Int J BioMed Public Health. 2018;1(4):169-174
doi 10.22631/ijbmph.2018.147137.1076

International Journal
of
BioMedicine and Public Health
http://www.ijbmph.com

Original Article

The effect of walnut thin shell extract on the growth of Penicillium species

Ehsan Haghi[1], Fariba Razeghi[1], Fereshteh Ahmadi[1], Nabi Shariati Far[1*]

Open Access

ARTICLE INFO

Article History:
Received 1 September 2018
Revised 24 November 2018
Accepted 24 November 2018

Keywords:
Penicillium
Walnut
Extract
Minimum inhibitory concentrations
Minimum fungicidal concentration

[1] Food Safety Division, Department of Environmental Health, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Correspondence:
Nabi Shariati Far. Food Safety Division, Department of Environmental Health, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
nshariati@tums.ac.ir

ABSTRACT

Introduction: Fungi broadly exist in the environment. Growing interest for natural and safe additives as well as concerns about synthetic fungicide have led to development of various fungicides. This study aimed to examine the effect of walnut thin shell ethanolic extract on the growth of Penicillium species.

Methods: Antifungal effects were assessed at four different concentrations ranging from 5 to 350 mg/ml. The extract dilutions were exposed to the desired fungi using the broth dilution method, and minimum inhibitory concentrations (MIC) of fungi were determined and compared with the effects of nystatin and fluconazole.

Results: The MIC for *Penicillium italicum* was 15 mg/ml for both walnut thin shell and fluconazole whereas it was 30 mg/ml for nystatin. Moreover, the minimum fungicidal concentration (MFC) for walnut thin shell, fluconazole and nystatin were 250, 30 and 60 mg/ml, respectively. The MIC for Penicillium expansum was 30 mg/ml for both walnut thin shell and fluconazole while it was 40 mg/ml for nystatin. The results also revealed that the MFC for these inhibitors was 300, 40 and 70 mg/ml, respectively. Furthermore, the MIC for *Penicillium digitatum* for both the shell walnut extract and nystatin was 40 mg/ml while it was 20 mg/ml for fluconazole. The MFC for walnut thin shell, fluconazole and nystatin were 325, 100 and 70 mg/ml, respectively.

Conclusion: Thin walnut shell extract has significant antifungal effects on Penicillium species and could be used as a natural antifungal. More research is required to assess the use of the extract for treatment of fungal infections.

Introduction

Fungi widely exist in natural environments and grow in various foods, including vegetables and fruits, causing spoilage and deterioration of these foods in some cases (1). Fungal pathogen growth is the major cause of economic loss during post-harvest handling of fruits (2). It is difficult to control fungal growth as they resist many conventional fungicides including benzimidazoles and dicarboximides (3). *Penicillium* is one of the largest fungal genera and includes some of the most commonly known filamentous fungi that can be found on many substrates and in various habitats. The P. *expansum*, that is regarded as the major producer of mycotoxin and patulin, is commonly found in decaying apples (4). Green and blue mold rots that are caused by *Penicillium digitatum* and *Penicillium italicum* respectively are probably the most common post-harvest diseases affecting citrus fruits. These pathogens may attack the fruit on the tree, in the packing house and during transit as well as in storage and in the market (5). Several methods have been used to solve post-harvest losses, including fungicide treatment and modified controlled atmosphere (6, 7). Post-harvest fungicide treatment is a primary method for controlling Penicillium infections. Thiabendazole and Imazalil were the most commonly used chemical fungicides to control post-harvest green and blue molds. Some Penicillium strains have become resistant to these chemical fungicides (8, 9). To overcome this resistance, along with

