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In vitro studying of probiotic inhibitory effects on some keratitis fungi

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ABSTRACT

Antifungal agents play an important role in the treatment of human serious diseases. Therefore, testing the susceptibility of some keratitic fungal isolates such as *Aspergillus parasiticus* AUMC 3944, *Aspergillus parasiticus* AUMC 3946, *Aspergillus flavus* AUMC 3939 and *Aspergillus flavus* OC10 to some probiotic organisms such as *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. The results revealed two novel antifungal compounds extracted from probiotics liquid cultures; butanol showed the best solvent for the extraction of antifungal compounds, moreover, *Saccharomyces cervisiae* showed the higher inhibitory effect than *Lactobacillus bulgaricus*. Scientifically, this study recommended the use of these extracts as a potent antifungal agent after their purification and identification.

***Keywords*:** Probiotics, Antifungal**,** fungal keratitis

Introduction

Fungal infections of the cornea (mycotic, fungal keratitis or kertomycosis) present as suppurative, usually ulcerative lesions. Such a corneal infection, poses a challenge to the ophthalmologist because of its tendency to mimic other types of stromal inflammation, and because its management is restricted by the availability of effective antifungal agents and the extent to which they can penetrate into corneal tissue (Thomas, 2003a,b). Fungal keratitis is one of the most important causes of ocular morbidity and visual loss in developing nations, where it may account for nearly half of corneal ulcers (Bharathi *et al*., 2003).

Aspergillus species were categorized as predominant causative species of fungal keratitis, and *Aspergillus flavus* was the most common species of fungal keratitis (Khairallah *et al*., 1992; Xie *et al*., 2008; Leema *et al.,* 2010 and Ismaiel *et al*., 2012).

As the microorganisms have developed resistance to many antibiotics and as a result immense clinical problem in the treatment of infectious diseases has been created, so much efforts should been devoted to the search for new antimicrobial materials from natural sources to be used in folk medicine to treat infections. The resistance of the organisms increased due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. So the present study focused on *Aspergillus flavus* isolated from keratitic patients in a special reference to its pathogenicity and its resistance with a common probiotics strains such as *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. In this study, we briefly described the inhibitory effect of these probiotics on the keratitic fungal isolates.

Materials and Methods

Microorganisms

The experimental fungal isolates:

Identified fungal isolates which were previously isolated from keratitis patients, were kindly obtained from Assiut University Mycological Center (AUMC), they were *Aspergillus parasiticus* AUMC 3944, *Aspergillus parasiticus* AUMC 3946, *Aspergillus flavus* AUMC 3939 and *Aspergillus flavus* OC10.

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Probiotic organisms:

i- *Saccharomyces cerevisiae*:

Identified *S. cerevisiae* (RCMB006001) was kindly provided from The Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar University.

ii- *Lactobacillus* (LAB) strain**:**

Lactobacillus bulgaricus (RCMB025001) was kindly provided from the Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar University.

Cultivation Conditions

Lactobacillus bulgaricus (RCMB025001) was transferred to grown in 250 *ml*-Erlenmeyer flasks containing 100 *ml* of sterilized deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) and was incubated at 37℃ for 48 h., the first liquid culture (LC) taken as it is. The supernatant of another liquid cultures was prepared by filtration with syringe filter (Zeites) to obtain acellular supernatant without pH adjustment (ASN), and acellular supernatant with pH adjustment at pH 6.5 (NASN). *Saccharomyces cerevisiae* (RCMB006001) was transferred to grown in 250 *ml*-Erlenmeyer flasks containing 100 *ml* of sterilized Sabouraud broth and was incubated at 30℃ for 3 days. The first liquid culture (LC) taken as it is. The supernatant of another liquid cultures was prepared to obtain acellular supernatant without pH adjustment (ASN), as follows; the cultures were centrifuged at (6000 rpm) for 10 min to obtained cell free filtrate, which was sterilized by membrane filtration (0.45 and 0.22 µm) (Aslim and Kilic, 2006) and acellular liquid culture with pH adjustment at pH 6.5(NASN). Both sterilized deMan, Rogosa and Sharpe (MRS) broth and sterilized Sabouraud broth were used as a negative control, and itraconazole dissolved in DMSO (dimethylsulphoxide) used as a positive control.

