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Research Article

In vitro assessment of antimicrobial and anti-inflammatory potential of endophytic fungal metabolites extracts

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ABSTRACT

Endophytes are endosymbiotic microorganisms that act as reservoir of novel bioactive secondary metabolites with antimicrobial, cytotoxic and anti-cancer activities. In the present study, the extracts of 26 different endophytic fungal strains were screened for their antimicrobial and anti-inflammatory activities. The results showed a wide variety of antimicrobial activities against 12 target microorganisms including three Gram (+) bacteria, three Gram (-) bacteria, 3 yeasts, 2 dermatophytic fungi and one keratinophilic fungus. Four fungal extracts (*Aspergillus versicolor*, *A. awamori*, *A. niger* and *Penicillium funiculosum*) displayed a broader antibacterial spectrum and inhibited the growth of all Gram (+) and Gram (-) bacterial species. The extracts of 8 endophytic fungi inhibited the growth of the two tested dermatophytic strains (*Trichophyton mentagrophytes* and *T. rubrum*). Only eight fungal extracts have an inhibition activity against the keratinophilic fungal strain (*Chrysosporium tropicum*). The anti-inflammatory assay showed that the extracts of *Emericella nidulans*, *Pleospora tarda* and *Penicillium funiculosum* had good activities in inhibition of protein denaturation reached to 83%, 82.5% and 81.4%, respectively. Also, *Emericella nidulans* and *Pleospora tarda* recorded the maximum inhibition effect on bovine serum albumin denaturation reached to 95% and 90.7%, respectively. On the other side, *Emericella nidulans* showed the maximum inhibition activity (69.5%) out of all tested endophytic strains against humun red blood cells membrane stabilization assay. In conclusion some secondary metabolites of endophytic fungi have a promising potential as antimicrobial and anti-inflammatory compounds.

Keywords: Endophytes; Fungi; Antimicrobial; Anti-inflammatory; Drug discovery.

1. INTRODUCTION

World health problems caused by drug resistant bacteria and fungi are increasing. Many pathogenic microorganisms have developed resistance due to the misusage or long-term usage of the same class of antibiotics. Intensive search for more effective antibiotics to deal with these problems is now ongoing [1]. The isolation of novel secondary metabolites from the endophytes is a progressive research field [2]. Endophytic microbes are fungi and bacteria that colonize internal tissues of living plants without causing any adverse effects on its host [3-5]. Endophytes are endosymbiotic microorganisms that act as reservoirs of novel bioactive secondary metabolites, such as alkaloids,

phenolic acids, quinones, steroids, saponins, tannins, and terpenoids that are of interest for specific medicinal applications [6, 7]. The bioactivity of the secondary metabolites of endophytic fungi includes antimicrobial, anti- inflammatory, anti-proliferative or cytotoxic activity towards human cancer cell lines, and activity against plant pathogens [8-10].

The researchers are currently paying more attention to the drug development from the endophytic fungi [11-15]. The search for new antimicrobial compounds is important as bacterial and fungal infection remains the main cause for morbidity and mortality worldwide due to microbial resistance against the present commercially antimicrobial drugs [16]. Moreover, due to risk of adverse effects encountered with the use of synthetic antibiotics, endophytic fungi may offer an alternative source for antimicrobial agent with significant activity against pathogenic and infective microorganisms [17].

Inflammation is a normal protective response to tissue injury which damaged by microbial agents, physical trauma or noxious chemical. Inflammation is associated with pain, increase of vascular permeability, membrane alteration and protein denaturation due to release of lysosomal enzymes, kinins, prostroglandins and histamine [18]. The lysosomal enzymes released during inflammation produced a variety of disorders, so stabilization of lysosomal membrane is important in limiting the inflammatory response [19]. The search for anti-inflammatory properties has been on the rise due to their potential use in the therapy of various chronic and infectious diseases [20]. The prevailing non-steroidal anti- inflammatory drugs (NSAIDs) in the treatment of diseases associated with inflammatory reactions has adverse effects which pose a major problem in the clinical use. The greatest disadvantage in the presently available potent synthetic anti-inflammatory drugs lies in their toxicity [21]. Moreover, long-term use of NSAIDs can cause peptic ulcer [22]. Therefore, it is necessary to develop a novel anti-inflammatory agent that could overcome the disadvantages of NSAIDs. Furthermore, identification of such agent from natural origin could confer safety and efficacy for the treatment of inflammation [23].

