Evaluation of Antifungal Activity of Some Plant Extracts and their Applicability in Extending the Shelf Life of Stored Tomato Fruits

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Abstract

Ethanolic extracts of tarragon (Artemisia dracunculus), rosemary (Rosmarinus officinalis L) and thyme (Thymus vulgaris L.) and the essential oil of oregano (Origanum vulgare L.) were tested against many fungi including A. niger, A. flavus, Penicillium spp., Rhizopus spp. and Fusarium spp. Oregano essential oil showed a very strong antifungal activity against Fusarium spp. (MIC: 0.8 mg/ml), A. niger (MIC: <1 mg/ml) and Penicillium spp. (MIC: 4.5 mg/ml) whereas other fungi showed more resistant. The results showed that Rhizopus spp. was the most sensitive fungus to the plant extracts whereas rosemary was the most effective plant. Application of essential oils or ethanolic rosemary extract with different concentrations (100, 500, 1000, 1500, 2000 ppm) and refrigeration storage (at either 5 or 25°C) for postharvest spoilage control of tomatoes for 4 weeks showed that the combination of two treatments (refrigeration with either oregano essential oil or rosemary extract) was more effective to keep quality of tomatoes.

Keywords: Artemisia dracunculus; Refrigeration storage; Ethanolic rosemary; Concentration

Introduction

Tomato (Lycopersicon esculentum) is an important commercial crop in the world. Nutritional values of tomato make it a widely accepted vegetable by consumers. However, tomato is a very perishable vegetable with a short shelf-life and high susceptibility to fungal disease during prolonged storage. Fruits are spoiled primarily by fungi due to their low pH which in addition to causing rot, may also contaminate the fruits by producing mycotoxins [1,2]. Tomato fruits are subject to attack by various fungal pathogens during harvesting, transportation and marketing postharvest disease caused by various pathogenic fungi. The main method to control fungi of post-harvest diseases is based on application of synthetic chemical products. Although the use of synthetic pesticides in plant protection had made a great contribution to plant protection, many are no longer used because of economic, environmental or health concerns, or due to development of resistant strains. However, nowadays consumers demand less use of synthetic chemicals and still expect food to be free from blemishes, microbial growth, toxins and other quality deteriorating factors [3,4]. Therefore, the scientific community at international level is looking for safer alternative products from plants to control pests during storage. Recently, medicinal plants and their extracts have gained importance as potential antimicrobial agents because they are generally assumed to be more acceptable and less hazardous than synthetic compounds [2]. Numerous studies have documented the antifungal properties of plant products [5-13]. In in vivo studies, emulsions of oils of thyme and oregano at 5000 ppm and 10000 ppm as dip treatments reduced disease development in tomatoes inoculated with Botrytis cinerea and Alternaria arborescens [14]. Additional experiments also concluded the fungicidal effect of essential oils against Alternaria alternata of tomatoes [12,15]. Al-Jabel Al- Akhdar province which is located in the eastern part of Libya has many widely predominant species of medical plants such as rosemary, oregano; thyme which is represents a rich source of potential disease control agents. Rosmarinus officinalis L. (family: Lamiaceae), commonly referred to as rosemary, belongs to mint family. It is a popular herb in many western countries, with global cultivation and an exceptionally wide usage in the Mediterranean countries from where it originated. Rosemary has a long list of claims pertaining to its medicinal usage including antibacterial [16] and antioxidant properties [17]. Oregano (Origanum vulgare L.), (Lamiaceae family), is widely known as a very versatile plant with many therapeutic properties (diaphoretic, carminative, antispasmodic, antiseptic, tonic) being applied in traditional medicine systems in many countries [18,19]. Thyme is (Thymus vulgaris L.) a perennial plant with a strong flavor. It is used as antiseptic and against inflammation. Tarragon (Artemisia dracunculus) is a perennial herb. It is traditionally used to prevent bacterial infection of wounds and to improve appetite, alleviate flatulence and to relief from the pain caused by arthritis and rheumatism [20]. The aim of this study was to assess the fungi toxic effect of some plant extracts against some fungi and to find out practical applicability of these extracts and essential oils in keeping quality of tomato fruits during storage.

