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Original Research Article

Inhibitory Effect of Natural Honey on The Growth of *Rhizopus stolonifer, Mucor spp.,* and *Aspergillus niger*

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Abstract: ***Background*:** Honey is frequently used as an antiseptic, prophylactic, and therapeutic agent for many infections. The chemical and physical properties of honey have an inhibitory effect on a lot of microorganisms including fungi. Rhizopus stolonifer, Mucor spp., and Aspergillus niger are common indoor molds that contaminate bread and eatable plants. During growth on bread, they multiply and produce large numbers of spores that can cause different types of infections, especially in immunocompromised patients. ***Objectives:*** 1) To evaluate the effect of natural honey on the growth of Rhizopus stolonifer, Mucor spp., and Aspergillus niger fungi. 2) To determine the minimal inhibitory concentration of honey that can suppress the growth of these fungi. 3) To assess the possibility of using honey as a natural replacement for chemical preservatives. ***Methodology*:** This experimental study was conducted in the College of Sciences at King Saud University between August and November 2021 in Riyadh, KSA. Multifloral honey was diluted in sterile distilled water volume to volume (%, v/v) in series concentrations: 5%, 15%, 30%, 45%, and 60%. The diluted samples of honey were then added to potato dextrose agar (PDA). Measured parts of the colonies of Rhizopus stolonifer, Mucor, and Aspergillus niger, were cultured on the PDA plates with the diluted honey. Two replicates were used for every concentration of each fungus. The inoculated plates, including the controls, were incubated at room temperature (25±2°C). Fungal growth was monitored daily for three months. ***Results*:** The 5%, 15%, and 35% concentrations of honey mixed with PDA did not inhibit the fungal growth, the fungi grew within the first 24h while the 60 % dilute concentration killed the three fungi and completely inhibited their growth. The fungi were monitored for 3 months; during that time, there was no growth. In conclusion: This study confirms that honey strongly inhibits the growth of Rhizopus Stolonifer, Mucor spp., and Aspergillus niger when used in a concentration of 60% or above. Accordingly based on these findings we determine that the minimal inhibitory concentration (MIC) of multi-floral honey on bread fungi is 60%.

Keywords: Inhibitory effect, honey, fungi**.**

INTRODUCTION

Honey is an organic supersaturated sweet substance produced by honeybees from the floral nectar of plants by water evaporation and salivary enzymatic activity. Honey is composed mainly of monosaccharides and other minor compounds including amino acids, enzymes, vitamins, and minerals with only 16-18% water [1]. Several factors contribute to the antifungal activity of honey including bee defensin-1, hydrogen peroxide (H2O2), flavonoids, phenolic compounds, and lysozyme. Moreover, the low pH (3.2–4.5) of honey has an inhibitory effect on the growth of many fungi. Additionally, the osmotic effect and low content of water in honey play major roles as antifungal mechanisms [2, 3]. The composition of honey varies according to the plant species from which the bee collected the nectar, but the main constituents are the same in all kinds of honey [4].

Some studies confirmed the presence of Rhizopus, Aspergillus, and mucor as food and water contaminants in Saudi Arabia [5-9].

Rhizopus stolonifer is a common bread and plant mold and is well known as an opportunistic pathogen that causes life-threatening diseases named mucormycosis (formerly zygomycoses), especially in immunocompromised people. Zygomycoses can manifest as pulmonary, rhinocerebral, gastrointestinal, or in the form of allergic diseases or cutaneous infections [10-13].

Aspergillus niger is a common environmental contaminant and is linked to external ear infections, cutaneous infections, and pulmonary diseases. Infection by Aspergillus niger can be severe and fatal in immunocompromised patients especially those who are using steroids [14-16].

Many studies documented these molds as causative agents of multiple types of infections in KSA [17-23].

This study was conducted to evaluate the inhibitory effect of natural honey on the growth of Aspergillus niger, Rhizopus stolonifer, and Mucor fungi.