For these reasons, in this study, the anti-bacterial, anti-dermatophytic and anti-keratinophilic fungi, anti-yeasts and anti-inflammatory activities of the extracts of 26 selected endophytic fungal strains were evaluated.

2. MATERIALS AND METHODS

2.1. Endophytic fungi

Total of 26 endophytic fungal strains were kindly provided by the Assiut University Myco-logical Centre (AUMC), Assiut University, Assiut Egypt. The fungal cultures were sub-grown on fresh slants have potato dextrose agar medium and incubated at 28 ± 2^0C for 10 days before used.

2.2. Preparation of fungal extract

Fungal strains grown on 50 ml of potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks. Cultures were incubated for 10 days at 28±2ºC. The mycelia and the fermentation broth of each fungal strain were blended with 150 ml ethanol in electric blender; the extracts were filtered using filter paper to remove the mycelia. Mislabel extracts were, individually, transferred into rotatory evaporator under reduced pressure at 35ºC till semisolid residue was obtained.

2.3. Determination of antimicrobial activities

2.3.1. Bacterial strains

Three strains of Gram (+) bacteria namely: *Bacillus subtilis* AUMC B-101, *Bacillus cereus* AUMC B-70 and *Staphylococcus aureus* AUMC 6538, in addition to three strains of Gram (-) bacteria namely *Escherichia coli* NCCB 50028, *Serratia marcescens* AUMC B-89 and *Klebsiella* sp. AUMC B-77 were obtained from the cultures collection of the AUMC. Bacterial cultures were cultivated on nutrient agar (NA) slants and incubated at 37^0C for 24 h before using.

2.3.2. Dermatophytic and keratinophilic strains

Two strains of dermatophytic fungi named *Tricophyton mentagrophytes* AUMC 2360 and *Tricophyton rubrum* AUMC 10337 in addition to one strain of keratinophilic fungus named

Chrysosporium tropicum AUMC 1804 were used to determine the anti-dermatophytic and keratinophilic activities of the fungal extracts. Also, these strains were kindly provided by the AUMC and cultivated on sabouraud dextrose agar (SDA) medium and incubated at 28 ± 2^0C for 7 days before using.

2.3.3. Yeast strains

Two strains of pathogenic *Candida* species (*Candida albicans* AUMC 9212 and *Candida parapsilosis* AUMC 9163) in addition to one strain of *Saccharomyces cerevisiae* AUMC 203 were obtained from AUMC. Yeast cultures were grown on SDA medium and incubated at 30 ± 2^0C for 48 h before used.

2.3.4. Agar well diffusion assay

The antimicrobial assay was performed by agar well diffusion method [24]. A spore suspension of each of the different tested bacterial and fungal strains was prepared. Petri dishes have NA for bacteria and sabouraud dextrose agar for fungi was prepared. One ml of spore suspension of each of the different bacterial or fungal strain was transferred to suitable number of dishes containing the appropriate medium. A sterile swab was used to distribute bacterial or fungal suspension on the solidified agar plates. The plates were allowed to dry for 15 minutes. Wells were then prepared in the plates with the help of a cork-borer (1 cm). A total of 100 µl of the test endophytic fungal extract were introduced into the well. The plates were incubated overnight at 37^0C for bacterial strains at 30^0C for 48 h for yeast strains and at 28^0C for 5 days for both dermatophytic and keratinophilic strains. Microbial growth was determined by measuring the diameter of inhibition zone. Ethanol as a negative control and chloramphenicol and clotrimazole as positive control for antibacterial and antifungal, respectively were used. All results were recorded as mean values of three replicates ± standard deviation.

