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Effects of aqueous extract of *Cinnamomum verum* on growth of bread spoilage fungi

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ABSTRACT

Food waste has been identified as a considerable problem and bread is the most wasted food. This study aimed to evaluate In-vitro anti-fungal activity of cinnamon extract on bread spoilage fungi and to determine its anti-fungal effect in the bread slices. At first, the MIC and MFC values of the extract were determined against Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Penicillium chrysogenum, Penicillium notatum and Rhizopus oryzae. Then, Aspergillus sp was selected to assess antifungal activities of different doses of cinnamon extract in bread slices. Cinnamon extract at a dose of 64 mg/ml completely inhibited all standard and bread isolated fungi. This concentration of extract also inhibited Aspergillus growth on bread slices and delayed colony formation but adversely affected the sensory characteristics of bread. Cinnamon extract at 32 mg/ml not only delayed fungal growth, but also improved bread shelf life and delayed its staling. Moreover, 32mg/ml of extract did not adversely affect bread aroma, flavor and texture. However, sodium acetate inhibited the growth of Aspergillus sp but is not recommended for fungal control because it is considered as chemical. Therefore 32 mg/ml of extract is recommended for increasing the shelf-life of flat bread.

Key words: Aqueous extract of Cinnamomum, Bread spoilage fungi**,** MIC, MFC

INTRODUCTION

Food waste is a serious problem in Iran and bread is the most wasted food products. Mold spoilage is one of the main causes of bread waste. In addition to food waste and economic losses, it can cause food safety problems due to aflatoxin production [1].

Aflatoxin B1 is the most common and highly toxic aflatoxin which is generally found in breads. After entering the body, it is metabolized to aflatoxin M1 and M2. Bansod et al [2010] observed that there was a linear relationship between the amount of aflatoxin M1 and M2 in cow's milk and aflatoxin B1 in cow's diet. Fungal aflatoxins are heat-stable and not inactivated by pasteurization [2]. They are also found in all products made from raw milk [3].

Today, due to increased use of bread waste in animal husbandry, much attention has been paid to find new methods to keep bread mold-free. Various chemicals have been suggested to overcome mold growth in cereal and breads [4]. Because of possible adverse effects of these chemicals on yeast activity, they are commonly used in products that don't rely on fermentation. Yeast fermentation has an important role in bread production and therefore, chemicals with minimal effects on yeast activity should be selected for breads preservation. Sodium acetate has been recognized as a suitable compound for prevention of mold spoilage in the breads. Sodium acetate is the sodium salt of acetic acid and contain 40% of free acetic acid [5]. Today, due to increased public concern about chemical preservatives researcher attention has been shifted toward natural products.

Cinnamon scientifically known *as Cinnamomum verum* is a small evergreen tree belonging to the family Lauraceae. It is a medium sized tree that reaches 5-7 meters in height. Cinnamon is native to Sri Lanka and its bark is widely used as a spice. Cinnamon has been reported to have antifungal, antiviral, antibacterial activity. Antibacterial and antifungal effects of cinnamon essential oil may be related to the ortho-methoxy cinnamaldehyde. It has been reported that fungal spores can't reproduce in medium containing cinnamon [6].

Studies conducted by different researchers showed that cinnamon essential oil has the ability to inhibit mold growth on bread [7]. Soleiman and Badea [2011] reported that cinnamon essential oil at a dose of 500 mg/ml can prevent the growth of *Aspergillus flavus, Aspergillus parasiticus, Aspergillus ochraceus and Fusarium moniliforme* in PDA medium[8].

Biological activities of Cinnamon oil have been attributed to its active compounds. The major components of cinnamon essential oil include trans-cinnamaldehyde, 3-methoxy-2-propanediol, methyl eugenol and cinnamic acid. The antifungal and antioxidant activity of these compounds have been demonstrated in previous studies. Trans-cinnamaldehyde, an aromatic aldehyde obtained from bark of cinnamon, exhibits significant inhibitory effect on Aspergillus [1]. Many species of Aspergillus sp, especially *A. flavus* and *A. parasiticus* produce poisonous fungal toxins called mycotoxins. These toxins are associated with cancer, neurological damage, birth defects, sterility, and immune dysfunction [9]. Aspergillus resistant to antifungal agents, bring a worrying clinical prognostic in immune suppressed people [4].