Materials and Methods

Plant material

The tested plants including rosemary (Rosmarinus officinalis L), thyme (Thymus vulgaris L) and tarragon (Artemisia dracunculus) were collected from Al-Jabel Al- Akhdar province in late spring of 2008. Plant material were freed from foreign materials and carefully rubbed between soft cloths to remove dust and subjected to shade drying.

Preparation of ethanolic extract of tested plant

Aerial parts of plant materials (dried leaves) were ground in a mortar and ethanolic extracts were prepared by mixing 100 g of ground leaves of each plant with 500 ml of ethanol 96% using blender for 3 min. and the slurries were filtered twice through cheese cloth and ethanolic extracts were subjected to evaporation by rotary evaporator (Rota

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Vapor, Buchi, Zürich, Germany) to remove ethanol. The dry materials were dissolved in known values of ethanol and kept at 4°C until use.

**Extraction of essential oil**

The essential oils were extracted by hydrodistillation, for 4 h, using a Clevenger-type apparatus according to the method recommended in British pharmacopoeia [21]. For this, fresh plant leaves of sage or oregano (100 g) were thoroughly washed with sterilized distilled water and cut into small pieces. The plant material was then placed in round-bottom flask of the Clevenger apparatus for distillation (the ratio of plant material to water was 1:3). The collected oil was dehydrated by anhydrous sodium sulfate (Bio-RAD) and then stored in dark bottles at 5°C until use.

**Fungi**

Fungi used in this study (kindly were donated by department of plant protection, Omer El- Mukhtar University) represented the most locally predominant fungi that are associated with fruit and vegetable post-harvest diseases. They included *Fusarium* spp, *A. flavus*, *Penicillium* spp, *A. niger* and *Rhizopus* spp. To prepare spore suspensions, spores from six- to ten-day-old colonies of *Fusarium* spp, *A. flavus*, *Penicillium* spp, *A. niger* and *Rhizopus* spp, were collected by adding 5 ml of sterile water containing 0.1% (v/v) Tween 80 (for better spore separation) to each Petri dish and rubbing the surface with a sterile L-shaped spreader (3 times). The suspension was collected and then centrifuged at room temperature [22].

**In vitro antifungal assay**

Small glass dishes (diameter: 5 cm) containing potato dextrose broth (Oxoid, Basingstoke, Hampshire, England) with 10% of the ethanolic extract of each plant inoculated (10^5 spore/ml) with each fungus separately were used to evaluate the inhibitory effect of each plant extract after incubation at 25°C for 5 days. To determine the inhibitory effect of plant extracts, dry weight of the mycelium of each fungus was obtained by drying fungal mycelium at 70°C then weighing and comparing with controls (distilled water or alcohol).

**Antifungal activity of essential oils**

Antifungal activity of essential oil, was determined by using the solid medium diffusion procedure. For this, 1 ml of mould homogenous suspension (approximately 10^5 spores/mL) was uniformly spread on Petri dishes containing solid potato dextrose agar medium (PDA, Oxoid, Basingstoke, Hampshire, England). After inoculum absorption by medium, well were made using sterile glass stems (diameter 6 mm) which were filled with 50 µL of the essential oil solution at different concentrations. The plates were incubated for 7-10 days at 25°C. At the end of the incubation period, diameter of inhibition zones was measured. Each assay was performed twice and the results were expressed as the average of the three replications.

**Determination of minimum inhibitory concentrations (MIC)**

MIC values were performed by using the poisoned food technique of Perrucci et al. [9] with graded concentrations of rosemary extracts or oregano essential oil. For this varying concentrations of essential oil were separately dissolved in 0.5 ml of 0.1% Tween 80 (Merck, Darmstadt, Germany) then mixed with 9.5 ml of melting (45°C) potato dextrose agar medium (PDA, Oxoid Ltd, Hampshire, UK) before solidified into Petri dish. The prepared plates were inoculated aseptically with 6 mm plugs of four-to-seven –day old fungal culture. Petri dishes were placed in containers with filter paper moistened with water maintaining high relative humidity (RH—95%) during the inoculation period. The containers were incubated at 25°C for 6 days. At the end of the incubation period, diameter of mycelial growth was measured and MIC was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm. The control sets were prepared similarly using equal amounts of sterilized distilled water in place of the oil.