Inoculation and detection of the inhibition zones:

Each fungal isolate was inoculated by diffusion method in Czapek Dox agar media (CDA), then poured in Petri dishes, after solidification, A well of 0.6 cm diameters was made on the surface of the medium using a sterile cork borer, then inoculate in each well 100 µg of each one of the following: LC *Saccharomyces cerevisiae,* ASN *Saccharomyces cerevisiae*, NASN *Saccharomyces cerevisiae*, LC *Lactobacillus bulgaricus*, ASN *Lactobacillus bulgaricus*, NASN *Lactobacillus bulgaricus*, MRS broth and Sabouraud broth as negative control, and 100µg pure Itraconazole dissolved in 1ml DMSO as positive control respectively.

All Petri dishes were put in refrigerator at 5 °C for 2 hours to make complete diffusion for the inoculated liquids; then incubated at 28 °C for 5 days.

The inoculum diffusion method were described by Eva *et al*. (2001); 0.1 ml ($1\times 10^6 \sim 1\times 10^8$ cfu/ml, adjusted by spectrophotometer OD530 were in range 0.07-0.27nm) of pathogenic microorganism was spread on sterilized Czapek Dox ag Czapek ar media then poured in Petri dishes and let for solidification.

After the incubation period, inhibition zone diameters (cm) were measured and compared with the controls well that contained; MRS broth, , Sabouraud dextrose (SD) broth, itraconazole dissolved in DMSO (Sigma). and then the inhibition zones were measured and compared. The inhibition zone was calculated by centimeter.

Extraction of antifungal compounds from probiotics cultures:-

According to Natarajan *et al*. (2010) A sterile MRS broth and Sabouraud dextrose (SD)broth were inoculated with an actively growing culture of *Lactobacillus bulgaricus* and *Saccharomyces cerevisi*ae respectively , incubated at 37°C for 48 hours and at 30°C for 3 days respectively. Cultures were then centrifuged at 6000 rpm for 15 minutes, and the supernatant was passed through a Millipore filter (0.2 µm porosity) to get a cells free filtrate.

For extraction of antimicrobial compounds the filtrates were firstly treated with hexan 1N. in order to de-fat in ratio (filtrate:hexan,5:1), Using separating funnel mix and shake 30 minutes and stand for 1-2 hours then separate and take the filtrate. Equal volumes of tested solvents were used as follows; mix an equal volume of fat-free filtrate with benzene solvent, mix and shaken in a separating

funnel for about 1-2 hours and leave standing over night, these steps repeated with other solvents (butanol, ethyle acetate, ethyle acetate:methanol 1:1/v:v and methelene chloride: methanol 2:1/ v:v). Then the organic layer was separated and collected. The solvent was removed in vaccum using a rotary vaccum evaporator. The residue after being collected used as inoculum (100µl) in the wells in Petri dishes of each pathogenic fungi (*Aspergillus parasiticus* AUMC 3944 , *Aspergillus parasiticus* AUMC3946, *Aspergillus flavus* AUMC3939, and *Aspergillus flavus* OC10, incubate at 28ºC for 5 days and then the inhibition zones of the filtrates were measured and compared. Then the inhibition zone was calculated .

All experiments were conducted in triplicates and the mean ± standard deviation (SD) of these triplicates was calculated.

Results

Diameters of the inhibition zones of fungal isolates (from patients suffering from keratitis) grown on the Czapek Dox agar (CDA) against *L. bulgaricus* grown on MRS and *S. cerevisia*e grown on Sabouraud dextrose were reported in Table (1).