2.3.5. Determination of anti-inflammatory activities

2.3.5.1. Protein denaturation assay

The anti-inflammatory activity of the endophytic fungal extracts was tested by the protein denaturation method as described by Padmanabhan and Jangle [25] with some modification. Briefly, the reaction included 10mµl of the fungal extract and 3 ml of phosphate-buffered saline (pH 6.5) which was vortex with 0.5 ml of egg albumin and incubated at 25ºC for 15 min. A denaturation reaction was induced in a 65ºC water bath for 12 min. After cooling, absorbance was measured at 660 nm by spectrophotometer using double distilled water as the blank. The percentage inhibition of protein denaturation was appraised by the following formula:

Inhibition% = (Ac - As/Ac) × 100

where Ac and As represent control and sample absorbance, respectively. In this assay, diclofenac sodium a powerful NSAID was used as a standard. The samples were analyzed in triplicates.

2.3.5.2. Albumin denaturation assay

According to method of Williams et al. [26] and Shah et al. [27], with minor modifications, a solution of 0.2% W/V of bovine serum albumin (BSA) was dissolved in Tris buffer saline and pH was adjusted using to glacial acetic acid to 6.8. A total of 2.5 ml of the 0.2% W/V BSA was transferred to tube containing 50 µl of fungal extract in test tube and 50 µl of standard (diclofenac sodium) in standard tubes. The solution was heated at 72°C for 5 minutes and then cooled at room temperature for 15 minutes. The control was taken without the extracts. The absorbance of solution was read at 660nm in spectrophotometer against blank and the percentage of inhibition was calculated using the following formula

Inhibition% = (Ac - As)/Ac×100

where: Ac and As represent control and sample absorbance, respectively. The samples were analyzed in triplicates.

2.3.5.3. Membrane stabilization assay

Preparation of human red blood cells (HRBCs) suspension

The blood was collected from healthy human who had not taken any NSAIDs for 2 weeks prior to the experiment. The fresh whole human blood (10 ml) was centrifuged at 3000 rpm for 10 min and washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline [28].

Human red blood corpuscles (HRBCs) membrane stabilization method

Observations and conclusion for anti-inflammatory activity of fungal extracts were made on the basis of stabilization of HRBCs membrane by hypotonicity induced membrane lysis as recorded by Shing and Kumar [29] and Dhamodaran and Rajeswari [30] with minor modification. Assay mixture was prepared by mixing 50µl of fungal extracts with 1.5 ml phosphate buffer (pH 7.4, 0.15 M), 1.5 ml hyposaline (0.36%) and 50 µl HRBCs suspension (10% v/v). The mixture was incubated in water bath at 56ºC for 30 min then cooled under running tap water. The samples were centrifuged at 2500 rpm for 5 min. Diclofenac sodium was used as positive control. The absorbance was measured at 560 nm in spectrophotometer. Hemolysis produced in the presence of distilled water was taken as 100%. Percentage of HRBCs membrane stabilization/protection was calculated using the following formula:

Stabilization % = (Ac - As)/Ac×100

where: Ac and As represent control and sample absorbance, respectively. The samples were analyzed in triplicates.

3. RESULTS AND DISSCUSSION

3.1. Antimicrobial activities

The crude ethanolic extracts of 26 endophytic fungal strains exhibited a wide variety of antimicrobial activities against 12 tested organisms: 3 Gram (+) bacteria (*Bacillus subtilis, B. cereus* and *Staphylococcus aureus*), 3 Gram (-) bacteria (*Klebsiella* sp., *Escherichia coli* and *Serratia marcescens*), 3 yeasts, (*Candida parapsilosis, C. albicans* and *Saccharomyces cerevisiae*) and two dermatophyte (*Trichophyton mentagrophytes* and *T. rubrum*) and one keratinophilic fungus (*Chrysosporium tropicum*) by agar well diffusion method. The results of antibacterial activity assay showed that, extracts of 22 endophytic fungi (84.6%) produced bioactive compounds that exhibited antibacterial activity against at least one test bacterium with inhibition zones ranging from 5 to 45 mm. Four (15.4%) fungal extracts (*Aspergillus versicolor, A. awamori, A. niger* and *Penicillium funiculosum*) displayed a broader antibacterial spectrum and inhibited the growth of all positive and negative bacterial species (Table 1). This result is better than those recorded by other several studies. In a preliminary study recorded by Wang et al. [31] on screening of some endophytic fungi for production of antimicrobial activities found that more than 50% of isolates displayed antimicrobial activity against at least one tested microorganisms. Gong and Guo [32] reported that 56% of endophytic fungi inhibited growth of at least one of the test organisms and 8% showed broad spectrum inhibition. Crude extracts of 75% of tested endophytic fungi tested by Kharwar et al. [33] showed antibacterial potential against one or more clinical human pathogen. Siqueira et al. [34] reported that only 16 out of 203 (7.9%) endophytic isolates showed antimicrobial activities with a wider action spectrum inhibiting Gram (+) and (-) bacteria and fungi. Tong et al. [35] found that 66 of 72 (92%) endophytic fungal isolates exhibited a significant inhibitory activity at least against one test microorganism with diameters of inhibition zones ranging from 9 to 26 mm for the test bacteria.