**Effects of rosemary extract and oregano oil on naturally infected development on tomato fruits**

Tomato fruits used in this study were obtained from a local market and selected for uniformity in size, appearance, ripeness and the absence of physical defects. Surface decontamination of tomato fruits by clipping used water was avoided as the study aimed to evaluate the effect of plant extracts and essential oils on reducing the development of natural fungal infection on tomato fruits. Different concentrations (100, 500, 1000, 1500, 2000 ppm) of ethanolic rosemary extract were prepared by dissolving the requisite amounts in sterilized distilled water. The same concentrations of oregano essential oil were prepared by dissolving the requisite amounts in 25 ml of 0.05% Tween-80 and then mixing with 475 ml of sterile distilled water. The control sets were prepared similarly using equal amounts of sterilized water in place of the essential oil [6]. Tomatoes were dipped into the solutions (rosemary or oregano essential oil) for 1 min at room temperature (~25°C) and air dried. Fruits were dipped into sterile distilled water, (to serve as control) and then air dried. Treated tomatoes (20 fruits per replicate) were placed in 1.5L plastic boxes and stored at either 5°C (for 4 weeks) or at 25°C (for 12 days). The percentage of infected fruits was recorded when about 50% of control fruits had decayed.

**Statistical analysis**

Statistical analysis of the data obtained in the present study was carried out in a completely randomized design layout with three replicates using SPSS software 20.0. Where significant difference between means was verified based on ANOVA, the comparison of means of different treatments was performed using Tukey's test at p=0.05.

**Results and Discussion**

**In vitro antifungal assay**

The inhibitory effects of alcohol extracts of rosemary, tarragon, thyme on the growth of each tested fungi was evaluated by determine dry weights of fungal mycelial grown in medium containing 10% of alcoholic extracts of each plant (Table 1). The results revealed that the antifungal activity greatly varied depending on plant and fungus species. It is evident from Table 1 that rosemary exhibited strong antifungal properties against some tested fungi as it significantly reduced mycelial dry weights of *Rhizopus* spp and *Fusarium* spp to 0.0261 and 0.060 gm respectively when compared to controls. Thyme extract caused a significant reduction in mycelial weight of *Rhizopus* spp whereas tarragon extract exhibited poor activity against tested fungi (Table 1). The lowest mycelial dry weights (0.0261, 0.0302 gm) were recorded for *Rhizopus* spp as results of the antifungal effects of rosemary and thyme extracts respectively suggesting the high susceptibility of the fungus to the tested plants. On the other hand, *A. niger, A. flavus* and *Penicillium* spp, were found to be more resistant to the plant extracts. The Minimum Inhibitory Concentration (MIC) values of rosemary were found to be 2.2 and 36.8 mg/ml for *Rhizopus* spp and *Fusarium* spp respectively confirming high sensitivity of *Rhizopus* spp to rosemary extract. Other researchers demonstrated that *Rhizopus*...
The inhibitory effect of different ethanolic plant extracts on the tested fungi. *Tested fungi were treated individually with ethanolic plant extracts. In each column, where the letters are the same, there is no significant difference between different treatments (P < %0.05) according to Tukey's multiple-range test. ± Indicate ranges in triplicate samples.

Table 1: The inhibitory effect of ethanolic plant extracts on the tested fungi. *Tested fungi were treated individually with ethanolic plant extracts. In each column, where the letters are the same, there is no significant difference between different treatments (P < %0.05) according to Tukey’s multiple-range test. ± Indicate ranges in triplicate samples.

Table 2: The inhibitory effect of oregano essential oil on tested fungi. *MIC: minimum inhibitory concentration: the lowest concentration shows an antifungal effect against tested fungus.

