direct relationship with the amount of extract were used. *Candida* species was the most sensitive fungi and R-(+) limonene had least antifungal effect (table 2). Orange peel extract had no inhibitory effect on fungi, and R-(+) limonene showed the most sensitive than the other fungi in paper disc diffusion method (table 3).

R-(-) limonene had the most antifungal effect in the method which fungal suspension was done using paper disc in culture media. Orange peel extract didn't show any antifungal effect, and *Aspergillus* sp. was more sensitive than the other fungi (table 4).

Table 1. The growth rate of fungi in the presence of inhibitors.

Fungi	(R)-(+)-Limonene (ml)	(S)-(-)-Limonene	Orange extract	Control	P value
Candida albicans	3±1.4	3±0	6±4.24	13±2.12	0.043
Penicillium sp.	4±0.71	2.5±0.71	2±0.71	12±7.07	0.135
Aspergillus sp.	2±0	3±1.41	6±0.71	10±4.95	0.114
Aspergillus niger	7±2.83	8±1.41	8±1.41	12±3.54	0.338

Table 2. The inhibitory effect of limonene and orange extract on fungi in the agar well diffusion method.

Fungi	(R)-(+)-Limonene			(S)-(-)-Limonene			Orange extract			P value
	10µl	20µl	30µl	10µl	20µl	30µl	10µl	20µl	30µl	
C. albicans	1±0	3±1.41	4±1.41	3.5±2.12	7±1.41	9.5±3.54	0±0	0±0	0±0	0.000
Penicillium sp.	1.5±0.71	2.5±0.71	3 ±1.41	0±0	0±0	0±0	0±0	0±0	0±0	0.000
Aspergillus sp.	5±1.41	5.5±2.12	7.5±3.54	13±0	13±0	15±0	0±0	0±0	0±0	0.000
A.niger	5±0	5±1.41	9±1.41	3.5±0.71	4±1.41	5.5±0.71	0±0	0±0	0±0	0.000

Table 3. The inhibitory effect of limonene and orange extract on fungi in the paper disc diffusion method.

Fungi	(R)-(+)-Limonene			(S)-(-)-Limonene			Orange extract			P value
	10µl	20µl	30µl	10µl	20µl	30µl	10µl	20µl	30µl	
C.albicans	3.5±2.12[b]	3.5±2.12	3.5±2.12	7±2.83	9.5±3.54	13±2.83	2±0	3.5±0.71	4.5±071	0.000
Penicillium sp.	0±0	0±0	0.5±0.71	1±0	2.5±0.71	3.5±2.12	0.5±0.71	3±1.41	3.5±0.71	0.016
Aspergillus sp.	0±0	0±0	1.5±0.71	2±0	3.5±0.71	6.5±0.71	0±0	0±0	0±0	0.000
A.niger	0±0	0±0	0.5±0.71	2±0	3.5±0.71	5±1.41	0±0	0±0	0±0	0.000

Table 4. The inhibitory effect of limonene and orange extracts on fungi in the paper disc diffusion method.

Fungi	(R)-(+)-Limonene			(S)-(-)-Limonene			Orange extract			P value
	10µl	20µl	30µl	10µl	20µl	30µl	10µl	20µl	30µl	
Candida albicans	1±2.12[b]	1.5±0.71	3.5±0.71	3.5±0.71	5±1.41	7±1.41	0±0	0±0	0±0	0.000
Penicillium sp.	0±0	1±0	1.5±0.71	2±0	2±0	3.5±0.71	0±0	0±0	0±0	0.000
Aspergillus sp.	0±0	0±0	0.5±0.71	6±0	7.5±0.71	9.5±2.12	0±0	0±0	0±0	0.000
Aspergillus niger	2±0	4±0	6±0	5±0	6±0	7.5±0.71	0±0	0±0	0±0	0.000

4. Discussion

The results of this investigation showed limonene has antifungal effect whereas orange peel extract did not showed this activity. Previous studies showed that limonene and orange peel extracts has anti microorganism effect especially on bacteria (Matsuoka et al., 1990; Raybaudi et al., 2006; Marostica et al., 2009). It has been demonstrated that limonene has a little antifungal effect (Chang et al., 2008). As limonene is volatile compound therefore this factor can reduce the antifungal effect of limonene or extracts during the course of study (Rangma et al., 1983; Pourbafrani et al., 2010). In other studies which performed on various organisms, it has been showed that limonene prevents the growth of fungal mycelium in two hours (Matsuoka ,1990). In a study which used paper disc diffusion method, the growth of various fungi such as *A. niger* and *penicillium* sp. was inhibited by limonene. Moreover it didn't have any inhibitory effect on *Candida albicans* (Yang et al., 2007).

Considering to high amount of limonene in orange peel (Pourbafrani et al., 2010), it is expected that the orange peel extract may have antifungal effect. In other study which used fungal suspension, the growth of *A. niger* inhibited by 3 microgram of orange peel extract (*Citrus sinensis*) containing 84.2% limonene (Sharma and Tripathi, 2008). The present study using disc diffusion method showed that the inhibitory effect of orange peel extract on fungal growth. These differences may be due to elimination of limonene in the extracts as the limonene is volatile component.

These results were similar to the results of investigations which used *Eugenia dysenterica*, (Cost et al., 2000). Therefore research on the other fungi at various temperature and also other methods, can elucidate this fact. According to the specific effect of the limonene on tested fungi and side effect of the antifungal chemical on environment (Aguin et al., 2006; Ishii, 2006), adding of this component to the foods prevents the foods and juices from decay.

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