This study aimed to evaluate inhibitory effect of cinnamon extract on different fungal strain in broth and bread model

MATERIALS AND METHODS

Preparation of *cinnamon extract*

The soaking procedure was used to prepare *Cinnamomum cassia* extract. 25 grams of cinnamon bark were dried in open air at room temperature and finely milled. The obtained powder was poured into a 1 liter flask and mixed with 300 ml of distilled water. The extract was filtered through a smooth filter paper and concentrated by using a rotary evaporator. The prepared extract was then mixed with Tyrode buffer containing KCl, NaCl, HEPES, NaH_2PO_4, H2O and glucose at a 1:1 ratio. The extract was sterilized by passing through a 0.4 micron filter and kept in a refrigerator at 4 °C [11].

Determination of the dry weight of the extract

One ml of extract was transferred into a pre-weighed container and heated at 50 °C to dryness. After 24, the container was re-weighed and extract dry weight was calculated from the weight difference between the final and initial weight of the container. The process repeated tree times and average was calculated [11].

Preparation of mold suspension for Inoculation

Aspergillus niger [PTCC 5012], *Aspergillus flavus* [PTCC 5006], *Aspergillus fumigatus* [PTCC 5009], *Penicillium chrysogenum* [PTCC 5021], *Penicillium notatum* [PTCC 5074] and *Rhizopus oryzae* [PTCC 5174] from Iranian Research Organization for Science and Technology, Tehran, Iran, were used in this study. They were cultivated on Sabouraud dextrose agar [SDA; Merck] containing Cholramphenicol antibiotic and incubated at 27 °C for 24 h. Mold suspension were prepared from cultivated molds and molds isolated from moldy bread. For this purpose, 500 ml of phosphate buffered saline [PBS: pH =7.2] was added to a 1ml micro-tube. A small amount of grown colonies on SDA was then suspended in PBS using an inoculating loop. After mixing, the number of cells was counted under microscope using a Neubauer lam, and a cell suspension with a density of 1×10^6 cell per ml that gives 100 cells per lam or absorbance of 0.08 at 630 nm was prepared [NCCLC, 2000].

Determination of MIC and MFC values using Macro Dilution Method:

Minimum inhibitory concentration [MIC] and minimum fungicidal concentration [MFC] are commonly used to assess the susceptibility of fungi to plant extracts, essential oils and antibiotics. They also help to find effective concentration of these agents against selected fungi. In this experimental study, serial dilutions of extracts were prepared in 10 sterile tubes. 1ml of various concentrations of the extract [0, 2, 4, 8, 16, 32, 64, 128, 256, 512 mg/ml] was added to each tube. Then 1 cc of each fungal suspension was added to each tube. A tube containing SDB medium and fungal suspension but no extract was considered as the negative control. Two positive controls were

used in this study. One of them had 1 mL of SDB medium, 1ml of fungal suspension and ml of fluconazole at its effective dose. Another had 1 mL of SDB medium, 1ml of fungal suspension and 1ml of serially diluted sodium acetate. All the tubes were then incubated at 25-30°c for 24-48 h. After incubation, the test tube with the lowest dilution displaying no visible growth when checked visually was considered as the MIC. Afterwards, 100 µl of content of each transparent tube exhibiting no turbidity were cultured on the SDA. After incubation, the first plate without fungal growth was considered as MFC point. All the tests were performed in three repetitions [NCCLC, 2000].

Preparation of breads

Flour used in this study was purchased from local market and active dried yeast was obtained from Iran-Mayeh factory, Tehran, Iran. One group of bread was prepared from flour, water, salt, active dry yeast and sodium acetate while another group was prepared from flour, water, salt, active dry yeast and aqueous extract of cinnamon 32 and 64 mg/ml. Amounts of raw materials required for preparing bread dough on the basis of flour include 55.7% water, 2% salt and 3% active dried yeast. Amount of water needed was determined based on Farinograph data. Sodium acetate and cinnamon extract concentrations were chosen based on preliminary tests and experimental cooking of bread. In order to determine best condition of baking, several preliminary experiments were performed before the main experiment.