The results showed that *S. cerevisiae* is the more effective probiotic than *L. bulgaricus*. The filtrates of both *S. cerevisiae* and *L. bulgaricus* as a liquid culture (LC) gave the highest inhibitory effect when compared with the same filtrate as acellular supernatant (ASN) or neutralized acellular supernatant (NASN). The results also showed that *Aspergillus parasiticus* 3946 was determined as the most sensitive tested fungi followed by *Aspergillus parasiticus* 3944, and *Aspergillus flavus* OC10. As displayed in Table (1) and Figures (1), (2), and (3).

Data given in Tables 2 and 3 indicated that n-butanol was a proper solvent for extraction the antimicrobial compounds of both *S. cerevisiae* and *L. bulgaricus*. With all fungal strains tested, the diameter of inhibition zone formed by n-butanol extract was significant (P≤0.05) as compared with those obtained by the other solvents tested, and the extraction of antifungal compound by n-butanol gave the highest inhibitory effect among the other used solvents which are (benzene, ethyle acetate, ethyle acetate: methanol 1:1/v:v and methelene chloride: methanol 2:1/v:v).

The solvent system composed of ethyl acetate and methanol exhibited satisfactory extraction potentiality recording an average of inhibition zone diameter of 0.9-1.0 cm with the fungal strains tested. The other non polar solvents were not appropriate for antimicrobial compounds extraction showing comparable diameters of inhibition zones with their negative controls.

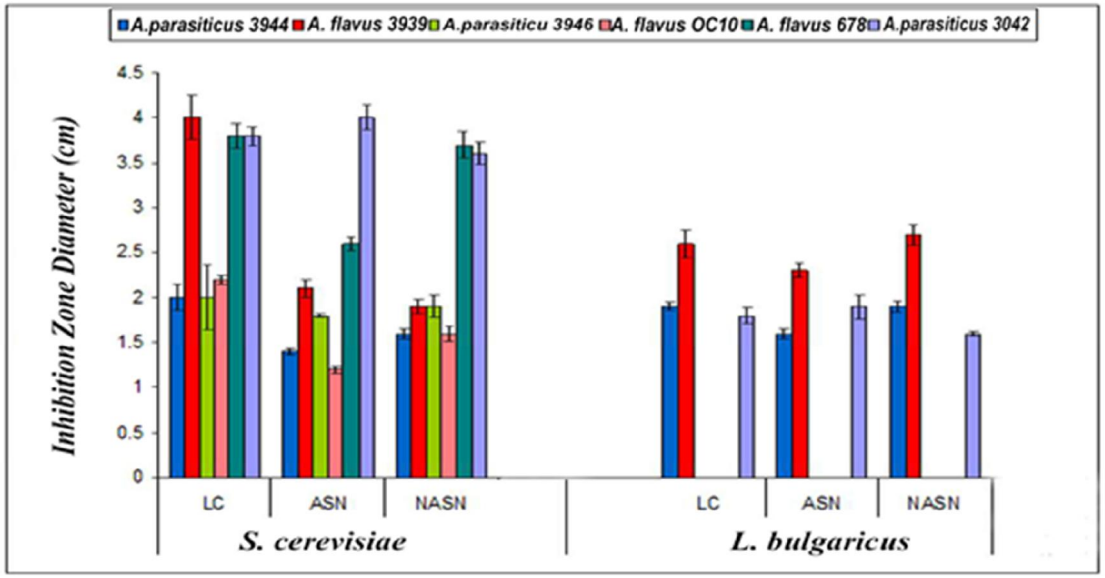

Fig. 1: Inhibitory effect of probiotic strains on fungal strains grown on CDA using well diffusion assay after incubation for 5 days at 28˚C. liquid culture (LC), acellular surpernatant (ASN) and neutralized acellular surpernatant (ASN) .