MATERIAL AND METHODS

Study Design:

This study was an experimental study conducted in the College of Sciences, King Saud University in Riyadh, KSA. During the period between November 2021 and March 2022.

Honey preparation

Multifloral honey was used in this study. The honey was tested for quality and purity in a certified laboratory. It was stored in airtight glass containers at room temperature (24°c) in a dry dark environment to preserve its antimicrobial effect, which is affected by significant changes in temperature.

The honey was diluted volume to volume in sterile distilled water to a series concentration of 5%, 15%, 35%, and 60% (%, V/V) to detect its minimum inhibitory concentration (MIC).

It was then mixed with the potato dextrose agar (PDA) depending on dilution into 95% ml of PDA mixed with 5% ml/cc diluted honey, 85% ml of PDA mixed with 15% ml/cc diluted honey, 65% ml of PDA mixed with 35% ml/cc diluted honey,40% ml of PDA mixed with 60% ml/cc diluted honey.

Honey Components

A sample of honey was analyzed in Honey Quality Laboratory, a certified laboratory in Riyadh. The tests showed that the used honey is pure, odorless, and 17.5% moist. The honey did not contain sucrose, insoluble particles, ash, molds, or yeasts. The acidity and diastase enzyme activity were within the accepted ranges of natural honey. According to the results of the tests, the honey used in the study is compliant with Saudi and Gulf Quality Standards of honey (101/1993-147/2008).

Tested Fungi

The Rhizopus stolonifer fungi used were obtained from the laboratory of the Department of Microbiology, of King Saud University. Aspergillus niger and Mucor species were isolated from moldy bread on which both the fungus had grown. They were inoculated on the potato dextrose agar and placed in the incubator at 25°-28°c for 24-48h. Rose Bengal was used to suppress bacterial growth in PDA and prevent fungal sample contamination.

Agar Preparation

The selected antifungal susceptibility testing (AFST) was Agar dilution to establish the minimum inhibitory concentration (MIC). The inoculation agar was prepared by dissolving 9.75 grams in 250 ml of distilled water. The mixture was sterilized in the autoclave at 121°C and 15 bar pressure for 30 minutes. The agar was left to cool to 45°C at room temperature. Series concentrations (5%, 15%, 30%, 45%, 60% ml/cc) of honey were added to the PDA and the flasks were placed on a stir plate for 2 minutes until the PDA had homogenously mixed with the honey, then were poured into the Petri dishes and left to cool and solidify.

Inoculum Preparation

Spores of Rhizopus stolonifer, Mucor, and Aspergillus niger from 48 hours old cultures were inoculated on the PDA with honey. Each fungus was inoculated in duplicate in each honey concentration. Cultures on PDA without added honey were used as positive controls. All the Petri dishes, including the controls, were incubated at 25^0C and observed for growth daily. The lowest concentration of honey that prevented bacterial growth was considered to be the minimum inhibitory concentration against that fungus.

Ethical Consideration:

The study proposal was approved by the Institutional Review Board of AlMaarefa University (IRB numbered 3/211).

RESULTS

The 5%, 15%, and 35% v/v concentrations of honey did not inhibit the growth of the three fungi. The fungi started to grow after 24 hours of incubation. The growth of the three fungi was completely inhibited at the 60% honey concentration; the fungi could not grow through the period of the research which extended to three months (Table 1).

Figure 1 shows the effect of the four concentrations of honey on the three fungi.