After weighing of raw materials, they were put into the mixer and mixed. The prepared dough was kept at an appropriate fermentation temperature for 3. Then, dough was divided into round pieces and kept at an appropriate temperature for another 45 minutes. After placing the dough balls on a baking tray, it was transferred to the oven having the temperature of 200 ºC. The time required for baking the breads was 25 minutes. After baking, the breads were transferred to the laboratory and cooled, and after reaching the desired temperature, they were cut into pieces 6 × 6 cm with a sterile knife and placed into sterile petri dishes.

Inoculation of bread slices with fungal cultures:

Center of bread slices were inoculated with Aspergillus sp using a sterile loop. Petri dishes containing inoculated bread slices were incubated at 25± 2°c. They were observed for colony formation and everyday colony diameters were measured in mm by ruler. The surface area of the mold was calculated from the measured diameter. Percent of moldy areas was calculated by dividing the surface of the mold to the surface of the bread. Measurements were continued until all the bread surface was covered with the mold. In this study, *Aspergillus sp* was selected for the assessment of antifungal activity of cinnamon extract because this genus showed the maximum growth rate when compared to the other strains. All the experiments were repeated three times [12].

Statistical analysis:

A completely randomized design was used in this study and experiments were repeated three times. Data were subjected to statistical analysis using $SPSS_{16}$ software. One-way ANOVA used to determine significant differences between means. $p<0.05$ was considered statically significant. Charts were drawn using a Excel $_{2007}$ software.

RESULTS

Antifungal effects of cinnamon extract

Effects of Cinnamon extract on Aspergillus strains:

Effects of different concentrations of Cinnamon extract on Aspergillus strains isolated from bread are shown in figure 1A. Results showed that different concentrations of aqueous extract of cinnamon bark had different effects on different strains of Aspergillus. In this study, growth of all stains were completely inhibited by Cinnamon extract at a dose of 64 mg/ml. Cinnamon extract at concentrations of 32 and 16 mg/ml completely inhibited *A. fumigatus* and *A. flavus* growth and significantly decreased A. *niger* growth. Aqueous extract of Cinnamon bark at doses of 4 and 8 mg/ml significantly decreased all stains growth compared to the control. *A. niger* showed relatively higher resistance to extract compared to the other strains. Effects of different concentrations of Cinnamon extract on standard strains of Aspergillus are shown in figure 1B. Aqueous extract of cinnamon bark at a dose of 64 mg/ml completely inhibited all standard stains of Aspergillus. Cinnamon extract at 32 mg/ml resulted in complete inhibition of the *A. flavus* and *A. niger*. It also resulted in 50% inhibition of the *A. fumigatus*. Among tested standard strains, *A. fumigatus* showed higher resistance compared to the other strain