The inhibitory effect of oregano essential oils on tested fungi are shown in Table 2. The results revealed that the essential oil isolated from oregano showed antifungal activity but differences in microbial susceptibility were registered. Oregano oil showed a very strong inhibitory effect against *Fusarium* spp. (inhibition zone: 6.25 cm) (Table 2) followed by *A. niger* (inhibition zone: 5.3 cm) whereas other tested fungi showed either no or less effect compared with *Fusarium* spp. However, sage essential oil showed no effect against all tested fungi (data not shown). Other studies [33,34] reported antifungal activity of oregano essential oil against *Aspergillus*, *Fusarium* and *Penicillium* species. Pastor et al. [35] demonstrated antifungal activity of oregano essential oil on the mycelium and spores of *A. niger, A. flavus* and *A.ochraceus*. The results of this study agree with those of other authors, such as Bouchra et al. [7] and Baratta et al. [36] who also showed that the antifungal activity of oregano essential oil on *A. niger* is much stronger than that of sage. Martos [37] found that oregano essential oil showed the highest inhibition of mold growth, followed by clove and thyme. Carmo et al. [31] demonstrated that oregano essential oil exhibited a strong antifungal property against *Aspergillus flavus* and *A. niger* which disagree with this study as oregano oil had no antifungal activity against *Aspergillus flavus*. This may be attributed to the variation on chemical composition between the same essences which due to the fact that there are some factors influencing the chemical composition of the oils such as climatic, seasonal and geographical conditions, harvest period and distillation techniques [38]. In this study the antifungal activity of oregano essential oil seemed to be species-dependent as the MICs were lower for *Fusarium* spp (0.8 mg/ml) and *A. niger* (<1 mg/ml) when compared to that obtained for *Penicillium* spp (4.5 mg/ml) (Table 2).

**Effects of oregano essential oil on natural infection development on tomato fruits**

When tomatoes were treated with different concentrations of oregano extract and stored at 25°C or 5°C, the results showed that one treatment (1500 ppm) (Figure 1) was substantially suppressed fungal infected tomato fruits by 100% at 5°C for 4 weeks of storage period (Figure 1). Moreover, a reduction of 80% in infected tomatoes stored at 5°C was achieved when fruits were treated with rosemary extracts at 100 or 1000 ppm although this reduction were not significant compared to control (Figure 1). Rosemary extracts at 500, 2000 ppm enhanced fungal growth on tomatoes by 40%. However, most rosemary treatments exhibited no effect in preventing fungal infection on tomatoes stored at 25°C as naturally fungal infected fruits depressed only by 20% at 100 and 1000 ppm of rosemary treatments (Figure 1).

**Effects of oregano essential oil on natural infection development on tomato fruits**

When tomatoes were treated with oregano essential oil and stored at 5°C the results showed that all examined concentrations (with the exception of 1000 ppm), were completely inhibited (100%) fungal infection development on tested tomato fruits (Figure 2). However, no reduction in the fungal infection was achieved when treated tomato fruits were stored at 25°C for 12 days (Figure 2). It seems that there was a synergistic effect of oregano and refrigeration in preventing infection development on stored tomatoes as evident by the complete decaying of oregano treated tomatoes stored at ambient temperature (25°C).

It is well established [39,40] that carvacol is the major component of oregano, and many authors [41-43] have attributed the antifungal properties of oregano to this compound. Soylu et al. [44] determined the chemical compositions of some essential oils by GC-MS analysis and the major compounds found in essential oils of thyme, oregano, rosemary, were carvacrol (37.9%), carvacrol (79.8) and borneol (20.4%) respectively. Carvacol action mechanism has not been firmly established, although interaction with the cell membrane of the pathogen is thought to be likely [40]. Conner and Beuchat [45] suggested that the antimicrobial activity of the essential oils of herbs...
and spices or their constituents such as thymol, carvacrol, eugenol, could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds. Davidson [46] reported that the exact cause effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined, although it seems that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material functionality.