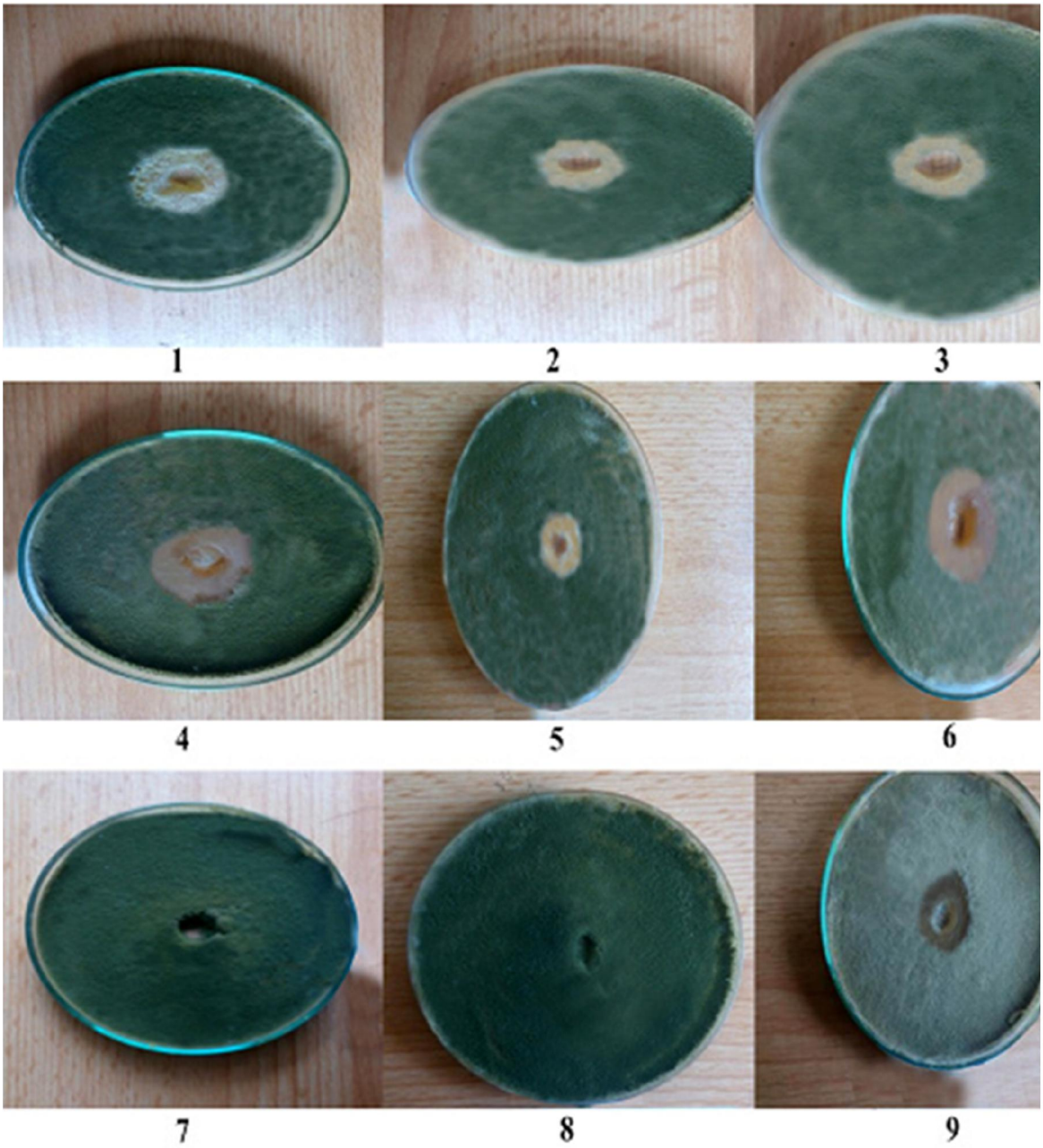

Fig. 2: Inhibitory effect of probiotics against *Aspergillus parasiticus* AUMC 3944 grown on Czapek Dox's agar incubated at 28ºC for 5days.
1: LC of *S. cerevisiae*, 2: ASN of *S. cerevisiae*, 3: NASN of *S. cerevisiae*.
4: LC of *L. bulgaricus*, 5: ASN of *L. bulgaricus*, 6: NASN of *L. bulgaricus* ,
7: MRS broth as negative control, 8: SD. broth as a negative Control, 9: Pure itraconazole (100µg/ml) a positive control.