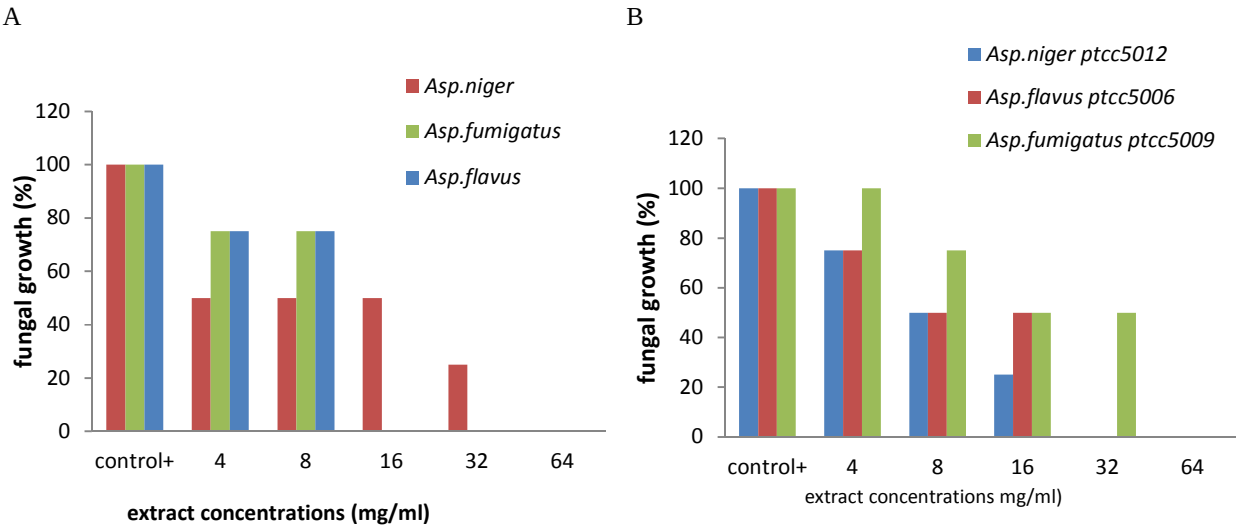

Figure 1: Effects of different concentrations of Cinnamon extract on: A] Aspergillus strains isolated from bread, B: standard stains of Aspergillus

Effects of Cinnamon extract on *Penicillium* strains:

Figure 2A, shows the effect of different doses of Cinnamon extract on standard strains of *Penicillium.* The lowest concentration of extract that completely inhibited the growth of *P. notatum* was 16 mg/ml while the lowest concentration that completely inhibited *P. chrysogenum* growth was 32 mg/ml. Cinnamon extract at doses of 4 and 8 mg/ml also reduced growth of *P. chrysogenum* and *P. notatum* in comparison to the control. Figure 2B, shows the effects of different doses of Cinnamon extract on *Penicillium* strain isolated from bread. Cinnamon extract at doses of 16 and 32 mg/ml completely inhibited *P. chrysogenum* but did not inhibit *P. notatum* completely.

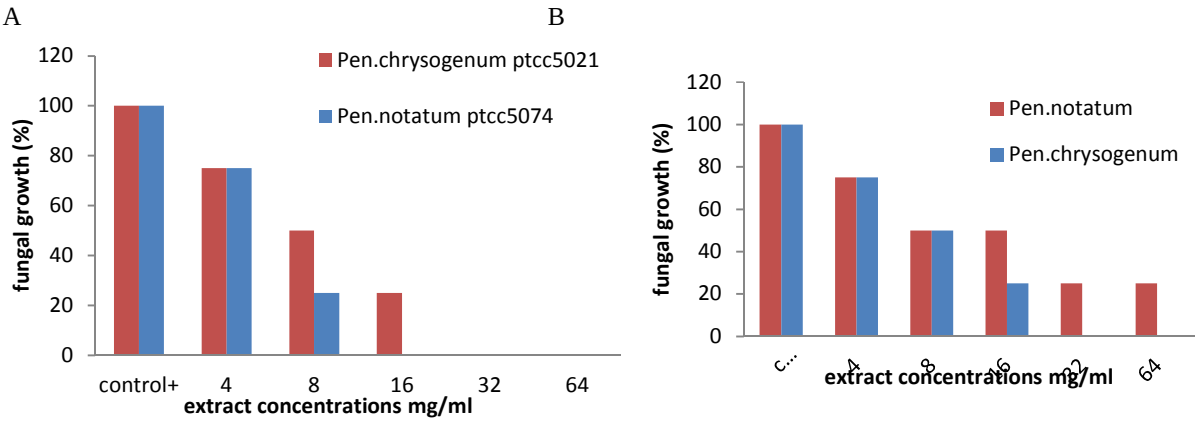

Figure 2: Effects of different concentrations of Cinnamon extract on: A] standard stains of Penicillium, B] Penicillium strains isolated from bread

Effects of Cinnamon extract on Rhizopus strains:

Figure 3, shows the effect of different doses of Cinnamon extract on standard strain of *Rhizopus.* Different concentrations of extract did not inhibit *R. oryzae* completely. However, Cinnamon extract at doses of 32 and 64 mg/ml reduced *R. oryzae* growth compared to the control.

