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Original article

Antifungal effect of edible film (CMC) containing aqueous and ethanolic mangrove plant extract on Citrus pathogens in vitro

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ABSTRACT

Plant extract have superior antimicrobial activity. Some plant extracts exhibit strong antifungal activity against undesirable fungi in foodstuffs during storage period. Generally, phenolic and terpene compounds are major contributors to this action. In this study, edible films (CMC) containing mangrove plant was evaluated for their activity against citrus pathogens. Mangrove leaves were dried in shadow and suitable condition, after extraction with ethanol 96 degree and water. Antimicrobial properties of carboxy methyl cellulose (CMC) films containing of 20, 40, 60 and 80 percent concentration of the extract against *Penicillium digitatum* and Alternaria *citri* was studied. The results show of the effect antimicrobial edible films containing of extracts ethanolic Avicennia marina at all concentrations have inhibition effect on growth of Alternaria citri, However aqueous extract of Avicennia marina leaves were able to inhibitory of growth the Alternaria citri only in 40, 60 and 80% concentrations and no antimicrobial activity was observed at 20 concentrations. The edible films containing mangrove extract presented the more effective impact on the growth of Penicillium *digitatum* than Alternaria *citri* ($p<0.05$). The results suggest that the food industry and consumers could use these films as wrappings to control surface contamination by mold pathogenic.

1. Introduction

Mangrove and mangrove associates contain biologically active antiviral, antibacterial and antifungal compounds (Bandaranayake, 2002). They provide a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins. Therefore, it is worth to screen mangrove plants for the presence of new antibacterial compounds to combat the pathogenic bacteria and fungi. A. marina (Forssk.) is commonly known as gray mangrove tree classified in the plant family Avicenniaceae, and is commonly used for ulcers.). Avicenniaceae family is a member of true mangrove plants, which has one genus, 11 species and several sub species. *Avicennia* marina (Forssk) Vierh is the most current species among these plants in Iranian mangrove forest. CMC is an anionic linear polysaccharide derived from cellulose. It is an important industrial polymer with a wide range of applications in flocculation, drug reduction, detergents, textiles, papers, foods, and drugs (Biswal and Singh., 2004). CMC is used primarily because it has high viscosity, is non-toxic, and is non-allergenic. The numerous hydroxyl and carboxylic groups in CMC enable water binding and moisture sorption properties. CMC hydrogel has a high water content, good biodegradability, and a wide range of applications due to its low cost (Nie etal., 2004).

Antimicrobial edible films may supply an effective way to control food-borne pathogens and spoilage microorganisms to thus enhance food safety and reduce product spoilage. The use of edible films as antimicrobial carriers represents an interesting approach for the external incorporation of plant extract onto food system surfaces. Mangrove plants are a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins. Extracts from different mangrove plants are reported to possess diverse medicinal properties such as antibacterial. Alternaria is a member of imperfect fungi, family Dematiaceae. It has different specious induced sever disease on plant, toxic substance, carcinogenic and produce allergic and respiratory disorder. Alternaria fungi cause two different diseases on citrus in Mazandaran province, North of Iran: Alternaria brown spot of tangerine hybrids and Alternaria black rot of the Navel orange fruits. Black rot of fruit, a post harvest disease is caused by Alternaria, occurs through all citrus growing regions, but is rarely abundant enough to cause economic losses in Iran. *Penicillium digitatum* is the most devastating pathogen of citrus fruit, being responsible for about 90% of production losses during post-harvest handling.In spite of the application of fungicides and the increased implementation of new biological control strategies green mold continues to exhibit high infection pressure on stored citrus commodities worldwide (Droby etal., 1998). The purpose of this study the antimicrobial effect of various concentrations of mangrove leaves on Alternaria *citri* and Penicillium *digitatum* is the most devastating pathogen of citrus fruit to increase the storage time of citrus fruit.

2. Materials and methods

2.1. Plant material

The leaves of *Avicennia Marina* were collected from the mangrove forests of *Qeshm* Iran, which extends from 26°50'N and 56°0'E. Branches and leaves of the plants chosen were cleaned with tap water, dried for 72h dried in shadow and appropriate condition, and then filtered using a 60-mesh sieve.