Table 1: Results of antifungal effect determined by agar dilution method of 5%, 15%, 35%, and 60% ml/cc series honey concentration against the studied fungi

Number of reputations	The diluted concentration of honey	After 24h of incubation	After 48h of incubation	After 72h of incubation	After 3months of incubation
2	5% v/v	Visible growth	Visible growth	Visible growth	Visible growth
2	15% v/v	Visible growth	Visible growth	Visible growth	Visible growth
2	35% v/v	Visible growth	Visible growth	Visible growth	Visible growth
2	60% v/v	No growth	No growth	No growth	No growth

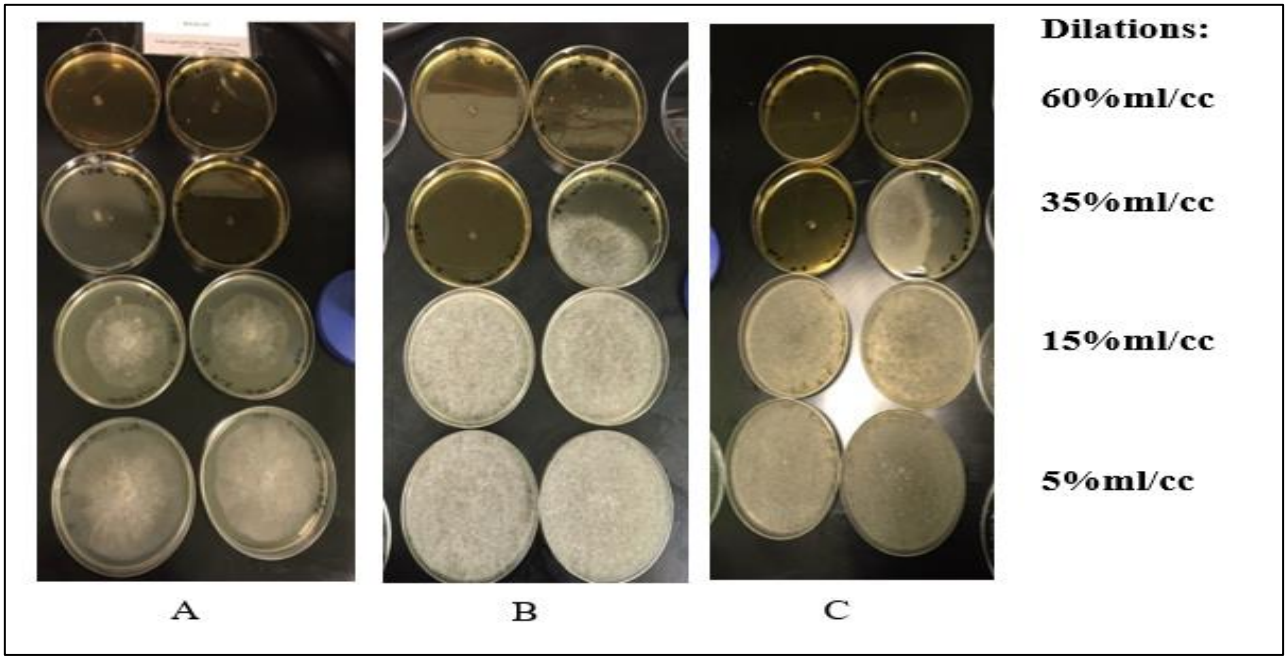

Figure 1: Evaluation of the antifungal activity of agar dilution by 5%, 15%, 35%, and 60% ml/cc concentration of honey against *Rhizopus stolonifera Fungi.* (A) fungal growth after 24 h, (B) Fungal growth after 48h, (C) Fungal growth after 72h

DISCUSSION

Honey is an organic substance produced by honeybees from the nectar of blossoms. Transformed in the stomach of bees into honey. In some countries, honey is used as an antiseptic therapeutic agent for burns and ulcers. A study report patient with advanced (COPD) are at risk for developing invasive aspergillosis (IA). The etiologic factors of FIs-CNS are moniliaceous moulds (Aspergillus spp. Causing skull-base syndrome) Mucoromycetes (Mucor spp., Rhizopus spp. causing stroke/infarction). Their common route of transmission is inhalation. In this study, we aimed to (i) Evaluate the effect of natural honey on inhibiting the growth of Rhizopus Stolonifer Fungi, Mucor Fungi, and Aspergillus niger Fungi; responsible for bread mold. (ii) To verify a clean environment free of fungal spores that lead to respiratory, neurological, and other diseases in humans. (iii) To identify a natural preservative that can be used as an alternative to the synthetic preservatives as potassium bromate which is possibly carcinogenic and potassium iodate which could cause thyroid-related problems. (iv) To determine a solution to rapid bread mold and reduce the economic disadvantage caused by moldy bread. The series concentrations of natural honey 5%, 15%, 35%, and 60% ml/cc used in this study and only 60% ml/cc showed an inhibitory effect on the growth of Aspergillus niger, Rhizopus Stolonifer, Mucor Fungi. The susceptibility of some of these fungi to honey is of significance. In the present study, the honey concentration of 60%ml/cc is considered the MIC.