In this study, after 4 weeks of refrigeration, the cold storage (at 5°C) of treated tomatoes with either rosemary or oregano essential oil was extended to another 2 weeks and the experiment was stopped when at least 50% of control fruits had decayed. At the end of 2 weeks (the total duration of cold storage was 45 days) percentage of naturally infected tomato fruits was reported (Table 3). In general oregano oil and rosemary treatments reduced the fungal infection development by 80% (1000 ppm) and 20% (1000, 1500, 2000 ppm) respectively after a round 45 days of cold storage at 5°C (Table 3). However it seems that rosemary treatments at 100, 500 ppm initiated fungal colony development on tomato fruits to reach that reported for control samples.

| Treatment | % naturally infected tomato fruits | Predominant fungi on infected tomatoes |
|-----------|-----------------------------------|---------------------------------------|
| Controls  | 100                               | A. solani                             |
| Rosemary (ppm) |                             |                                       |
| 100       | 100                               | A. solani                             |
| 500       | 100                               | A. solani                             |
| 1000      | 80                                | Alternaria spp.                       |
| 1500      | 80                                | Alternaria spp.                       |
| 2000      | 80                                | Alternaria spp.                       |
| Oregano essential oil (ppm) |                             |                                       |
| 100       | 60                                | A. solani                             |
| 500       | 80                                | A. solani                             |
| 1000      | 20                                | A. solani                             |
| 1500      | 80                                | A. solani                             |
| 2000      | 40                                | A. solani                             |

Table 3: % of naturally fungal infected tomato fruits after 45 days of storage at 5°C. *After 4 weeks of refrigeration, cold storage at 5°C of treated tomatoes with either rosemary or oregano essential oil was extended to another 2 weeks. Experiment was stopped when 50% of control fruits had decayed then the percentage of naturally infected tomato fruits was calculated.

(100%) (Table 3). Tzortzaki [22] found that cinnamon essential oil promoted the spore germination of *Aspergillus niger* by 40% compared with control. In present study the most predominant fungi on naturally infected tomatoes at the end of storage period (45 days at 5°C) was identified and found to be *Alternaria solani* or *Alternaria* spp (Table 3). It is well known that among of the most important fungi causing post-harvesting diseases of plant are *Aspergillus* spp. *Alternaria* spp. and *Rhizopus stolonifer* [24]. *Alternaria solani* is the most destructive and the main pathogen causing early blight disease and yield losses in numerous ecumenically important crops such as potato, tomato and eggplant crops [10,24,47]. The resistance of *A solani* to plant extract has been reported by researchers [24] and controlling plant diseases caused by *Alternaria* spp can be difficult because it spread so readily [48]. In this study the variation on the percentage of naturally infected tomato fruits after 45 days of cold storage indicated the effectiveness of oregano treatments in retarding infection with *Alternaria solani* and *Alternaria* spp and extending the cold storage of tomato fruits (Table 3) [49].

In this study the results showed that some lower concentrations were more effective than higher. This phenomenon was reported by other researchers; Tzortzaki [22] revealed that % spore germination of *Botrytis cinerea* was significantly lower (90%) at 50 ppm of cinnamon oil than that reported at 100 ppm (94%). Similarly a concentration of 25 ppm reduced % spore germination of *A.niger* to around 50% whereas a higher concentration of 50 ppm reduced the % spore germination to only 18% [10]. Bosquez-Molina [23] observed that increasing concentrations of thyme or Mexican lime essential oils led to greater infection in treated papayas. In current study, after 45 days of cold storage of tomatoes the efficacy of oregano treatments in preventing natural fungal infection on refrigerated stored tomatoes was found to be higher than that of rosemary extract as lowest percentage of infected tomatoes (20%) was achieved at 1000 ppm of oregano oil (Table 3). However, the same concentration of rosemary failed to prevent decaying of refrigerated fruits. This may be attributed to the fact that essential oils usually exhibit a stronger antimicrobial activity than alcoholic extracts [13,50,51]. This discrepancy in exerting the antibacterial potential may be caused by a variation that concerns the chemical composition as this one is determined at a greater extent by the distillation and extraction technique or by the different geographical chemotype [50].
Conclusion

Oregano oil and rosemary showed promising prospects for the utilization of natural plants or their oils and extracts in post-harvest disease control. In vivo experiments, oregano essential oil showed enhancement in the shelf life of cold stored tomatoes by reducing post-harvest disease control. Therefore essential oils can be used as a potential source of sustainable ecofriendly botanical fungicides, after successful completion of wide range trials. Further studies are required to determine the active components of oregano essential oil and rosemary extract that responsible for the antifungal property and the potentially use for fumigation in cold storage or for active packing.

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