GAZİOSMANPAŞA BİLİMSEL ARAŞTIRMA DERGİSİ (GBAD)
Gaziosmanpasa Journal of Scientific Research
ISSN: 2146-8168
http://dergipark.gov.tr/gbad
Araştırma Makalesi (Research Article)

Cilt/Volume : 7
Sayı/Number: 3
Yıl/Year: 2018
Sayı/Pages: 173-180

***Alınış tarihi (Received)*: 20.11.2018**
***Kabul tarihi (Accepted)*: 28.12.2018**

Baş editor/*Editors-in-Chief:* ***Ebubekir ALTUNTAŞ***
Alan editörü/*Area Editor*: ***Köksal PABUÇCU/Bülent TURAN***

Varietal Differences in Antimicrobial Activities of Walnut (*Juglans regia* L.) Leaf Extracts

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ABSTRACT: In this study, antibacterial and antifungal activities of leaf extracts of five cultivars of walnut (*Juglans regia* L.) on eleven bacteria and nine fungi species were determined using agar well diffusion method. Five bacterial species *(Pseudomonas gingeri, P. syringae*, *Staphylococcus aureus, Bacillus cereus* and *Yersinia enterocolitica)* were generally found sensitive to the walnut extracts. However, antibacterial effects of the aqueous extracts were weaker than the ethanolic ones. The most sensitive bacterium to all of the treatments was determined to be *S. aureus.* The walnut leaf extracts have generally showed significant antifungal effect on three species (*Candida albicans*, *Botrytis cinerea*, *Geotrichum candidum*) with both ethanolic and water extracts. The most sensitive fungus species to all of the treatments was seen to be *Candida albicans* and the most resistant fungi was *Aspergillus niger*. Among the walnut cultivars, generally, Şebin cultivar extract was seen to have higher antimicrobial effects than the others. MIC values of juglone and the extract for *S. aureus* bacterium were 0.006 and 0.05 mg/ml, and for *C. albicans* fungus were 0.003 and 0.025 mg/ml, respectively.
***Keywords*:** Antibacterial activity; Antifungal activity; Juglone; Leaf extracts; Walnut cultivars.

ÖZET: On bakteri ve dokuz fungus türü üzerinde beş farklı ceviz (*Juglans regia* L.) varyetesinin yaprak özütlerinin antibakteriyel ve antifungal etkileri, agar çukur diffüzyon yöntemi kullanılarak belirlenmiştir. Juglon pozitif kontrol olarak kullanılmıştır. Beş bakteri türü *(Pseudomonas gingeri, P. syringae*, *Staphylococcus aureus, Bacillus cereus* ve *Yersinia enterocolitica)* ceviz özütlerine duyarlı bulunmuştur. Bununla beraber, sulu özütlerin etkisi alkolik özütlere göre daha zayıf bulundu. En duyarlı bakteri türü *S. aureus* idi. Ceviz yaprak özütleri genellikle üç fungus (*Candida albicans, Botrytis cinerea* and *Geotrichum candidum*) üzerinde önemli antifungal etki göstermişlerdir. Antifungal etkinin antibakteriyel etkiden daha kuvvetli olduğu görülmüştür. En duyarlı fungus *C. albicans* idi. Buna mukabil, en dirençli fungus *Aspergillus niger* idi. Ceviz özütleri içinde Yalova-3, Yalova-4 ve 1974/7 varyetelerine ait özütler en zayıf antimikrobiyal etki gösterdiler. Oysa en kuvvetli antimikrobiyal etkiyi Şebin vayetesinin özütü göstermiştir. Bunu ise Yalova-2 varyetesi izlemiştir. *S. aureus* bakterisi için Şebin vayetesi özütü ile juglonun MIC değerleri, sırasına göre, 0.006 and 0.05 mg/ml olarak belirlenirken, *C. albicans* fungusu *için 0.003 and 0.025 mg/ml olarak belirlenmiştir.*

Anahtar kelimeler: Antibakteriyel etki, antifungal etki, ceviz varyeteleri, ceviz özütleri, juglon

1. Introduction

Plants sometimes exude some organic chemicals to the environment from their different parts such as root, stem, leaf, fruit and seed. The chemicals named allelochemical affect other plants, insects and microorganisms live around the allelochemical producing plant. An

allelochemical of a plant may be noxious to another and helpful to a third organism; but they are usually toxic and induce stress and may even cause death. This event has been called as allelopathy (Rizvi and Rizvi 1992, Kocacaliskan and Terzi, 2001, Cheema et al. 2013).