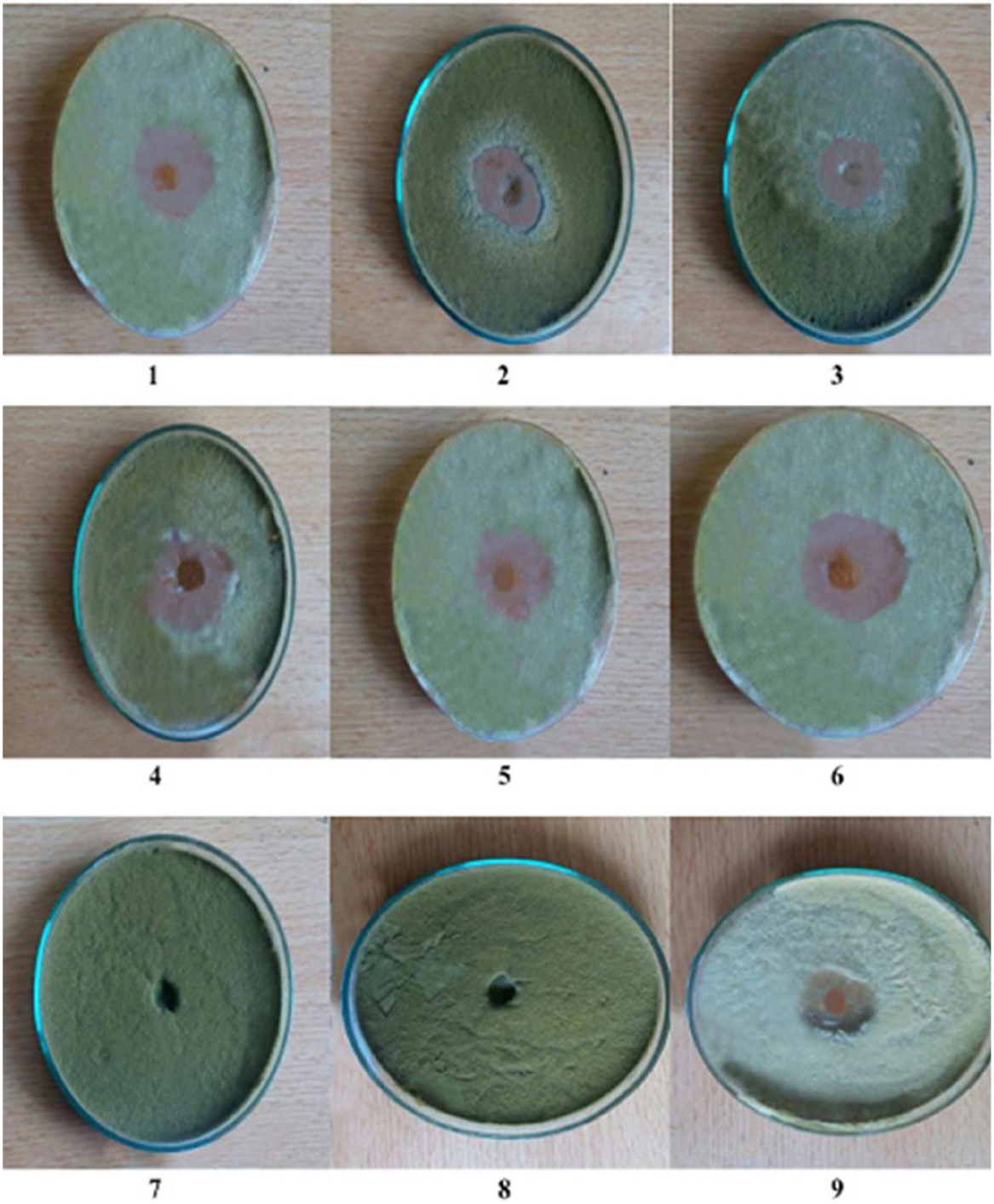

Fig. 3: Inhibitory effect of probiotics against *Aspergillus flavus* AUMC 3939 grown on Czabek Dox's agar incubated at 28°C for 5days.
1: LC of *S. cerevisiae*, 2: ASN of *S. cerevisiae*, 3: NASN of *S. cerevisiae*.
4: LC of *L. bulgaricus*, 5: ASN of *L. bulgaricus*, 6: NASN of *L. bulgaricus* ,
7**:** MRS broth as negative control, 8:SD broth as a negative Control, 9: pure itraconazol (100µg/ml)a positive control.

Table 1: Inhibitory effect of liquid culture (LC), acellular surpernatant (ASN) and neutralized acellular surpernatant (ASN) of probiotic strain against keratitic fungal causatives after incubation for 5 days at 28°C:

Fungal strains	Probiotics						Controls		
	Diameter of inhibition zone (cm)*						Diameter of inhibition zone (cm)*		
	Saccharomyces cerevisiae			*Lactobacillus bulgaricus*			Positive control	Negative controls	
	LC	ASN	NASN	LC	ASN	NASN	Itraconazole (100 µg/ml)	Sabauraud's broth	MRS broth
***A.flavus* AUMC 3939**	4.0±0.25 [a]	2.1±0.10 [c]	1.9±0.08 [c]	2.6±0.16 [a]	2.3±0.08 [a]	2.7±0.11 [a]	1.9±0.08 [c]	0.0[d]	0.0[d]
***A.flavus* OC10**	2.2±0.05 [b]	1.2±0.04 [f]	1.6±0.08 [d]	0.0[c]	0.0[d]	0.0[d]	1.8±0.08 [c]	0.0[d]	0.0[d]
***A.parasiticus* AUMC3944**	2.0±0.15 [b]	1.4±0.04 [e]	1.6±0.05 [d]	1.9±0.05 [b]	1.6±0.06 [c]	1.9±0.06 [b]	1.9±0.08 [c]	0.0[d]	0.0[d]
***A.parasiticus* AUMC 3946**	2.0±0.36 [b]	1.8±0.02 [d]	1.9±0.12 [c]	0.0[c]	0.0[d]	0.0[d]	1.9±0.08 [c]	0.0[d]	0.0[d]

*Diameter was calculated without subtracting the diameter of the well (0.6cm)., **LC** :liquid culture, **ASN**: acellular supernatant , **NASN**: neutralized