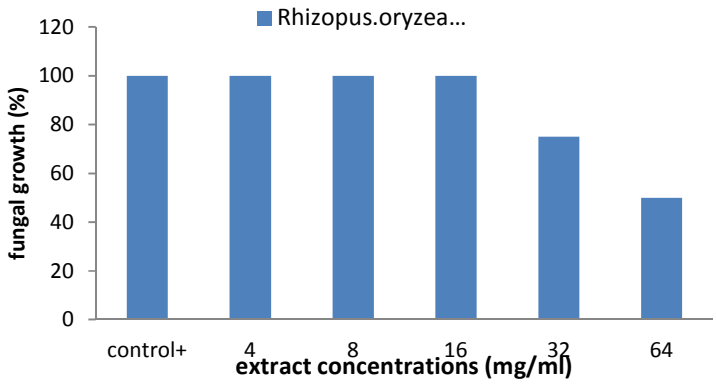

Figure 3: Effects of different concentrations of Cinnamon extract on standard strain of Rhizopus

Antifungal effects of sodium acetate

Effects of sodium acetate on Aspergillus strains:

Effects of different concentrations of sodium acetate on standard strains of Aspergillus are shown in figure 4A. At higher concentrations sodium acetate completely stopped the growth of the standard strains of Aspergillus. Sodium acetate at concentrations of 0.32 and 0.16 mg/ml completely stopped the growth of tested fungus. There was significant difference between inhibitory effects of different concentrations of sodium acetate [$p<0.05$]. As shown in figure 4B, 0.32 mg/ml of sodium acetate suppressed the growth of all three strains of Aspergillus isolated from bread. Sodium acetate at a concentration of 0.16 mg/ml completely inhibited the growth of *A. fumigatus* but failed to inhibit other tested strains. Sodium acetate showed significant dose-dependent inhibitory effects on the fungal growth and their growth significantly decreased by increasing sodium acetate concentrations.

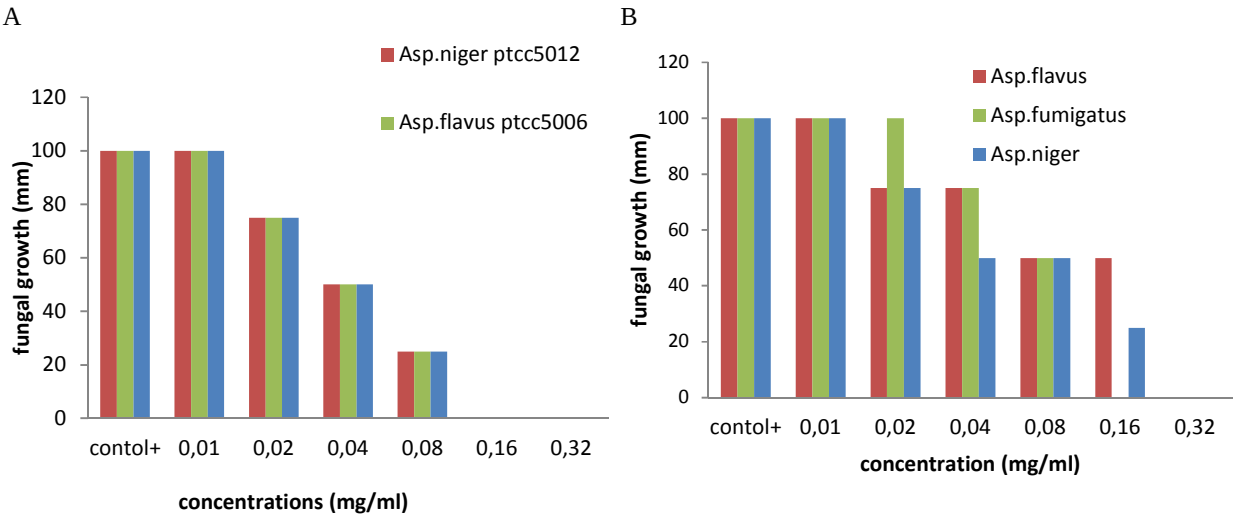

Figure 4: Effects of different concentrations of sodium acetate on: A] standard strain of Aspergillus, B] on Aspergillus strains isolated from bread

Effects of sodium acetate on Penicillium strains:

As shown in figure 5A, Sodium acetate at concentrations of 0.32, 0.16 and 0.08 mg/ml completely stopped the growth of standard strain of *P. chrysogenum* but failed to stop *P. notatim* growth. According to the statistical analysis, the inhibitory effect of sodium acetate significantly increased by increasing its concentrations [$p<0.05$]. Different concentrations of sodium acetate did not completely inhibit the standard strain of *P. notatum*. Inhibitory effects of different concentrations of sodium acetate on Penicillium strains isolated from bread are shown in figure 5A. The growth of *P. chrysogenum* was stopped at the concentrations of 0.32, 0.16 and 0.08 mg/ml but *P. notatim*

growth was stopped at the concentrations of 0.32 and 0.16 mg/ml. Finally, fungal growth significantly reduced by increasing sodium acetate concentrations.