The importance of the researches on antimicrobial activity of natural plant substances such as allelochemicals is increasing. Because these compounds are biodegradable in nature and for this reason they are environmentally and toxically more reliable than synthetic ones. Although synthetic compounds are a source of environmental pollution and detrimental for human health, they are being extensively used for controlling diseases (Vyvyan, 2002, Macías, et al. 2003).

The walnut family contains several species such as *J. nigra* and *J. regia* and the species are common on the world. Several parts of the walnut tree especially leaves are using because of its pharmacological properties like antifungal, antibacterial, antiviral, trypanocidal, antimalarial, antiinflammatory, and antipyretic properties including anticancer activity and cosmetic industries, as well (COSMULESCU and TRANDAFIR 2011). Since their ease of availability, using of the leaves are more common. The leaves of walnut have been taken account as a source of health support compounds, and have also been intensively used in supportive medicine for its antidiarrheic, antihelmintic, depurative and astringent properties. On the other hand, its keratolytic, hypoglycaemic, hypotensive and sedative activities have been determined, as well (COSMULESCU et al., 2014**).**

Juglone (5-hydroxy-1,4-naphthoquinone) is a quinone as an allelochemical from seconder metabolites has been found in all parts of the walnut tree and it has also been reported that the amounts of juglone were in range of green peel>leaves>bark (Sytykiewicz 2011). Because of its chemical reactivity juglone can be toxic for surrounding plant species in the same field. Therefore, juglone has been accepted to have walnut pathogenic defense mechanism (EL HADRAMI et al., 2005).

Although some articles have been published on the Juglone's biological activity, little is known about its toxic effect mechanism(s) on plant cells growth (RUDNICKA et al., 2014). Juglone has been shown to have herbicidal effect on some weeds such as field poppy, creeping thistle and henbit (TOPAL et al., 2007) and antioxidant effects on some enzymes such as catalase and superoxide dismutase (ALTIKAT et al., 2013). Its antiviral and antimicrobial activities have also been detected (MONTENEGRO et al., 2010; TAN et al., 2012).

Since its juglone content and antimicrobial activities, extracts of walnut leaves are used in allelopathic investigations (Clark et al. 1990, Dama et al. 1998, MONTENEGRO et al., 2010; TAN et al., 2012). Recently its antifungal and antibacterial activities have been enhanced using PLGA nanoparticle system, as well (ARASOGLU et al., 2016; 2017).

In this study, it has been aimed to research first time antimicrobial activities and to reveal varietal differences in antimicrobial effects of the five cultivars of walnut, *Juglans regia* L. Cv Şebin, Cv Yalova-2, Cv Yalova-3, Cv Yalova-4 and Cv 1974/7 which are developed and grown in Turkey. For this aim, antimicrobial activities of ethanolic and aqueous walnut leaf extracts obtained from the varieties were tested on eleven bacterial and nine fungal species. Pure juglone was used as a positive control.

2. Material and Methods

Nine pathogenic bacteria play roles in causing human diseases, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* NRRL B-3711, *Enterococcus faecium* NRRL B-3502, *E. faecalis* ATCC 29212, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris, Staphylococcus epidermidis, Yersinia enterocolitica* (clinical isolates), and two phytopathogenic bacteria *Psedomonas gingeri, P. syringae* pv. Phaseolicola (wild types) were used for testing antibacterial activity. Nine microfungi species, *Aspergillus flavus* ATCC 9807, *A. fumigatus* NRRL 163, *A. niger* ATCC 10949, *A. parasiticus* NRRL 465, *Botrytis cinerea* (AHU 9424), *Candida albicans* Y- 12983, *Fusarium graminearum, F. solani, and Geotrichum candidum* (wild types) were chosen for testing antifungal activity. Bacterial and fungal strains were taken from American Type Culture Collection (ATCC, Rockville, MD, USA), Northern Regional Research Laboratory (NRRL, USDA, Peoria, Illinois/USA) and Biology Department of Osmangazi University (Eskisehir/Turkey).