acellular supernatant, calculated mean is for triplicate measurements from two independent experiments ± SD, [a-f] Means with different superscripts in the same column are considered statistically different (LSD test, $P \leq 0.05$),Values having the same alphabetical letter(s) did not significantly differ at the 0.05 level of significance, according to Duncan's multiple range test .

Table 2: Inhibition zone diameter of antifungal compound extracted from *Saccharomyces cerevisiae* dissolved in different solvents on pathogenic fungi

	Inhibition zone diameter (cm)*									
Fungal strains	**Compound dissolved in benzene**	**Benzene**	**Compound dissolved in butanol**	**Butanol**	**Compound dissolved in Ethyl acetate**	**Ethyl acetate**	**Compound dissolved in Ethyl acetate:methanol (1/1;v/v)**	**Ethyl acetate:methanol (1/1;v/v)**	**Compound dissolved in Methelene chloride:methanol (2/1;v/v)**	**Methelene chloride:methanol (2/1;v/v)**
***A.flavus* AUMC 3939**	1.0 ±0.02 ^{d}	0.9±0.05 ^{a}	1.9±0.40 ^{b}	0.7±0.08 ^{a}	0.7±0.07 ^{b}	0.6±0.05^{c}	1.1±0.12 ^{ab}	0.7±0.05 ^{c}	0.8±0.02 ^{b}	0.6±0.05 ^{a}
***A.flavus* OC10**	0.8 ±0.02 ^{b}	0.8±0.06^{ab}	2.4±0.18 ^{a}	0.8±0.05 ^{a}	0.7±0.02 ^{b}	0.7±0.01^{b}	1.2±0.03 ^{a}	0.7±0.02 ^{c}	0.9±0.05 ^{a}	0.6±0.06 ^{a}
***A.parasiticus* AUMC 3944**	0.9± 0.03 ^{a}	0.9±0.06 ^{a}	2.1±0.20	0.7±0.07 ^{a}	0.7±0.05 ^{b}	0.6^{c}±0.0^{c}	1.0±0.04 ^{ab}	0.7±0.06 ^{c}	0.7±0.04 ^{c}	0.6±0.04 ^{a}
***A.parasiticus* AUMC 3946**	0.9 ±0.06 ^{a}	0.8±0.08^{ab}	1.9±0.09 ^{b}	0.7±0.10 ^{a}	0.8±0.04 ^{a}	0.7±0.06^{b}	1.2±0.15 ^{a}	0.8±0.06 ^{b}	0.7±0.03 ^{c}	0.6±0.03 ^{a}

