RESEARCH ARTICLE

Antifungal Activity of *Eucalyptus Microtheca* Leaves Extract Against Aflatoxigenic Fungi

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ABSTRACT

Eucalyptus trees are evergreen, fast-growing, and widely cultivated in Iraq. Its leaves, buds, capsules, and even seeds contain several compounds that have antimicrobial activity.

Fresh leaves were collected and let dry in the shade at room temperature, then alcoholic, and aqueous stock solution (200mg/ml) of leaves extract was prepared in 10% DMSO from which different concentrations were done. Antibiotic susceptibility test was performed by the disk diffusion method using FLU, KCA, MCL, ECN, and ITC antibiotics. Leaves extracts were also examined for its antifungal activity then the MIC and MFC were determined using the microdilution method. KCA, ECN, and MCL were the most effective antifungal drugs on most isolates. Only two isolates were resistant to all antibiotics, and one isolate showed sensitivity to all antibiotics under study.

The methanolic and ethanolic extract of *Eucalyptus* leaves extract showed the highest inhibitory influence on fungal growth in comparison with the aqueous extract. Furthermore, Alcoholic extracts showed MIC at 50mg/mL and MFC at 100mg/ml. The aqueous extract *of Eucalyptus* had no inhibitory effect on the growth of all *Aspergillus* isolates.

The results of the present research showed the potential antifungal activity of the *Eucalyptus microtheca* leaves extract against the aflatoxigenic *A. niger* and *A. flavus*, which is an indication of the fungicidal value of the plant extract. This research suggests that the plant extract may possess some compounds with antifungal properties against fungi, and it can be used as a safe and economical alternative against aflatoxigenic fungal food and feed contamination.

Keywords: Antifungal, *Eucalyptus*, Leaves extract.

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INTRODUCTION

Eucalyptus trees are evergreen, fast-growing, and widely cultivated in Iraq as part of the green Iraq project.

Its leaves contain several compounds that are important in defense against vertebrate herbivores, insects, protection against stress of ultraviolet (UV) radiation, and cold. *Eucalyptus* leaves represent important sources of compounds like flavonoids, tannins, glycosides, saponines, alkaloids and essential oils with biological activities such as bacteriostatic, fungistatic and anti-inflammatory. Terpenoids give *Eucalyptus* its characteristic smell, which forms most of the essential oil. *Eucalyptus* plant parts extract showed high antimicrobial activity and used in many pharmaceuticals, toothpaste, and soaps.[1,2]

It was reported that the extract of *Eucalyptus* leaves at concentrations range from 25, 50 and 75mg/mL on growth inhibition of mold that includes (*Aspergillus* spp, *Penicillium* spp., *Alternaria* spp. and *Trichoderma* spp.), showed increasing in mold growth inhibition (average 70.1%) and it's proportional with the concentration.[3]

Very few studies manipulated the *Eucalyptus* effect on mold growth. However, In another study, the antifungal activity of *Eucalyptus microtheca* leaves crude extract was tested by agar well diffusion method against *P. digitatum* and *A. niger* fungi. Alcoholic extracts inhibited the growth of *P. digitatum* and *A. niger* fungi significantly more than their aqueous extract. The methanolic extract showed higher inhibition activity than ethanolic extract.[4] In an, another study, the leaves ethanolic extract of *Eucalyptus* at both concentrations of (1 and 0.5mg/ml) inhibited the growth of *A. flavus* and *Penecillum* significantly as compared with control.[5]

Using acetone (30%), the *Eucalyptus* capsule crude extract showed strong anti-microbial activity where the MIC for fungi were 18mg/ml for *Mucor* sp. and 4mg/ml for *R. stolonifer*.[6]

Antifungal, anti-aflatoxigenic, and anti-aflatoxin activity of alcoholic extract of plants were reported and are represented as the best safe and economical alternative against fungal and aflatoxins contaminated food and feedstuff.[7]

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This research is evaluating the antifungal activity of alcoholic and aqueous extracts of *Eucalyptus microtheca* leaves against aflatoxigenic *Aspergillus* spp*. in vitro.*

MATERIALS AND METHODS

Aflatoxigenic fungi were previously isolated from dry fruits and examined for aflatoxins production by TLC and HPLC,[8,9] (data not shown).

Leaves Extract preparation

Fresh leaves were collected and let dry in the shade at room temperature. A bout100g of grinded dry leaves powder was mixed with 250mL of solvent (absolute ethanol or methanol or hot distilled water) and shacked at 190rpm for 24h. The mixture was filtered using gauze then centrifuged at 5000xg for 10minutes. The supernatant was dried at 45ºC by rotary evaporator.[10] The dried extract was weighed , and 200mg/mL stock solution was prepared in 10% Dimethyl Sulfoxide (DMSO) (SDFCL, India) from which different concentrations were done. The extracts were stored at -20ºC until use after filtration using Millipore filter (0.22µm).