2.1. Plant material and preparation of aqueous and ethanolic extracts

Walnut leaf extracts were prepared from the leaves of five walnut cultivars (*Juglans regia* L. Cv Şebin, Yalova-2, Yalova-3, Yalova-4, 1974/7) generated in Turkey. Since juglone content in the leaves of walnut tree is in the highest level in August (Turan, 2008), the leaves were picked in the middle of August from the trees in the walnut garden of Agricultural Faculty of Uludağ University, Bursa, Turkey, and dryed at 70^{o}C in an oven for 48 h. Later 10 g dried leaf was homogenized using a Waring Blender in 100 ml of distilled water or ethanol. Then the homogenates were filtered and centrifuged at 20 000 rpm for 10 min. Supernatants were taken and, water and ethanol were removed from the supernatants by lyophilizing or evaporating, respectively. Then the treatments were prepared at the concentrations of 0.05 mg/ml of aqueous and 0.1 mg/ml of ethanolic walnut leaf extracts by dissolving in DMSO.

2.2. Antimicrobial activity determination

The extracts of the walnut leaves were studied for their antibacterial and antifungal activities by the well diffusion method. Since it is a modification of the disc diffusion method, the well diffusion method was preferred for the screening of antimicrobial effects (Wilkinson 2006). This method is widely used to evaluate the antimicrobial activity of plant extracts (Magaldi et al. 2004, Valgas et al. 2007).

Human pathogenic bacterial strains were inoculated in tubes containing Nutrient Agar (NA) medium and incubated at 37^{o}C for 24 h. On the other hand, phytopathogenic bacteria were inoculated in Petri dishes containing Yeast Malt Agar (YMA) medium and incubated at 25^{o}C for 72 h. Then they were diluted about 10^{8} cfu/ml. After that, 0.1 ml of cell suspension was delivered into the tube containing 5 ml of Mueller Hinton Agar (MHA) (Oxoid, UK) medium and mixed. They were flood-inoculated onto the entire surface of solidified MHA medium in Petri dishes. Then, the holes with a diameter of 10 mm were punched aseptically with a sterile cork borer, and a volume (0.1 ml) of extract solutions at desired concentration were introduced into the wells. For their diffusion they were kept in a refrigerator for 30 min. Then, the agar plates were incubated at 37^{o}C for 24 h and at 25^{o}C for 72 h, human pathogenic and phytopathogenic ones, respectively (CLSI 2012a).

Approved standard method (M51-A) developed by the CLSI for evaluating the susceptibility of filamentous fungi to antifungal agents was used. Each fungi was inoculated in tubes including Potato Dextrose Agar (PDA) medium at 25^{o}C for 72 h and cells/spores obtained

were diluted about 10^7 cfu/ml for yeast cells and 10^6 spore/ml for filamentous fungi. Then 0.1 ml of cell/spore suspension was added into the tube containing 5 ml of Sabouraud Dextrose Agar (SDA) (Oxoid, UK) medium and mixed. After that, they were flood-inoculated on to the surface of SDA medium in Petri dishes. Later, 0.1 ml of the treatments was added into the wells which were 10 mm diameters. For their diffusion they were incubated in a refrigerator for 30 min and incubated at 25°C for 72 h (Ilhan et al. 2007, CLSI, 2010).

Antimicrobial activites of walnut leaf extracts and juglone were evaluated with the inhibition zone against the test organism. The diameters of the inhibition zones (DIZ) were measured as milimeters. The experiments were repeated three times. Duncan's multiple range test was used for comparing the means of DIZ values of the walnut cultivars and juglone. Juglone treatment was used as positive control at 0.1 mg/ml concentration. Test organisms were assumed as sensitive to the treatments if the DIZ was larger than 17 mm, less sensitve if the DIZ was between 14-17 mm, and resistant if the DIZ was smaller than 14 mm (Kocaçalışkan et al. 2006).