the consumer worries about eating foods treated with synthetic fungicides and environmental issues, it is important to discover new and novel antifungal agents (4, 10). Furthermore, there is a need for discovering new strategies to develop fungicides to control fruit post-harvest diseases. Studies demonstrated the effectiveness of certain natural compounds including derivatives of benzoic or cinnamic acid as antifungal or anti mycotoxigenic agents. Furthermore, several researches have shown anti-inflammatory and antioxidant effects for walnut shell extract (11). Moreover, the antimicrobial effect of volatile compounds, phenols and tannins in walnut green shells has also been proven (12). Molecular structures and different inter-species sensitivity affect characteristics of phenols (13). The role of methanol extract of green walnut shells in preventing the growth of different fungi including dermatophytosis was examined to observe whether green walnut extract could be used as an alternative to synthetic antifungal agents. Walnut hulls is proved to have an inhibitory effect on four selected species of fungi (Microsporum canis, Trichophyton mentagrophytes, Epidermophyton floccosum and Candida albicans) and prohibited 60% of fungal growth (14). Furthermore, the antibacterial effect of walnut thin shells on Bacillus subetilis and Salmonella typhimurium was specifically examined, which resulted in the restricted growth of microorganisms (15).Therefore, in order to prevent chemical contamination of fruits with synthetic antifungal, this study was conducted to examine the effect of ethanolic extract obtained from walnut thin shell (*Juglans regia* L.) on standard and isolated species of *Penicillium expansum*, *Penicillium digitatum* and P. *italicum* compared to nystatin and fluconazole.

Methods

Material

Nystatin powder (Royan Darou Co., Iran) and Fluconazole (Pars Darou, Iran), Potato-dextrose agar fungi growth medium (Merck, Germany), Sabouraud Dextrose Broth growth medium for MIC (Merck, Germany).The *Penicillium expansum* (ATCC No. 42710) *Penicillium italicum* (ATCC No. 66636) and *Penicillium digitatum* (ATCC No. 201167) were used. The standard species of fungi were prepared from fungus bank located in School of Public Health, Tehran University of Medical Science.

Preparation of walnut thin shell ethanol extract

To prepare the extract, Iranian walnuts (*Juglans regia* L.) with no trace of mold were obtained from a region in the west of Iran, which is famous for good quality walnuts. Walnut thin shells were removed and dried at room temperature. The dried walnut thin shells were powdered by an electrical grinding machine. Prior to taking the extract, the powder was sterilized under the influence of UV ray using a laminar hood. Afterwards, 20 grams of dried powder was mixed with 100 cc of ethanol 80% and restored at room temperature (about 22 °C) for 24 hours. Then, the extract was filtered by filter papers and poured into the rotary device (to remove ethanol). The obtained alcoholic extract was dried at 40 °C. Subsequently, 1 gram of dried alcoholic extract was added to 5 cc of dimethyl sulfoxide solvent and shook for 2 minutes. Afterwards, the extract was filtered again and sterilized by syringes with a 0.22 micron diameter filter (16).

Preparing the fungi suspension

The fungi were primarily grown on the potato-dextrose agar (PDA) and stored for three days at 25 °C. After three days, the plates were examined macroscopically. Slides were also prepared from the cultures to determine the microscopic form of fungi. New fresh culture penicillium conidia were solved in distilled water; the turbidity of the above suspension was adjusted spectrophotometrically at 530 nm to match the turbidity of 0.5 McFarland standard with 10^6 ml cells (17).

Preparing nystatin and fluconazole suspension

Standard nystatin powder and fluconazole were prepared. Dimethyl sulfoxide and distilled water were used to solve nystatin (insoluble in water) and fluconazole (soluble in water), respectively. Then, the walnut extract, nystatin and fluconazole were diluted in a 22 series to compare the MIC and MFC between thin walnut shell extract, fluconazole and nystatin.