*Diameter was calculated by cm., without subtracting the diameter of the well (0.6 cm)., calculated mean is for triplicate measurements from two independent experiments ± SD, $^{a-f}$ Means with different superscripts in the same column are considered statistically different (LSD test, $P \leq 0.05$),Values having the same alphabetical letter(s) did not significantly differ at the 0.05 level of significance, according to Duncan's multiple range test.

Table 3: Inhibition zone diameter of antifungal compound extracted from *Lactobacillus bulgaricus* dissolved in different solvents on pathogenic fungi:

	Inhibition zone diameter*									
Fungal isolates	**Compound dissolved in benzene**	**Benzene**	**Compound dissolve in butanol**	**Butanol**	**Compound dissolved in Ethyl acetate**	**Ethyl acetate**	**Compound dissolved in Ethyl acetate:methanol (1/1;v/v)**	**Ethyl acetate:methanol (1/1;v/v)**	**Compound dissolved in Methelene chloride:methanol (2/1;v/v)**	**Methelene chloride:methanol (2/1;v/v)**
***A.flavus* AUMC 3939**	0.9±0.05^{a}	0.9±0.05^{a}	2.1±0.12^{a}	0.7±0.01^{a}	0.8±0.04^{b}	0.6±0.07^{c}	1.0±0.03^{a}	0.7±0.02	0.8±0.04^{b}	0.6±0.08^{a}
***A.flavus* OC10**	0.8±0.02^{b}	0.8±0.04^{b}	2.0±0.08^{ab}	0.8±0.05^{a}	0.9±0.04^{a}	0.7±0.04^{b}	1.0±0.11^{a}	0.7±0.04	0.8± 0.06^{b}	0.6±0.01^{a}
***A.parasiticus* AUMC 3944**	0.8±0.07^{b}	0.9±0.03^{a}	2.0±0.04^{ab}	0.8±0.03^{a}	0.7±0.06^{c}	0.6±0.05^{c}	1.0±0.02^{a}	0.7±0.04	0.8±0.03^{b}	0.6±0.05^{a}
***A.parasiticus* AUMC 3946**	0.9±0.04^{a}	0.8±0.02	1.9±0.05^{b}	0.7±0.03^{a}	0.7±0.02^{c}	0.7±0.02^{b}	0.9±0.05^{a}	0.8±0.01^{b}	0.9± 0.02^{a}	0.6±0.05^{a}

*Diameter was calculated by cm. ,without subtracting the diameter of the well (0.6 cm)., calculated mean is for triplicate measurements from two independent experiments ± SD, a-f Means with different superscripts in the same column are considered statistically different (LSD test, P ≤0.05),Values having the same alphabetical letter(s) did not significantly differ at the 0.05 level of significance, according to Duncan's multiple range test.

Discussion

As the microorganisms have developed resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created (Davis, 1994), so much efforts should been devoted to the search for new antimicrobial materials from natural sources to be used in folk medicine to treat infections (Gibbons, 1992, Menna and Sethi, 1994, Arora and ohlan, 1997, Arora and Bhardwaj, 1997).