Minimum inhibitory concentration (MIC) determination

Microdilution method was used to define MIC values using a 96 well plate (CLSI 2010, CLSI 2012a, CLSI 2012b). A serial dilution in the range of 0.4 to 4000 µg/ml was prepared transferring from the stock extract solution into the wells containing 100 µL of Sabouraud dextrose broth (SDB).
The inoculums were adjusted as described previously. One hundred µL of the inoculums was transferred to all the wells and the plate was incubated at 27°C for 24h. MIC values were detected by adding 20 µL of 0.5% trifenil tetrazolium chloride (TTC) aqueous solution. The MIC was defined as the lowest concentration of juglone or the extract that inhibited any visible microorganismal growth (Savaroglu et al. 2011).

S. aureus and *C. albicans* were used in MIC determination as test organisms since they were the most sensitive among the bacterial and fungal species, respectively. On the other hand, Şebin cultivar's extract was used in MIC determination since it has the most antimicrobial effect among the walnut varieties.

2.3. Thin layer chromatography (TLC) assay

This method was used to show if juglone is exist in all the leaves of the walnut cultivars studied and to determine its R_f value. Toluen: ethylasetate: methanol: buthanol solvents mixture (29.1:0.9:5:5) was used to move and separate juglone on silikagel covered plate. The spots on the plate were visualized under UV light (Ponder and Tadros, 1985).

3. Results and Discussion

As seen in Table 1, both ethanolic and aqueous walnut leaf extracts and juglone were generally found to have antibacterial activities against five bacterial species (*P. gingeri, P. syringae, S. aureus, B. cereus* and *Y. enterocolitica*). Since no teratment applied were showed any antibacterial activity on the other six species studied, whose DIZ values were zero, these data were not shown on the Table 1.

Table 1. Antibacterial activities of walnut (*J. regia* L.) leaf extracts and juglone on five bacterial species. Values are diameter (mm) of inhibition zones (DIZ), as mean of three replicates. Means with different letters within each column are significantly different (Duncan, 5%).

Bacterial strains	***Pseudomonas gingerie***	***Pseudomonas syringae***	***Staphylococcus aureus***	***Bacillus cereus***	***Yersinia enterocolitica***
Walnut cultivars			Ethanolic extracts (0.1 mg/ml)		
Şebin	14.6^{+} b	12.0^{-} d	22.8* b	18.5* b	18.6* b
Yalova-2	14.6^{+} b	17.3* b	21.0* b	18.3* b	18.0* b
Yalova-3	15.0^{+} b	15.3^{+} c	18.3* c	18.3* b	18.0* b
Yalova-4	14.3^{+} b	14.6^{+} cd	19.6* bc	16.0^{+} c	15.0^{+} c
1974/7	14.0^{+} b	14.6^{+} cd	18.6* c	15.6^{+} c	16.0^{+} c
			Aqueous extracts (0.05 mg/ml)		
Şebin	14.0+ b	13.3- d	16.6+ d	14.3+ d	0
Yalova-2	14.0+ b	13.3- d	16.6+ d	14.3+ d	0
Yalova-3	13.0- c	13.0- d	14.3+ e	13.6- d	0
Yalova-4	13.0- c	13.0- d	13.3- e	14.0+ d	0
1974/7	14.0+ b	12.6- d	15.0+ de	15.3+ c	0
			Juglone (0.1 mg/ml)		
	29.3* a	21.6* a	29.3* a	29.0* a	28.3* a

*Sensitive (DIZ above 17 mm). $^{+}$Less sensitive (DIZ between 14-17). $^{-}$Resistant (DIZ below 14 mm)

Bacteria of *S. aureus* and *B. cereus* species are Gram-positive and they were found more sensitive against to the treatments than the other species**.** *P. gingeri*, *P. syringae* and *Y. enterocolitica* which Gram-negative were less sensitive. On the other hand, the bacteria of *E. faecium*, *E. faecalis*, *S. epidermidis* are Gram-positive and *S. typhimurium*, *P. vulgaris*, *E. coli* are Gram-negative. But all of them were completely resistant to the treatments. This indicates that juglone and walnut leaf extracts have no selective effects on Gram-positive and Gram-negative bacteria.

Although juglon's antibacterial effects was higher than the extracts, antibacterial effects of ethanolic extracts were generally seen to be higher than aqueous extracts of walnut leaves. This result may be because of juglon's higher solubility in ethanol than water. Furthermore, the extract of Yalova-2 cultivar was generally found to have more antibacterial activity than the other cultivars. For example, antibacterial effect of the extract of Yalova-2 cultivar on *S. aureus* and *P. syringae* bacteria was significantly higher than the other cultivars. This may be originated from the juglone content of this cultivar. Because, it has previously been shown that juglone content of the leaves of Yalova-2 cultivar (3.51 mg/g leaf) was higher than those of the other walnut cultivars studied (Turan, 2008).