Determining the MIC of fungus growth

An antifungal susceptibility test was carried out using the broth microdilution method based on the Clinical and Laboratory Standards Institute (CLSI M38-A2) guidelines. For determining the inhibitory concentration on the fungi, 22 dilution series of walnut thin shell ethanolic extract ranging from 350 mg/ml to 5-liter concentration were prepared and then added to the wells. Moreover, 22 dilution series for nystatin and fluconazole were prepared separately. For determining the MIC, 200 micro liters of Sabouraud Dextrose broth was added to 24 microdilution plate wells. Subsequent-

ly, 200 micro liters of walnut thin shell extract, nystatin and fluconazole were injected into 22 dilution series. Of the 24 wells, two wells were considered as growth control: one as a positive control and the other as a negative control. The negative control contained media whereas the positive control consisted of media and microbial suspension. Subsequently, 50 micro liters of fungi suspension were added to all the wells except the negative control according to McFarland standard. These steps were repeated for nystatin and fluconazole. All the tests were repeated for three times. At last, microdilution plates were incubated at 25 °C for 72 hours and were then observed for the presence of visible growth. The turbidity of each well, which reflects the growth, was compared with the turbidity of the positive well. Negative reaction was defined as the observation of no growth and no turbidity (18).

Determining the MFC of fungi growth

A sample was obtained from tubes with no fungi growth with a sterile loop, and was transferred to culture media containing the PDA and cultivated based on the streak culture method. Then, the sample was incubated at 25 °C for 72 hours. Afterwards, the plates were observed for fungi growth. The minimum extract concentration in plates that resulted in no fungi colonies was considered as the MFC (19).

Results

The results of the growth-inhibiting effect are shown in Tables 1, 2 and 3. The MIC for *Penicillium italicum* was 15 mg/ml for both thin walnut shell and fluconazole and 30 mg/ml for nystatin. Moreover, the MFC was 250 mg/ml for walnut thin shell extract, but was 30 and 60 mg/ml for fluconazole and nystatin, respectively (Table 1). Regarding the *Penicillium expansum*, the MIC was 30 mg/ml for both walnut thin shell extract and fluconazole and 40 mg/ml for nystatin. Furthermore, the MFC was 300 mg/ml for walnut thin shell extract, but was 40 and 70 mg/ml for fluconazole and

Table 1. Minimum inhibitory concentration for Penicillium italicum (mg/ml)

Concentrations	Extract	Nystatin	Fluconazole
5 mg/ml	+	+	+
10 mg/ml	+	+	+
15 mg/ml	_	+	_
20 mg/ml	_	+	_
30 mg/ml	_	_	_ *
40 mg/ml	_	_	_
50 mg/ml	_	_	_
60 mg/ml	_	_ *	_
70 mg/ml	_	_	_
80 mg/ml	_	_	_
90 mg/ml	_	_	_
100 mg/ml	_	_	_
125 mg/ml	_	_	_
150 mg/ml	_	_	_
175 mg/ml	_	_	_
200 mg/ml	_	_	_
225 mg/ml	_	_	_
250 mg/ml	_ *	_	_
275 mg/ml	_	_	_
300 mg/ml	_	_	_
325 mg/ml	_	_	_
350 mg/ml	_	_	_
Control +	+	+	+
Control -	_	_	_

Table 2. Minimum inhibitory concentration for Penicillium expansum (mg/ml)

Concentrations	Extract	Nystatin	Fluconazole
5 mg/ml	+	+	+
10 mg/ml	+	+	_
15 mg/ml	+	+	_
20 mg/ml	+	+	_
30 mg/ml	_	+	_
40 mg/ml	_	_	_ *
50 mg/ml	_	_	_
60 mg/ml	_	_	_
70 mg/ml	_	_ *	_
80 mg/ml	_	_	_
90 mg/ml	_	_	_
100 mg/ml	_	_	_
125 mg/ml	_	_	_
150 mg/ml	_	_	_
175 mg/ml	_	_	_
200 mg/ml	_	_	_
225 mg/ml	_	_	_
250 mg/ml	_	_	_
275 mg/ml	_	_	_
300 mg/ml	_ *	_	_
325 mg/ml	_	_	_
350 mg/ml	_	_	_
Control +	+	+	+
Control -	_	_	_

nystatin, respectively (Table 2). The MIC for *Penicillium digitatum* was observed to be 40 mg/ml for both the shell walnut extract and nystatin and 20 mg/ml for fluconazole. furthermore, the MFC for these inhibitors were 325, 100 and 70 mg/ml, in that order (Table 3).