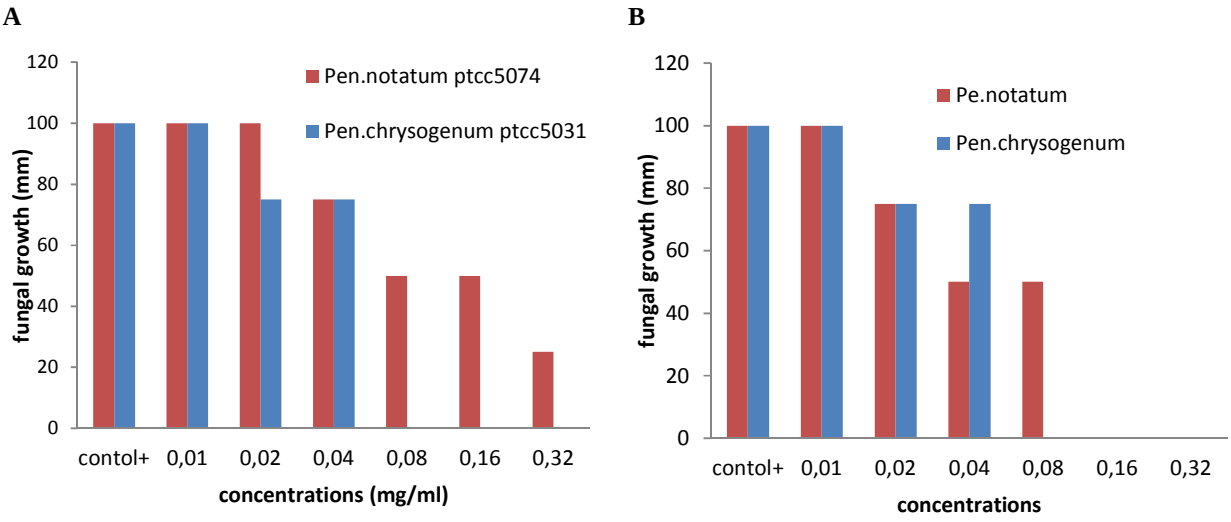

Figure 5: Effects of different concentrations of sodium acetate on: A] standard strain of Penicillium, B] Penicillium strains isolated from bread

Effects of sodium acetate on Rhizopus strains:

Figure 6, shows the effect of different doses of sodium acetate on standard strain of Rhizopus. As shown is figure 6, sodium acetate at doses of 0.32, 0.16 and 0.08 mg/ml completely inhibited the growth of *R. oryzae*. Same effect was also observed on the growth of fungus isolated from bread [Data not shown]. Inhibitory effect of sodium acetate was increased by increasing its concentration.

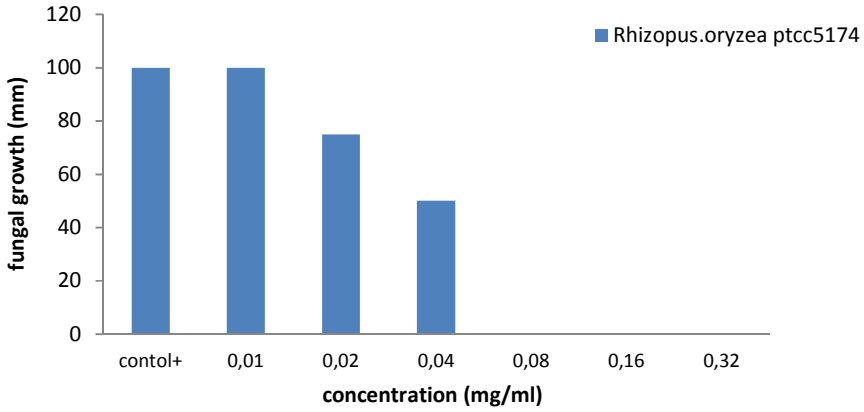

Figure 6: Effects of different concentrations of sodium acetate on Rhizopus *oryzae*