Allelochemicals may be preferred in controlling plant diseases mainly from point of environmental protection. Because they are friendly to the environment and safer than synthetic chemicals since their degradability is easy as compared to synthetic chemicals in nature. For example, juglone has been shown to be degraded rapidly in soil by bacterial or abiotic processes (Ponder and Tadros 1985, Schmidt 1988).

All the bacteria studied in this study are important pathogenic species on either human or plants. For examples; as sensitive bacterial species against to the treatments in this study; *S. aureus* causes several infections which may be result in several diseases such as boils, impetigo, food poisoning, cellulitis and toxic shock syndrome (Rongpharpi et al. 2014). *B. cereus* is a cause of mainly food poisoning and it is also a cause of serious and potentially fatal non-gastrointestinal tract infections (Bottone 2010). On the other hand, *Pseudomonas* spp. bacteria are phytopathogenic and *P. gingeri* causes the brown blotch disease in cultivated *Agaricus* mushroom (Abou-Zeid 2012). *P. syringae* pv. *phaseolicola* is the cause of bean

halo-blight disease (Tsiamis et al. 2000). *Y. enterocolitica* most often causes enterocolitis, acute diarrhea, terminal ileitis, and pseudoappendicitis but, if it spreads systemically, can also result in fatal sepsis. Its infection results in the disease yersiniosis, which is a zoonotic disease appearing in humans, as well as a wide order of animals such as cattle, deer, pigs, and birds (Huovinen et al. 2010).

Both aqueous and ethanolic walnut leaf extracts were found to have significant antifungal activities against three fungi species (*C. albicans, B. cinerea, G. candidum*) whereas juglone had significant antifungal activity on all the fungi studied (Table 2). The most sensitive species to all of the treatments was seen to be *C. albicans* and it was followed by *B. cinerea* and *G. candidum*. The most resistant one was *A. niger* that no activity was seen with the aqueous extracts.

Table2. Antifungal activities of leaf extracts of walnut (*J. regia)* cultivars and juglone against nine fungi species determined by the agar well diffusion method. Values in the table are diameter (mm) of inhibition zones (DIZ), as mean of three replicates. Means with different letters within each column are significantly different (Duncan, 5%).

Fungal strains	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus parasiticus*	*Fusarium solani*	*Fusarium graminearum*	*Geotrichium candidum*	*Botrytis cinerea*	*Candida albicans*
Walnut cultivars	Ethanolic extracts (0.1 mg/ml)								
Şebin	13.0^{-} b	17.3^{+} b	14.0^{+} b	12.8^{-} b	14.3^{+} b	14.0^{+} b	15.9^{+} c	18.6* b	26.7* ab
Yalova-2	12.0^{-} b	15.6^{+} b	13.0^{-} bc	12.6^{-} b	14.3^{+} b	14.0^{+} b	15.3^{+} c	18.6* b	24.8* b
Yalova-3	13.3^{-} b	14.6^{+} c	12.0^{-} c	12.6^{-} b	14.0^{+} b	12.6^{-}c	14.0^{+} d	17.5* bc	24.6* b
Yalova-4	12.0^{-} b	13.3^{-} c	12.0^{-} c	12.0^{-} b	12.0^{-} c	12.3^{-} c	15.3^{+} c	18.0* b	23.0* c
1974/7	12.0^{-} b	13.6^{-} c	12.3^{-} c	12.3^{-} b	12.0^{-} c	13.3^{-} bc	16.0^{+} bc	16.3^{+} c	20.7* d
	Aqueous extracts (0.05 mg/ml)								
Şebin	0.0^{-}	13.3^{-} c	14.6^{+} b	12.6^{-} b	12.3^{-}c	13.3^{-} bc	16.6^{+} bc	16.6^{+} c	21.0*d
Yalova-2	0.0^{-}	12.6^{-} d	14.0^{+} b	13.0^{-} b	12.3^{-} c	13.3^{-} bc	17.0* b	18.6* b	20.7* d
Yalova-3	0.0^{-}	12.6^{-} c	14.0^{+} b	12.6^{-} b	12.0^{-} c	12.3^{-} c	17.0* b	18.0* b	20.7* d
Yalova-4	0.0^{-}	12.6^{-} c	12.6^{-} c	12.6^{-} b	12.0^{-} c	12.0^{-} c	16.3^{+} bc	18.6* b	18.3*e
1974/7	0.0^{-}	12.6^{-} c	12.6^{-} c	12.3^{-} b	12.0^{-} c	12.6^{-} c	17.3* b	16.6^{+} c	17.7* e
	Juglone (0.1 mg/ml)								
	28.6* a	32.6* a	28.6* a	28.0* a	29.3* a	22.7* a	28.6* a	26.6* a	28.7* a