2.2. Extract preparation

Maceration method was used to prepare extracts. The amount 50 gram of *Avicennia Marina* leaves powder was Added to 250 ml ethanol 96 degree or distilled water. The ethanolic extract mixture was preserved at laboratory temperature for 24 hours and was stirred every few hours with a glass rod. The aqueous mixture was boiled for 20 minutes with low flame until the cream colored liquid was obtained. The collecting supernatant was centrifuged by 3000 rpm for 10 min. The resulting extract (supernatant) volume has reached to the original with ethanol or distilled water, and then samples were stored into the dark container at refrigerator temperature after filtering by 0.45 µ Whatman filter paper (Ahmad and beg., 2001).

2.3. Preparation of film solutions

Films were prepared according to Wang et al. (2007). CMC, were solubilised with distilled water. Glycerol was added as plasticizer to each solution at a constant glycerol: powder ratio of 1:2 (w/w). Glycerol solutions (glycerol and distilled water) were preheated at the designated heating temperature for 5 minutes. All solutions were

stirred continuously on a magnetic stirrer hotplate, until powders were completely dissolved. Solutions were placed in 60 and 80°C water bath, held for 30 minutes and subsequently cooled to 40°C.

2.4. Test microorganisms

Two type of mold as a spoilage microorganism *Alternaria citri* and *penicillium digitatum* as a pathogen which are mainly important in the fruit and vegetables area have been selected *Alternaria citri* and *penicillium digitatum* has been isolated from rotten orange using a different dilution method.

2.5. Inucolum preparation

Fungi *Alternaria citri* and *penicillium digitatum* was maintained on Sabouraud dextrose agar. Sterile distilled water containing 0.05% Tween 80 was added to the surface growth and spores and hyphae were scraped off with a sterile wire loop. A spectrophotometer set at 530 nm used to adjust the suspension to 90% transmittance. This resulted in a concentration of about 1×10^6 CFU/ml (Collins *et al*., 1995).

2.6. Antimicrobial activity

The antimicrobial assay was performed by methods viz. agar disc diffusion method for solvent extract. The SDA agar was inoculated with 100 µl of the inoculums (1×10^8 cfu/ml) and poured into the Petri plate. For agar disc diffusion method, the disks (7 mm diameter) cut from the films were placed on SDA (Merck) plates were saturated with 100 µl of the test compound allowed to dry and was introduced on the upper layer of the seeded agar plate. The plates were incubated72 h at $27C^{o}$. Microbial growth was determined by measuring the diameter of zone of inhibition. For each fungi strain, controls were maintained where pure solvents were used instead of the extract. The result was obtained by measuring the zone diameter. The experiment was done three times and the mean values are presented (He and Zhou 2007).

2.7. Statistical analysis

All the assays were carried out in triplicates. The experimental results were expressed as mean ± standard deviation. The data were analysed using one way analysis of variance (ANOVA) using SPSS version 17.

3. Results

The results of the edible films antimicrobial effects of ethanolic and aqueous *Avicenna marina* extracts, by "the agar diffusion method" are presented in (Tables 1, 2 and Figure 1, 2). The results show that mangrove leaf ethanolic extracted at all concentrations (20, 40, 60 and 80%) had the inhibitory effect on *Alternaria citri* and *Penicillium digitatum*. The results show that mangrove leaf aqueous extracted at all concentrations (20, 40, 60 and 80%) had the inhibitory effect on *Penicillium digitatum*. The results show that mangrove leaf aqueous extracted at concentrations (40, 60 and 80%) had the inhibitory effect on *Alternaria citri* ,However, 20% concentration aqueous extracts, have no significant antimicrobial effect on *Alternaria citri* and it is not able to prevent the growth of fungi on culture.

Table 1
average diameter (mm) of microbial free zone area of edible films by ethanolic *Avicenna marina* leaves extract on *Alternaria citri* and *Penicillium digitatum* (disk agar diffusion method)

Microorganism	***Alternaria citri***			
Avicenna marina concentration	20	40	60	80
Average diameter (mm) of microbial free zone area	7.5±0/5774	8.4±0/2887	10.1±0/5774	11.9±0/2887
Microorganism	***Penicillium digitatum***			
Avicenna marina concentration	20	40	60	80
Average diameter (mm) of microbial free zone area	8.2±0/2887	9.7±0/5774	11.6±0/2887	13.1±0/5774