It is evident that honey has an inhibitory effect on the growth of Aspergillus niger Fungi. This is similar to a study done in Egypt, production of aflatoxins (AFs) and ochratoxin A (OTA) by Aspergillus flavus and Aspergillus ochraceus, respectively, have indicated that 3 mg -100 ml media of honey-derived silver nanoparticles AgNPs have reduced the aflatoxin (AF) G1, G2, B1, and B2 production by A. parasiticus to 77.55, 62.91, 58.76 and 66.56%, respectively and ochratoxin A (OTA) by A. ochraceus to 79.85% with significantly inhibitory effect on mycelial growth. The percentage of reduction depends on the AgNPs concentration [1]. It is evident that honey can inhibit the growth of Aspergillus niger Fungi, and honey-derived silver nanoparticles AgNPs reduce the production of aflatoxin (AF) G1, G2, B1, and B2.

The result shows that honey at dilution of 60% has an inhibitory effect on Fungal growth such as Rhizopus Stolonifer, Aspergillus spp., and Mucor fungi,

this is in a line with a study done in South America, showed that honey in its most concentrated form is very effective against pathogenic organisms. In some cases, diluted honey, up to 50% produces the same effect. Further, since honey is a cheap, easily available, and non-toxic antimicrobial agent due to its properties, it can be very effectively used for medical purposes [3]. It is evident that diluted honey up to 50%-60% ml/cc can produce the same inhibitory effect that most concentrated forms can produce. Honey is an effective antifungal at dilution of 60% ml/cc therefore, it can be used at this dilution to achieve an economic benefit.

The result shows that the inhibitory effect of honey depends on the concentrations, a series of 5%, 15%, and 35% ml/cc are not able to inhibit fungal growth while in 60% ml/cc dilution, fungal growth is inhibited. This is against to a study done in Nigeria, results obtained reveal that the honey samples showed varying levels of inhibitory activity at various concentrations 20%, 40%, 60%, and 80% against the fungi tested with zones of inhibition increasing with increasing honey concentration. The samples of honey used in the study showed a broad spectrum and promising antifungal activity [24]. This difference is mainly due to the flowers from which bees gather nectar to produce honey may contribute to the difference in the antimicrobial activities. The composition of honey varies according to the plant species on which the bee forages, but the main constituents are the same in all kinds of honey.

This study has some limitations including, a lack of previous studies in the research area a couple of studies performed in Egypt, Nigeria, South America, and Australia, limiting time to perform the experiment and repeat the trials knowing how long the fungus will remain without any growth or spread in the environment (PDA) [25-27]. Conducting more experiments on the components of honey and working to find and extract the distinctive feature of honey that prevents the growth of any germs or fungi. However, a major strength of this study is that it addressed, for the first time in the research area.

CONCLUSION AND RECOMMENDATION

In summary, this study confirms that honey strongly inhibits the growth of Rhizopus Stolonifer, Mucor spp., and Aspergillus niger when used in a concentration of 60% or above. Accordingly based on these findings we determine that the minimal inhibitory concentration (MIC) of multi-floral honey on bread fungi is 60%.

We recommend trying other diluted concentrations of honey by experimenting with less than 60% ml/cc of honey and more than 35% ml/cc of honey to see if there is a positive result that prevents fungi from growing and spreading.

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