The Gram (+) bacteria tested in the present study appeared to be more susceptible to the inhibitory effect of the crude extracts than Gram (-) bacteria. This result is in agreement with previous study by Chareprasert et al. [36] who found that most of the bioactive metabolite compounds from endophytic fungi were more effective against Gram (+) than Gram (-) bacteria and pathogenic fungi. The result in Table 1 showed that the negative control did not show any inhibition while antibiotic control showed mean zones of inhibition ranging from 5 to 45 mm in the agar well diffusion assay. Crude extract of *Aspergillus terreus* exhibited

inhibition zone of 10 mm against *Staphylococcus aureus* and 9 mm against each of *Escherichia coli* and *Klebsiella* pneumonia of 9 tested crude extracts of endophytic fungal species against the bacteria and fungi by well diffusion method. Moreover, endophytic fungi isolated from *Salvadora oleoides Decne* showed potent antimicrobial activity against *Salmonella typhi, Escherichia coli, Klebsiella pneumoniae* and *Aspergillus niger* [38]. Conclusively, Bugni and Ireland [39] found that *Aspergillus genera* are a major contributor of antimicrobial compound of fungal origin. Ogidi et al. [15] returned the antimicrobial activity of *Lenzites quercina* to the presence of fatty acids and other phytochemicals.

Table 1. Antibacterial activities of the ethanolic extracts of 26 endophytic fungi against three different strains of each of Gram (+) and Gram (-) bacteria

	Fungal strains	Gram negative bacteria			Gram positive bacteria		
		***Klebsiella* sp. AUMC B-77**	***E. coli* NCCB 50028**	***S. marcescens* AUMC B-89**	***B. subtilis* AUMC B-101**	***B. cereus* AUMC B-70**	***S. aureus* AUMC 6538**
Alternaria	*A. alternata* AUMC 6836	-ve	-ve	-ve	-ve	-ve	-ve
	A. alternata AUMC 8840	-ve	-ve	-ve	-ve	-ve	-ve
	A. alternata AUMC8841	-ve	-ve	-ve	-ve	-ve	-ve
Aspergillus	*A. awamori* AUMC 8855	15 ± 0.09	20 ± 0.07	30 ± 0.1	25 ± 0.3	25 ± 0.1	20 ± 0.2
	A. fumigatus AUMC8872	-ve	-ve	-ve	17 ± 0.02	29 ± 0.1	40 ± 0.2
	A. niger AUMC8852	20 ± 0.03	-ve	-ve	30 ± 0.4	30 ± 0.2	30 ± 0.007
	A. niger AUMC8856	20 ± 0.003	25 ± 0.1	35 ± 0.3	35 ± 0.02	43 ± 0.08	20 ± 0.1
	A. oryzae AUMC8863	30 ± 0.4	20 ± 0.02	-ve	-ve	25 ± 0.04	-ve
	A. versicolor AUMC6872	33 ± 0.03	40 ± 0.1	30 ± 0.04	4± 0.001	37 ± 0.2	20 ± 0.01
	Circinella muscae AUMC8861	-ve	-ve	30 ± 0.01	-ve	-ve	-ve
	Chaetomium globosum AUMC8862	-ve	-ve	40 ± 0.3	25 ± 0.05	15 ± 0.4	-ve
Fusarium	*F. lateritium* AUMC6833	-ve	-ve	5 ± 0.09	15 ± 0.01	20 ± 0.1	-ve
	F. oxysporum AUMC6827	-ve	-ve	-ve	5 ± 0.02	7.5 ± 0.05	-ve
	F. semitectum AUMC6816	-ve	-ve	-ve	10 ± 0.3	10 ± 0.01	-ve
	F. scirpi AUMC8858	-ve	-ve	-ve	15 ± 0.1	-ve	-ve
	F. subglutinans AUMC 8839	-ve	-ve	20 ± 0.08	-ve	-ve	-ve
	Gliocladium solani AUMC 6802	- ve	-ve	- ve	-ve	5 ± 0.2	-ve
Emericella	*E. nidulans* AUMC 8854	28 ± 0.2	-ve	15 ± 0.04	-ve	-ve	30 ± 0.02
	E. rugulosa AUMC8867	-ve	-ve	20 ± 0.05	-ve	-ve	-ve
	Exophiala costellanii AUMC8865	-ve	-ve	-ve	15 ± 0.1	-ve	-ve
	Papulaspora irregularis AUMC8843	-ve	-ve	-ve	-ve	-ve	-ve
Penicillium	*P. aurantiogriseum* AUMC8847	-ve	30 ± 0.07	40 ± 0.2	15 ± 0.05	20 ± 0.1	15 ± 0.3
	P. funiculosum AUMC8850	28 ± 0.1	15 ± 0.01	14 ± 0.2	16 ± 0.08	40 ± 0.001	40 ± 0.05
	P. raistrickii AUMC7265	-ve	15 ± 0.04	15 ± 0.1	15 ± 0.06	18 ± 0.01	-ve
	Penicillium sp. AUMC8859	18 ± 0.1	-ve	20 ± 0.006	18 ± 0.01	14 ± 0.06	-ve
	Pleospora tarda AUMC 8871	-ve	-ve	-ve	-ve	20 ± 0.03	-ve
Standard (chloromphanicol)		5 ± 0.001	45 ± 0.06	40 ± 0.01	32 ± 0.1	30 ± 0.05	25 ± 0.08