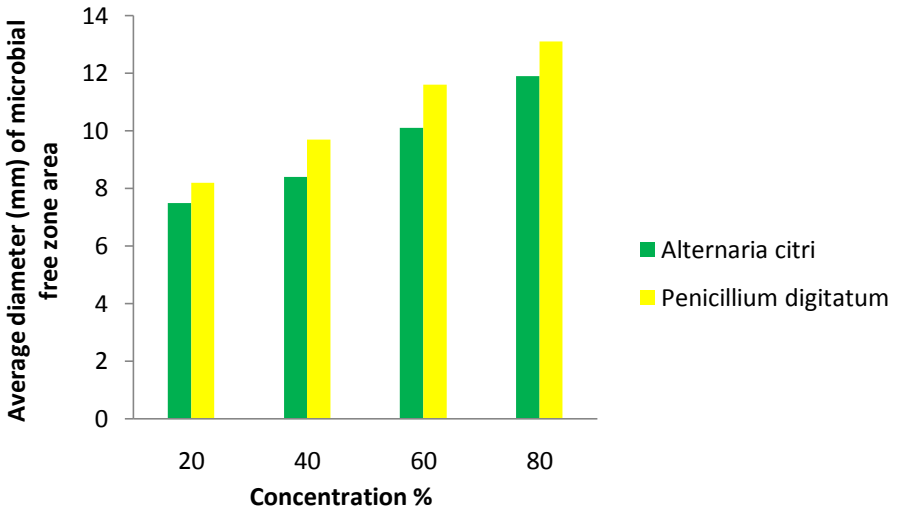

Fig. 1. Antifungal activity of edible films by ethanolic Avicenna marina leaves extract.

Table 2
average diameter (mm) of microbial free zone area of edible films by aqueous *Avicenna marina* leaves extract on *Alternaria citri* and *Penicillium digitatum* (disk agar diffusion method)

Microorganism		***Alternaria citri***		
Avicenna marina concentration	20	40	60	80
Average diameter (mm) of microbial free zone area	_	7.6±0/2887	8.9±0/5774	10±0/2887
Microorganism		***Penicillium digitatum***		
Avicenna marina concentration	20	40	60	80
Average diameter (mm) of microbial free zone area	7.2±0/2887	8.4±0/5774	9.6±0/2887	11.1±0/5774

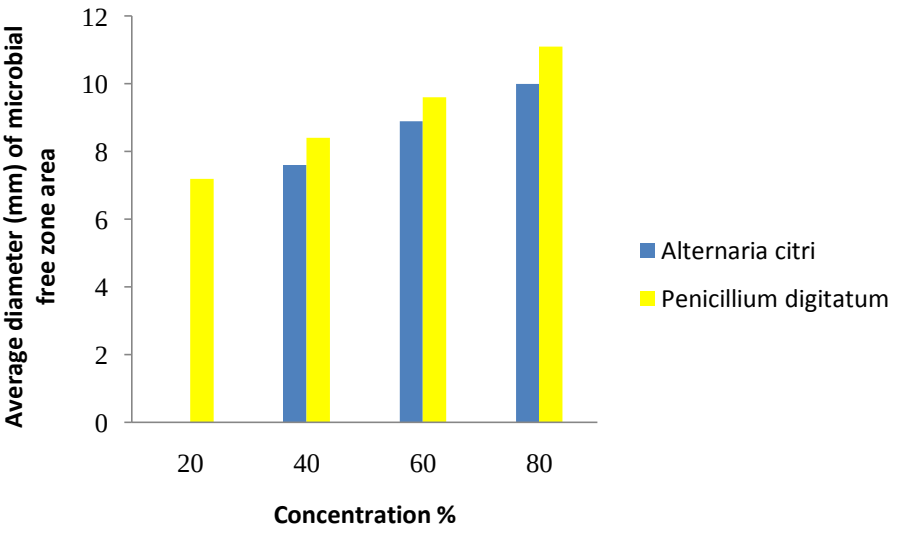

Fig. 2. Antifungal activity of edible films by aqueous Avicenna marina leaves extract.

4. Discussion

The use of edible antimicrobial films on fruit and vegetable can offer the following advantages to the consumer: prevention of moisture loss; thereby avoiding cross contamination; and browning (discoloration); reduction in microbial load; and prevention of losses of volatile flavors and foreign odor pickup.