Discussion

The MIC for *Penicillium digitatum* was observed to be 40 mg/ml for walnut thin shell extract and nystatin and 20 mg/ml for fluconazole. Furthermore, the MFC for these inhibitors were 325, 100 and 70 mg/ml, in respective order. According to the findings of this study, thin walnut shell extract had an acceptable inhibitory effect on *Penicillium expansum* and *Penicillium italicum* and a notable effect on *Penicillium digitatum*. The findings of this study revealed a similar effect for fluconazole on P. *italicum*. However, this effect was higher on P. *italicum* and P. *expansum* compared to nystatin. Furthermore, the inhibitory effect of walnut thin shell extract was weaker on P. *digitatum* compared to fluconazole but was similar to that of nystatin. With regard to the MFC, the walnut thin shell extract was not sufficiently effective compared to fluconazole and nystatin. An active substance called juglone (5-hydroxy-1,4-naphthoquinone) is one of the strongest phytotoxic and allelopathic chemical compounds in the leaves, roots and husks of *Juglans regia* L. (20, 21). Juglone restrains the growth and development of many plants (22). It was proven that natural phenolic compounds which are found in walnuts have very potent antifungal agents with little or no toxic effects. These agents have the potential be used as antifungal agents alone or in synergism with the currently used antifungal agents (23). The antifungal effects of aqueous and methanolic extracts of fruit peel of walnut (Juglans regia) and fluconazole against four Candida species were evaluated. The MIC of fluconazole, aqueous and methanolic extracts of walnut fruit peel for different Candida species were 0.001- 0.032, 6.25-50, 3.125-25 mg/ml, respectively. The MFC of fluconazole, and methanolic extracts of fruit peel of walnut were 0.001-0.032 and 6.25-25 mg/ml, respectively. These findings were in line with the findings by Naseri et al. and Arji et al. (24, 25). The antibacterial and antifungal effects of the methanolic extract of walnut leaves were investigated in a research. The results showed that the growth preventing effects of walnut extract was more powerful against Candida albicans compared to Candida glabrata and Candida krusei (26). In a study by Yigt et al., antifungal and antibacterial activities of aqueous and methanolic extracts of leaves and husk of walnut were assessed. The results showed that the MIC values of alcoholic extracts was 1.5 mg/ml against all Candida species (27). The effect of ethanolic extract of walnut thin shell on the growth of Aspergillus species was examined in a previous study which reported that the MIC of 15 mg/ml for Aspergillus fumigatus and 61.5 mg/ml for Aspergillus flavus (28). These studies indicated that walnut fractions could restrict the growth of fungus, which were in line with the findings of the current study. The previously reported MIC and MFC values were lower than those in our study, which may be due to the difference in levels of phenolic acids or different sensitiveness of fungi species to phenolic compounds. Thin shell around walnut is full of phenolic and antioxidant compounds. The walnut thin shell serves as a defective layer to protect fatty acids especially polyunsaturated fatty acids against free radicals (29). Several phenolic compounds with antioxidant properties have been identified in J. regia leaves. Some phenolic acids produced by plants as secondary products and could damage mitochondrial DNA and cell walls and lead to microorganism death (29, 30) while some other prod-

Table 3. Minimum inhibitory concentration for Penicillium digitatum (mg/ml)

Concentrations	Extract	Nystatin	Fluconazole
5 mg/ml	+	+	+
10 mg/ml	+	+	+
15 mg/ml	+	+	+
20 mg/ml	+	+	–
30 mg/ml	+	+	–
40 mg/ml	–	–	–
50 mg/ml	–	–	–
60 mg/ml	–	–	–
70 mg/ml	–	–	–*
80 mg/ml	–	–	–
90 mg/ml	–	–	–
100 mg/ml	–	–*	–
125 mg/ml	–	–	–
150 mg/ml	–	–	–
175 mg/ml	–	–	–
200 mg/ml	–	–	–
225 mg/ml	–	–	–
250 mg/ml	–	–	–
275 mg/ml	–	–	–
300 mg/ml	–	–	–
325 mg/ml	–*	–	–
350 mg/ml	–	–	–
Control +	+	+	+
Control -	–	–	–

ucts can interfere with the metabolic pathways, reverse the effect of MDR, when given in the combination with cytotoxic agents, and inhibit the activity of ABC transporters (that make fungal pathogens resistant to drugs) (23). A large number of different chemical compounds exist in herbal extracts with similar antimicrobial function, but an unknown mechanism.