Effects of Cinnamon extract on Aspergillus sp in bread slices:

Colony formation of Aspergillus growing on bread slices in the presence and absence of Cinnamon extract are shown in figure 7. Cinnamon extract at a dose of 64 mg/ml showed the highest inhibitory activity and delayed colony formation for 5 days. Cinnamon extract at 16 and 32 mg/ml delayed fungal growth for 4 days but after this period the growth of the fungus initiated at the lower speed compared to the control. Statistical analysis showed significant difference between inhibitory effects of different concentrations of cinnamon extract on Aspergillus in bread slices.

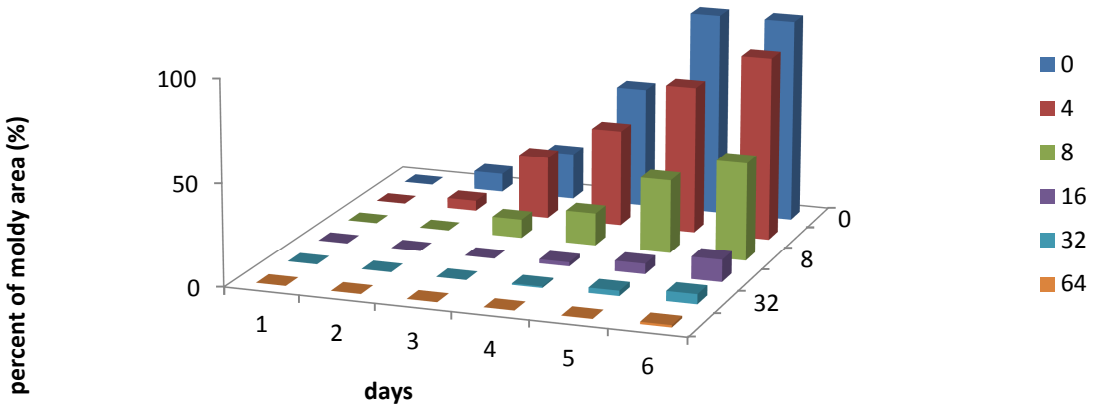

Figure 7: Colony formation of Aspergillus growing on bread slices in the presence and absence of Cinnamon extract

DISCUSSIONS

In our study, as expected, sodium acetate showed good antifungal activity and inhibited the growth of all tested fungi [figure 4-6]. Charalambous [1986] and Barret [1990] [3,13] showed that sodium acetate is an effective growth inhibitor of bread spoilage molds. A study conducted by Plegg [1986] and Glabe [1998] [14, 15] also showed that sodium acetate can prevent fungal contamination of livestock and poultry feed. In recent years, natural products such as herbal extracts and essential oils have attracted public attention due to increased concerns about chemical preservatives. Therefore, replacement of chemical compounds with herbal based preservatives is highly recommended. Essential oils and their active constituent, were shown to inhibit more than 15 types of bacteria and fungi [16: 17; 5].

In this study, in vitro antifungal activity of cinnamon extract was evaluated against standard fungi and fungi isolated from breads. According to the results of this study, aqueous extract of cinnamon at a concentration of 64 mg/ml completely inhibited all standard and bread isolated strains of Aspergillus. Antifungal effects of plant essential oils and extracts have been evaluated in previous studies.

Kalemba et al [2003] [16] investigated the inhibitory effect of 24 plant essential oils against 7 fungal and 9 bacterial species. All tested essential oils showed inhibitory effect against *Mycobacterium Smegmatis* with MIC values ranged from 5.62 to 400 µg/ml. 13 essential oils showed inhibitory effect against *Staphylococcus aureus*, 17 essential oils showed inhibitory effect against *Candida albicans* and 16 essential oils showed antifungal effects against *A. niger*. 3 essential oils at concentrations ranging from 200 to 400µg/ml showed inhibitory effects against *Streptococcus faecalis*. Furthermore, Inhibitory effects of 11 essential oils against *A. niger* were determined by broth dilution assay. From which the 5 essential oils [cinnamon, dill, coriander, garlic and onion] showed significant inhibitory effects against A. niger.