All results were recorded as mean values of three replicates ± standard deviation.

Table 2. Antifungal activities of the ethanolic extracts of 26 endophytic fungi against some strains of yeasts, dermatophytic and keratinophilic fungi

Fungal strains		Yeasts			Dermatophytic fungi		Keratinophilic fungi
		***C. parapsilosis* AUMC 9163**	***C. albicans* AUMC 9212**	***S. cerevisiae* AUMC 203**	***T. mentagrophytes* AUMC 2360**	***T. rubrum* AUMC 10337**	***C. tropicum* AUMC 1804**
Alternaria	*A. alternata* AUMC 6836	-ve	-ve	-ve	-ve	-ve	-ve
	A. alternata AUMC 8840	-ve	-ve	-ve	15 ± 0.1	-ve	-ve
	A. alternata AUMC 8841	-ve	-ve	-ve	-ve	-ve	-ve
Aspergillus	*A. awamori* AUMC 8855	-ve	-ve	-ve	30 ± 0.001	20 ± 0.3	20 ± 0.1
	A. fumigatus AUMC 8872	-ve	-ve	-ve	20 ± 0.005	35 ± 0.3	5 ± 0.03
	A. niger AUMC 8852	-ve	-ve	-ve	35 ± 0.02	30 ± 0.1	40 ± 0.09
	A. niger AUMC 8856	-ve	-ve	-ve	20 ± 0.1	15 ± 0.5	20 ± 0.01
	A. oryzae AUMC 8863	-ve	-ve	-ve	45 ± 0.07	35 ± 0.01	20 ± 0.3
	A. versicolor AUMC 6872	-ve	-ve	-ve	40 ± 0.03	-ve	-ve
	Circinella muscae AUMC 8861	-ve	-ve	-ve	-ve	-ve	-ve
	Chaetomium globosum AUMC 8862	-ve	-ve	-ve	-ve	-ve	-ve
Fusarium	*F. lateritium* AUMC6833	-ve	-ve	-ve	-ve	-ve	-ve
	*F. oxysporum*AUMC6827	-ve	-ve	-ve	40 ± 0.03	-ve	-ve
	F. semitectum AUMC6816	-ve	-ve	-ve	-ve	-ve	-ve
	F. scripi AUMC 8858	-ve	-ve	-ve	5 ± 0.03	-ve	-ve
	F. subglutinans AUMC8839	-ve	-ve	-ve	-ve	-ve	-ve
	Gliocladium solani AUMC 6802	-ve	-ve	-ve	-ve	-ve	-ve
Emericella	*E. nidulans* AUMC 8854	-ve	-ve	-ve	30 ± 0.2	25 ± 0.06	2.5 ± 0. 3
	E. rugulosa AUMC 8867	-ve	-ve	-ve	-ve	15 ± 0.1	-ve
	Exophiala costellanii AUMC 8865	-ve	-ve	-ve	20 ± 0.08	10 ± 0.04	-ve
	Papulaspora irregularis AUMC 8843	-ve	-ve	-ve	-ve	-ve	-ve
Penicillium	*P. aurantiogriseum* AUMC 8847	-ve	-ve	-ve	35 ± 0.05	-ve	5 ± 0.2
	P. funiculosum A UMC 8850	-ve	-ve	-ve	45 ± 0.1	25 ± 0.4	30 ± 0.02
	P. restickii AUMC 7265	-ve	-ve	-ve	-ve	20 ± 0.2	-ve
	Penicillium sp. AUMC 8859	-ve	-ve	-ve	-ve	10 ± 0.03	-ve
	Pleospora tarda AUMC8871	-ve	-ve	-ve	-ve	-ve	-ve
Standard (clotrimazole)		35 ± 0. 30	25 ± 0.10	10 ± 0.03	30 ± 0.07	25 ± 0.20	15 ± 0.01