Conclusion

The results of the current study showed that walnut thin shell extract has significant antifungal effects. The growth preventive effect of the extract was indicated for P. *expansum*, P.*digitatum* and P. *italicum*. Regarding the growing interest of the community, health authorities and consumers in natural and safe additives as well as concerns about overuse and resistance to antibiotics, this extract can be used as a natural and effective fungicide in food products and other cases to promote public health.

Ethical disclosure

Not applicable.

Acknowledgment

The authors appreciate Dr. Sassan Rezaie for all of his assistance.

Author contributions

E H, F R, F A and N Sh F contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Conflict of interest

The authors have no conflicts of interest with the material presented in this paper.

Funding/support

None declared.

References

1. Era MS, Sakai Sh, Tanaka A, Kawahara T, Kanyama T, Morita H. Antifungal activity of fatty acid salts against Penicillium pinophilum. Japan J Food Engin. 2015;16(2):99-108. doi:10.11301/jsfe.16.99
2. Spadaro D, Garibaldi A, Lodovica Gullino M. Control of Penicillium expansum and Botrytis cinerea on apple combining a biocontrol agent with hot water dipping and acibenzolar-S-methyl, baking soda, or ethanol application. Postharvest Biol Technol. 2004;33(2):141-51. doi:10.1016/j.postharvbio.2004.02.002
3. Elad Y, Yunis H, Katan T. Multiple fungicide resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of Botrytis cinerea in Israel. Plant Pathol. 1992;41(1):41-6. doi:10.1111/j.1365-3059.1992.tb02314.x
4. He L, Liu Y, Mustapha A, Lin M. Antifungal activity of zinc oxide nanoparticles against Botrytis cinerea and Penicillium expansum. Microbiol Res. 2011;166 (3):207-15. doi:10.1016/j.micres.2010.03.003
5. Plaza P, Usall J, Teixidó N, Viñas I. Effect of water activity and temperature on germination and growth of Penicillium digitatum, P. italicum and Geotrichum candidum. J Appl Microbiol. 2003;94(4):549-54 doi:10.1046/j.1365-2672.2003.01909.x
6. Scherrer Montero C, Beatriz Antes R, Loss Schwarz L, Cunhados Santos L, Piresdos Santo R, João Bender L, et al. Complementary physical and chemical treatments as an alternative to fungicide use to control postharvest decay incidence and fruit quality of Montenegrina tangerines. Crop Prot. 2010;29(10):1076-83. doi:10.1016/j.cropro.2010.06.014
7. Romanazzi G, Lichter A, Mlikota Gabler F, L.Smilanick J. Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. Postharvest Biol Technol. 2012;63(1):141-7. doi:10.1016/j.postharvbio.2011.06.013
8. Spotts RA, Cervantes LA. Population, pathogenicity and benomyl resistance of Botrytis spp., Penicillium spp. and Mucor piriformis in packinghouses. Plant Dis. 1986;70:106-8.
9. Baraldi E, Mari M, Chierici E, Pondrelli M, Bertolini P. Studies on thiabendazole resistance of Penicillium expansum of pears: pathogenic fitness and genetic characterization. Plant Pathol. 2003;52(3):362-70 doi:10.1046/j.1365-3059.2003.00861.x
10. Zamani M, Sharifi Tehrani A, Ahmadzadeh M, Hosseininaveh V, Mostofy Y. Control of penicillium digitatum on orange fruit combining pantoea agglomerans with hot sodium bicarbonate dipping. J Plant Pathol. 2009;91(2):437-42.
11. Bhatia K, Rahman S, Ali M , Raisuddin S. In vitro activity of JuglansRegia L. bark extract and its protective effect on cyclophosphamide-induced urotoxicity in mice. Redox Rep. 2006;11(6):273-9 doi:10.1179/135100006X155030
12. Jin Z, Qu ZY. Studies on hydrolysable tannin constituents in seed of Juglans regia. Zhongguo Zhong Yao Za Zhi. 2007;32(15):1541-4. PMID:17972584
13. Zabka M, Pavela R. Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi. Chemosphere. 2013; 93(6):1051-6.doi:10.1016/j.chemosphere.2013.05.076
14. Salamat F, Keivani S, Emami M, Amin GH. Evaluation of Juglansregia pericarp on antifungal susceptibility with broth dilution method. J Islamic Azad Univ Tehran Med Branch. 2007;16(4):201-5.
15. An Jun L, Yue Wei W, Zen Yuan Z, Yan W, Ying C.