In the study conducted by Fateh *et al* [2010] inhibitory effects of aqueous and alcoholic extract of *Allium hirtifolium* was evaluated against *Aspergillus sp*, *Penicillium sp*, *Rhizopus sp*, and *candida albicans*. *A. hirtifolium* showed strong inhibitory effect with MIC values higher than Miconazole[18].

Moghtader and Salari [2011] [19] found that herbal essential oils can inhibit *A. flavus* at a concentration of 0.32 mg/ml. In our study cinnamon extract at 32 mg/ml inhibited *A. flavus* growth. It is reported that essential oils show stronger and broader activity compared to the extracts.

According to the results of present study, cinnamon extract at doses of 64 and 32 mg/ml completely inhibited standard strain of *P. notatum* and *P. chrysogenum* but only inhibited P. *chrysogenum* isolated from breads

Inhibitory effects of cinnamon extract and essential oils against different fungi were shown in previous studies. Cinnamon essential oil has been reported to show antibacterial, antifungal and antioxidant effects [20]. Results of recent studies showed that, the addition of cinnamon extract to the PDA medium can prevent growth of *Penicillium patulum, Penicillium citrinum, Penicillium roqueforti, Aspergillus parasiticus* and *Aspergillus flavus.* They also found that cinnamon extract can inhibit yeast growth [21-23].

Negi et al [2012] [24] reported that the inhibitory effect of cinnamon extract on *P. notatum* growth is related to its active components such as cinnamaldehyde and eugenol. We also found that Cinnamon extract not only inhibits *P. notatum* but also inhibits other standard strain of saprophytic fungi obtained from Iranian Research Organization for Science and Technology except for *R. oryzae.* No inhibitory effects on *R. oryzae* growth was observed in this study.
In our study antifungal activity of cinnamon extract were also investigated in bread slices. Due to importance of Aspergillus sp, it was chosen for studying the anti-fungal activity in bread slices. The results of present study showed that aqueous extract of cinnamon at a dose of 64 mg/ml was able to inhibit Aspergillus growth on bread slices. This concentration of extract extended the lag time of colony formation by 5 days. Cinnamon extract at 16 and 32 mg/ml delayed fungal growth for 4 days but after this period the growth initiated at significantly lower speed compared to the control. Furthermore, the highest concentration of extract that did not cause any deleterious changes in the flavor and quality of bread was 32 mg/ml. According the results of previous studies, cinnamon extract can be added to various food products as a preservative [25, 26]. Our research also showed that it can be used to extend shelf life of different types of bread especially flat breads.

The results of our study indicated that aqueous extract of cinnamon can prevent mold growth on bread. If there weren't technical and legal constraints, degree of inhibition increases by increasing concentration of extracts. These results were consistence with other studies [27, 28]. Cinnamaldehyde, a main constituent of cinnamon essential oils and extracts shows antifungal activity by destroying fungal cell wall enzymes. Eugenol, another compound that found in small quantities has been shown to exhibit antimicrobial and antifungal effects. Eugenol is reported to increase the permeability of the cytoplasmic membrane [29]

Kalantari et al [2012] [3] found that Cinnamon essential oil has potent inhibitory effects on pathogenic and saprophytic strains of Aspergillus. This result was in harmony with our results. We showed that the aqueous extract of cinnamon can be added to retard mold growth and improve the flavor and aroma of bread.

CONCLUSION

Considerable antifungal activity of cinnamon extract has been demonstrated in this study. Lower concentrations of cinnamon extract showed antifungal activities lower than sodium acetate but higher concentrations of extract presented antifungal activities similar to the sodium acetate. Regarding its effectiveness, herbal nature and fewer side effects, cinnamon extract can be used to inhibit mold growth and increase bread shelf life. WHO and FAO recommendations, growing concerns about food safety and quality issues and increasing demand for less-processed food or food without chemical preservatives lead to extensive researches for finding new natural preservative. Cinnamon extract not only increases bread shelf life and inhibits mold growth but also improve sensory properties of breads. Ideally, if consumers were perfectly informed about effectiveness and health promoting effects of cinnamon extract, the health and safety issues would be considered more important than sensory properties.

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