All results were recorded as mean values of three replicates ± standard deviation

The extracts of 8 endophytic fungi (30.8% of tested strains) were affected on the two tested dermatophyte strains (*Trichophyton mentagrophytes* and *T. rubrum*) and gave inhibition zones ranged from 10-45 mm. Other 5 extracts were inhibited *Trichophyton mentagrophytes* only with inhibition zones ranged from 5-40 mm and other 3 fungal extracts were inhibited the growth of *Trichophyton*

rubrum with zones between 10-20 mm. Only 8 fungal extracts have an inhibition activity against the keratinophilic fungus (*Chrysosporium tropicum*). On the other hand, the extracts of all tested endophyte have no activities against any of the three tested yeast strains (Table 2). Seven endophytic extracts show inhibition zones against all the three tested dermatophytic and keratinophilic strains. *Penicillium funiculosum* and *Aspergillus oryzae* show the highest inhibition zone (45 mm) against *Tricophyton mentagrophytes*, *Aspergillus oryzae* and *Aspergillus fumigatus* appeared their highest activities against *Tricophyton rubrum* with inhibition zone reached to 35 mm. *Aspergillus niger* formed the highest inhibition zone against *Chrysosporium tropicum* which reached to 40 mm (Table 2).

Recently, studies were done on the effect of endophytic extracts against both dermatophytes and yeasts. Tong et al. [35] examined methanolic and ethyl acetate extracts of 72 endophytic fungal isolates against yeast and fungi and recorded that only moderate antiyeast and antifungal activities were observed for both with diameter of inhibition zone less than 16 mm on disc diffusion assay. Seddek [40] found that about 50% of the crude ethanol and aqueous extracts of 57 endophytic fungal isolates on the growth of 6 human pathogenic fungi representing 3 species of *Candida* (*C. albicans*, *C. glabrata* and *C. krusei*) and 3 dermatophytic fungi (*Trichophyton rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum*) had no inhibition activities against all the 3 dermatophytic fungi while 72% of the extract affected the tested 3 species of *Candida*. Pharamat et al. [41] examined the antimicrobial activity of 73 endophytic fungi against *Saccharomyces cerevisiae* and *Candida albicans* and found that 11 (15.1%) and 7 (9.6%) of isolates produced inhibition zones ranged from 9 to 30 mm against the 2 tested yeast species, respectively. Kalyanasundaram et al. [37] reported that the crude extract of *Aspergillus terreus* inhibited *Trichophyton rubrum*, *Candida albicans* and *Trichophyton mentagrophytes* growth with inhibition zones reached to 8, 4 and 3 mm, respectively. Also, the inhibitory effect of *Clitocybe nebularis* on *Trichophyton mentagrophytes* with inhibition zones of 9-11 mm [42]. Medicinal product from plants and mushrooms could be continually sourced and adequately utilized to treat dermatophyte infections [43, 44]. This antifungal activity may be attributed to the presence of glucanase [45] or ganoduric protein [46].