The resistance of the microorganisms increased due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. So the present study focused on *Aspergillus flavus* isolated from keratitic patients in a special reference to its pathogenicity.

The results in the present study showed that *Saccharomces cerevisiae* was the highest active antifungal probiotic followed by *Lactobacillus bulgaricus*.

The present results showed that the using of probiotics as a liquid culture (LC) has the greatest antifungal effect than using them as acellular supernatant without pH adjustment (ASN) or even as neutralized acellular supernatant (NASN). The same result was reported by Denkova R. *et al*. (2013). The present results are partially agree with that reported by Abbaszadeh *et al*. (2015), where their study reported that both liquid culture (LC) and supernatant of *Lactobacillus Caseie* exhibited high antifungal activity on *A. parasiticus* and *A. flavus*. These results may explain the high metabolic aptitude of *Saccharomyces cerevisiae* and in case of *Lactobacillus bulgaricus*; there is a high concentration of lactic acid production during the growth phase, which suggesting that this metabolic aptitude contributed to explain the highlighted antifungal phenotype.

While Padmaja and periyar (2016) studied the antifungal and anti-aflatoxigenic effect of four different *Lactobacillus* probiotics strains against *A. flavus* and *A. parasiticus*, it was demonstrated that *lactobacillus delbrueckii* subsp. *Lactis* showed maximal antifungal and anti-aflatoxigenicactivity against *A. flaves* whereas *A. parasiticus* was inhibited by *Lactobacillus brevis* with less antifurgal and anti-aflatoxigenic activity.

After that significant inhibitory effect of the tested probiotics on pathogens (Keratitis fungi) isolation and purification of the antimicrobial compounds from probiotics liquid culture (as mentioned in details at materials and methods).

Research which concerns the usefulness of probiotics show increasing interest based on the rise of their publications, products and the awareness of the public of their benefits. There is increasing interest concerning probiotics from the public, researchers, governmental organizations (such as the WHO/ FAO) and medicinal and food companies.

The present results completely agree with that reported by Adam *et al*. (2018) in the fact that lactic acid bacteria (LAB) have the ability to control the growth of various fungi. Where they explained this antagonistic effect is owed to low molecular weight compounds produced by LAB, such as organic acids (acetic acid – Lactic acid), hydrogen peroxide, proteinaceous compounds, reuterin, hydroxyl fatty acids, and phenolic compounds also lactic acid lowers pH, which inhibits the mycelia growth .

One possible explanation for the inhibitory effect of *Saccharomyces cerevisiae* to reduce the growth of the tested fungi is that the production of ethanol by *Saccharomyces cerevisiae*, which it has been suggested that the interaction results in changes in membrane properties equivalent to a decrease in membrane fluidity; this fact run paralleled with that reported by Susan and Rose (1979), and that explained by Ingram (1976) as his results suggested that decreased fluidity may be brought about by ethanol molecules replacing water molecules associated with the polar headgroups on phospholipids, thereby decreasing their repulsion, or by becoming located in the interior of the bilayer and restricting movement of fatty-acyl chains. It is believed that there are precise requirements for fluidity in biological membranes, with the activities of individual membrane-bound proteins being regulated by the fluidity in the domain of the membrane in which they occur (Haest *et al*., 1972).

The results in the present study also reported that the crude butanol extract of both probiotics showed the highest antifungal activity which completely agree with that reported by Khandan and Ramanayaka (2015).

Conclusion

This study revealed that butanol extracts of probiotic organisms; *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* on the screened fungal isolates be promissing in treating human fungal kiratitis especially *Aspergillus parasiticus* AUMC 3944, *Aspergillus parasiticus* AUMC 3946, *Aspergillus flavus* AUMC 3939 and *Aspergillus flavus* OC10. Further studies are needed to isolate, characterize and elucidate the structure of the bioactive compounds of probiotic organisms for antifungal drugs formulation.

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