*Sensitive (DIZ above 17 mm). $^{+}$Less sensitive (DIZ between 14-17 mm). $^{-}$Resistant (DIZ below 14 mm).

There was almost no important difference between antifungal effects of ethanolic and water extracts of walnut leaves at 0.1 and 0.05 mg/ml concentrations, respectively. On the other hand, Yalova-2 cultivar was generally found to have more antifungal activity than the others. This result may be originated from the juglone content of this cultivar as mentioned above (Turan, 2008).

As a result of thin layer chromatography, it has been shown juglone is exist in all of the walnut varieties at 0.9 Rf in the same solvent system used. Of course, after initial screening of walnut leaf extracts, more detailed studies of their effects against test microorganisms should be conducted (Cowan, 1999). At this stage, more specific medium can be used and MIC can be effectively compared to that of pozitive control substance, juglone. For this purpose, the Şebin cultivar leaf (ethanolic) extract and the susceptible test organism, *C. albicans* were selected and determined MIC values of juglone and crude extract. MIC values for C. albicans of juglone and walnut leaf extract were found to be 0.003 and 0.025 mg/ml, respectively. These values are related to the DIZ values of juglon and leaf extracts (Table 1).
Although allelochemicals were generally tested against clinically important microorganisms, their effects on phytopathogenic microorganisms have not been researched in detail. In this

study, all of the fungi tested are pathogenic species for plants or human. *C. albicans* causes infections mainly in vaginal, oral and finger-nail tissues (Calderone and Fonzi 2001). *B. cinerea* is a necrotrophic fungal pathogen that attacks over 200 different plant species, and the disease is manifested by necrotic areas with extensive fungal growth, giving the characteristic appearance of grey mould (Govrin and Levine 2000). *G. candidum* fungus is a saprophyte and is found on several fruits and vegetables. It causes wound infection resulting sour rot disease in *Citrus* fruits (Baudoin and Eckert 1985). It has also been reported to cause a disease in immunosuppressed people and clinically it is similar to candidiasis and may causes vaginal, oral, skin or systemic infection (Verghese and Ravichandran 2003). *F. solani* causes the potato tuber dry rot disease of stored potatoes (Daferera, Ziogas et al. 2003). *Fusarium* head blight is a devastating disease of wheat and barley common on the world caused by *F. graminearum* (Bai and Shaner 2004). The genus *Aspergillus* includes etiologic agents of aspergillosis disease. It ranges from allergic reactions to invasive pulmonary infection. *A. fumigatus* is the major cause of the disease, followed by *A. flavus, A. niger* and the other species (Person et al. 2010).

4. Conclusion

In conclusion, the results indicate that juglone and ethanolic walnut leaf extracts, especially those of Yalova-2 cultivar, may be used in controlling the diseases caused by bacteria tested. In addition, the results indicate that juglone for all the fungi species studied and walnut leaf extracts at least for *C. albicans*, *G. candidum* and *B. cinerea* species may be used in controlling fungal diseases caused by fungi tested. Therefore, juglone and the walnut leaf extracts may be important treatments for controlling diseases caused by bacterial species tested to be sensitive in this sudy, especially the most sensitive species *(Staphylococcus aureus)* to all of the treatments, and the treatments may reduce the synthetic drug dependence. Also, it is suggested that juglone and walnut leaf extracts may be important treatments to control several diseases caused by especially the fungal species tested in this study and reduce the dependence to synthetic drug and chemicals.

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