Antifungal Assays

Antibiotic Susceptibility Test

The disk diffusion method was conducted using Muller Hinton agar (MHA) (Himedia, India) in triplicates.[11] FLU, KCA, MCL, ECN, and ITC (Liofilchem, Italy) antifungal drugs have been tested against aflatoxigenic mold isolates. About 5×10^3 spore/ml of spore suspension was spread on MHA and allowed to dry for 5minutes. Thereafter, antibiotics disks were placed on the surface of each inoculated MHA plate. The plates were incubated at 35ºC then read after 24 to 72 hour. The inhibition zone around each disc was measured in millimeter using a Vernier caliper.

Leaves extract antifungal test

The plant extract was tested against aflatoxigenic mold isolates to check its anti-fungal activity. The same procedure for disk diffusion mentioned above was followed using 6 mm sterile paper discs saturated with the plant extract stock solution.

MIC determination using the microdilution method

MIC and MFC were determined using the microdilution method[12,13] with some modification. Microplates of Flat-bottomed 96-well were used where 100µL of Sabouraud dextrose broth (SDB) ‎(Himedia, India)‎ were added to each well. A 100µL of plant extract stock solution was added into the first row. Two-fold serial dilutions were done along the rows to obtain 100 to 0.78mg/mL. About $5x10^3$ spore/mL was added to each row. The inoculated plates were incubated at 28ºC for 72h. MIC was determined as the lowest concentration that visibly inhibits fungal growth.

MFC was determined by inoculating about 10µL of each MIC well on SDA and incubated at 28ºC for 3–7days. The lowest concentration with no fungal growth defined as MFC.[14] The last three columns of each test plate included growth control, sterility control, and positive control. All tests were done in triplicates.

RESULTS AND DISCUSSION

KCA, ECN, and MCL were the most effective antifungal drugs on most isolates. This agrees with the results reported by.[15]

In Table 1, the result showed that isolates (5 and 6) were resistant to all antibiotics, while isolate 2 showed sensitivity to all antibiotics under study. Furthermore, only this isolate was FLU sensitive with 30.7mm inhibition zone diameter.

Most isolates were ITC resistant which disagrees with a study by.[16,17]

KCA showed high antifungal activity against most isolates in comparison with previous studies by.[18,19] and more active compared with FLU.[15,20]

Through our study, the methanolic and ethanolic extract of *Eucalyptus* showed the strongest inhibitory influence on fungal growth in comparison with the aqueous extract, that agrees with the previous study reported by.[21] Furthermore, Alcoholic extracts showed MIC at 50mg/ml and MFC at 100mg/mL (Table 2 and Figure 1). Although, ethanolic extract was found with both inhibitory and fungicidal activity at higher concentration compared with what was reported by.[5]

The aqueous extract *of Eucalyptus* had no inhibitory effect on the growth of all *Aspergillus* isolates, which does not agree with a study by.[3,4] Add to that; alcoholic extract showed inhibitory effect to all *Aspergillus* isolates that agree with the results reported by.[4]

CONCLUSION

The results of the present research showed the potential antifungal activity of the *Eucalyptus microtheca* leaves extract against the aflatoxigenic *A. niger* and *A. flavus*, which is an indication of the fungicidal value of the plant extract. This research suggests that the plant extract may possess some compounds with antifungal properties against fungi, and

Table 1: Antibiotic sensitivity of aflatoxigenic fungal isolates

		Inhibition Diameter (mm) of Aspergillus isolates									
Antibiotics	*µg*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
ECN	10	/	33.2	30.2	/	/	/	24.3	/	30.3	30.3
ITC	50	/	13.3	14.1	7.1	/	/	/	/	13.6	/
FLU	25	/	30.7	/	/	/	/	/	/	/	/
KCA	10	13.2	33.2	31.75	/	/	/	25	/	23	26
MCL	10	/	24.8	15.5	/	/	/	18.2	/	/	15.3

Figure 1: Microdilution antifungal activity and MIC test of *Eucalyptus* leaves alcoholic extract. A-H (100-0.78mg/mL). 1-9: aflatoxigenic isolates, 10: Positive control (KCA), 11: Growth control, 12: Sterility control.

Table 2: Antifungal activity of *Eucalyptus microtheca* leaves extract.

Type of extract	*mg/mL*	*Mold isolates*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Ethanol	MIC	50	50	50	50	50	50	50	50	50	50
	MFC	100	100	100	100	100	100	100	100	100	100
Methanol	MIC	50	50	50	50	50	50	50	50	50	50
	MFC	100	100	100	100	100	100	100	100	100	100
Aqueous	MIC	-	-	-	-	-	-	-	-	-	-
	MFC	-	-	-	-	-	-	-	-	-	-

it can be used as a safe and economical alternative against aflatoxigenic fungal food and feed contamination.

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