Extraction and antimicrobial activities of polysaccharide from walnut kernel pellicle. Mod Food Sci Technol. 2010;26(4):160-5.
16. Sharafati-Chaleshtori F, Shrafati-Chaleshtori R, Momeni M. Comparison of the antimicrobial effects of theethanolic and aqueous extracts of Scrophularia striata onEscherichia coli O157:H7 in vitro. J Shahrekord Univ Med Sci. 2009;10:32-37.
17. Evans EGV, Richardson MD. Medical mycology a practical approach. England: Oxford University Press; 1989.
18. Wayne PA. Reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard. CLSI document M27-A2;2002.
19. Wayne PA. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard- Second edition. CLSI document M27-A2;2002.
20. Tomaszkiewicz-Potępa A, Vogt O. Juglon: biological active metabolite from plants of family juglandeceae. Wiadomości Chemiczne. 2004;58 (11-12):881-94.
21. Hejl AL, Koster KL. Juglone disrupts root plasma membrane H+-ATPase activity and impairs water uptake, root respiration, and growth in soybean (Glycine max) and corn (Zea mays). J Chem Ecol. 2004;30(2):453-71.
22. Piskorski R, Ineichen S, Dorn S. Ability of the Oriental Fruit Moth 9. Grapholita molesta (Lepidoptera: Tortricidae) to Detoxify Juglone, the Main Secondary Metabolite of the Non-host Plant Walnut. J Chem Ecol. 2011;37(10):1110-6. doi:10.1007/s10886-011-0015-4.
23. Ansari MA, Anurag A, Fatima Z, Hameed S. Natural phenolic compounds: a potential antifungal agent. Microb. 2013:1189-95.
24. Arji P, Naseri A, Rakhshandeh H, Najafzadeh MJ. Investigation of antifungal activity of methanol and aqueousextracts of walnut (Juglans regia) leaves and peel against candida species. J Birjand Univ Med Sci. 2015;22(2):115-24.
25. Naseri A, Shamsian SA. In vitro anti-candidal effects of aqueous and methanolic extracts of walnut (Juglansregia) tree fruit peel in comparision with fluconazole. Int J Med Res Health Sci. 2016;5(6):72-6.
26. Citoglu GS, Altanlar N. Antimicrobial activity of some plants used in folk medicine. J Fac Pharm Ankara. 2003;32(3):159-63.
27. Yigit D, Yigit N, Aktas E, Ozgen U. Antimicrobial activity of walnut (Juglansregia L.). Turkish Soc Microbiol Res. 2009;39:7-11.
28. Haghi E, Rezaie S, Molaee Aghaee E, Sadighara P, Ahmadi F. The effect of ethanolic extract of walnut thin shell on the growth of Aspergillus spp. J Food Safe Hyg. 2016;2(3-4):84-9.
29. Ayoughi F, Barzegar M, Sahari MA, Naghdi Badi H. Antioxidant activity of dill essential oil (Anethum graveolens) in soybean oil, in comparison with chemical antioxidants. J Med Plants. 2009;2 (30): 71-83.
30. Mombeini T, Mombeini M, Aghayi M. Evaluation of pharmacological effects of Origanum genus (Origanum spp.). J Med Plants. 2009;4(29):18-35.