Molds have powerful arsenal of hydrolytic enzymes which can cause a high degree of deterioration of edibles and responsible for considerable economic losses. Food contamination with fungi especially its toxigenic strains producing of secondary metabolites is a major concern, which has received worldwide attention due to their deleterious effects on human and animal health as well as their importance in international food trade (Kumar etal., 2010). Plants are rich in a wide variety of phytochemicals like tannins, terpenoids, alkaloids, flavonoids, antimicrobial peptides, etc., that have been found to have antimicrobial activities (Weimann and Heinrich, 1997; Atindehou *et al*., 2002; Edeoga *et al*., 2005). The results showed of the effect antimicrobial edible films containing of extracts aqueous and ethanolic *Avicennia marina* at all concentrations have inhibition effect on growth of *Penicillium digitatum*. The results showed of the effect antimicrobial edible films containing of extracts ethanolic *Avicennia marina* at all concentrations have inhibition effect on growth of *Alternaria citri,* However aqueous extract of *Avicennia marina* leaves were able to inhibitory of growth the *Alternaria citri* only in 40, 60 and 80% concentrations and no antimicrobial activity was observed at 20 concentrations, the advantages of using an edible film with extract plant for food products are that it may be easy to use and it may be able to enhance quality and extend the shelf life while reducing packaging waste. Moreover, in this regard, extract plant is a valuable component for processing biodegradable packaging which can extend shelf-life and inhibit pathogens and spoilage. A pathogen, gram negative bacteria *Escherichia coli* and a rotten fungi, *Penicillium digitatum* has been affected by mangrove extract using disc inhibitory zone (Amirkaveei and Behbahani, 2011).

The results show that mangrove leaf extracts at all concentrations (20, 40, 60 and 80%) had the inhibitory effect on *Alternaria citri* and *Penicillium digitatum,* antifungal metabolites Mangrove plant leaves include alkaloids, flavonoids and related compounds, varied fatty acids, oxygen heterocyclics, proanthocyanidins, Quinones, stilbenes, terpenoids and triterpenoid saponins (Bandaranayake, 2002). The latex showed no against bacteria and yeast but some against fungi. The leaves were a rich source of a different class of terpenoids and with stilbenes, inhibited histamine release from rat mast cells and were active against Bacillus and Staphylococcus (Bandaranayake, 2002).

Also, ethanol extract compared to the aqueous extract was more effective and has a greater deterrent. The reason of these phenomena may be extracting more effective materials extracted by ethanol from *Avicennia marina* (Alizadeh Behbahani etal., 2012). These results are consistent with the findings of a study by Mahasneh (2002) on Qataris mangrove species and it was found the aqueous mangrove extract, did not have a significant antimicrobial effect, and the butanol extract, is able to inhibit *Pseudomonas aeruginosa*. Tian *et al*., 2009 investigated the antibacterial effects of “Galla chinesis” (a medicinal plant native to China) reported that the juice extracted by the solvent ethyl acetate, ethanol and water are the highest antibacterial effect. In this study the plant extracts were showed that gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Bacillus Subtlis*) were more sensitive than gram negative bacteria (Escherichia coli, Shygyla Dysantrya). This result was consistent with the findings of this study (Tian *et al*,. 2009). Mangroves are widely used by mangrove dwellers for bush medicine, e.g. A. illicifolius is used for skin disorders, boils and wounds. Many medicines derived from mangroves (ashes or bark infusions) can be applied for skin disorders, e.g. Lumnitzera racemosa and sores, including leprosy. They have been reported to treat different kinds of diseases (headaches, boils, ulcers and diarrhoea). Common uses of mangroves in bush medicine are reviewed by Bandaranayake (Bandaranayake, 2002).

Xanthone is an active substance in these plants. These compounds have toxicological characteristics, such as, anti-tumor, anti-inflammatory, anti-fungal. The fact the results of this study showed that extract of mangrove show antifungal properties justify their traditional use as medicinal plants.

5. Conclusion

Fungal contamination of food products is a chronic problem in developing countries and it leads to a decline in quality and quantity of foodstuffs. Some plant or spice extract plant can be used food preservatives due to their strong antimicrobial activity. The application of edible or coating films containing extract plant can improve food

safety by eliminating fungal spread, and they also leave no detectable residues after storage. The incorporation of extract plant in edible films would be an alternative antifungal application to control of fungal growth.

Avicennia marina plants may have potential medicinal importance it can suggest that *Avicenna marina* leaf extract in In-vitro, have considerable antimicrobial ability over the studied strains. In addition, more studies are needed in In-Situ be done, to identifying the effective dose of the extract on the microorganisms, and finally introduce the extract as a natural and novel antimicrobial compound.

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