3.2. Anti-inflammatory activity

In the present study, only 8 strains were selected for examined their anti-inflammatory activities (Table 3). The extracts of 3 of them (*Aspergillus niger*, *Aspergillus awamori* and *Penicillium funiculosum*) were recorded as effective on all Gram (+), Gram (-) bacteria and dermatophytic and keratinophilic fungi in the previous expriment. Other 3 extracts (*Aspergillus fumigatus*, *Aspergillus oryzae* and *Emericella nidulans*) appeared an inhibition effect on all dermatophytic and keratinophilic fungi and some of bacterial strains under study. The 7 selected extract (*Aspergillus versicolor*) was recorded as inhibition for all bacterial strains and only one of dermatophyte. The last selected extract (*Pleospora tarda*) had no inhibitory activity against all tested bacterial and fungal strains with exception of Gram (+) *Bacillus cereus.*

As part of the investigation on the mechanism of the anti-inflammation activity, ability of the selected 8 fungal extracts to denaturated protein was studied (Table 3). All the fungal extracts and the standard were tested at 10 µl/ml concentration. *Emericella nidulans*, *Pleospora tarda*, and *Penicillium funiculosum* extracts showed higher activities with inhibition % of protein denaturation reached to 83%, 82.5% and 81.4%, respectively. On the other hand, the extracts of all the 5 *Aspergilli* under study showed lower activities and inhibited protein denaturation by 65-79.9% (Table 3).
Standard diclofenac sodium recorded 77.4% inhibition of protein denaturation. Denaturation of proteins is well documented cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs like salicylic acid have shown dose dependent ability to inhibit thermally induced protein denaturation [47]. The inhibitory effect on bovine serum albumin denaturation by the ethanol extracts of the tested endophytes is shown in Table 3. Maximum inhibition was 95% was observed by ethanol *Emericella nidulans* extract at 50 µl/ml followed by *Pleospora tarda* (90.7% at the same concentration).

Table 3. Anti-inflammatory activity of ethanolic extracts of selected endophytic strains

Fungal strains	Protein denaturation	Albumin denaturation	HRBCs membrane stibilization
	% inhibition	% inhibition	% inhibition
Aspergillus awamorii AUMC 8855	77.9 %	53.9 %	62.8 %
Aspergillus fumigatus AUMC 8872	75 %	62.7 %	52.8 %
Aspergillus niger AUMC 8856	71.7 %	82.4 %	59.3 %
Aspergillus oryzae AUMC 8863	79.9 %	86 %	66.8 %
Aspergillus versicolor AUMC 6872	65 %	69 %	63.4 %
Emericella nidulans AUMC 8854	83 %	95 %	69.5 %
Penicillium funiculosum AUMC 8850	81.4 %	57.7 %	52.7 %
Pleospora tarda AUMC 8871	82.5 %	90.7 %	56.5 %
Standard of diclofenac sodium	77.4 %	87.4 %	74.3 %

All results were recorded as mean values of three replicates ± standard deviation

The standard anti-inflammatory drug, diclofenac sodium showed 87.4% inhibition. The results recorded in this study are better than those recorded by Govindappa et al. [48] who found that methanol extract at 200 µg/ml concentration of *Aspergillus niger* showed 79% inhibition followed by *Aspergillus alternata* (78.6%) and *Penicillium* sp. 65.84%.

Since HRBCs membrane is similar to these lysosomal membrane components [49] and its stabilization implies that the extract may as well stabilize lysosomal membranes. So, the prevention of hypotonicity induced RBCs membrane lysis was taken as a measure in estimating the anti-inflammatory property of the secondary metabolites of fungi. Thus, HRBCs membrane stabilization has been used as a method in estimating the anti-inflammatory property [50, 51]. Stabilization of HRBCs membrane was studied for establishes the mechanism of anti-inflammatory action of ethanolic extracts of different 8 endophytes. All the tested extracts were effectively inhibiting the heat induced hemolysis. These results provide evidence for membrane stabilization as a mechanism of their anti-inflammatory effect. This effect may possibly inhibit the release of lysosomal content of neutrophil at the site of inflammation. The extracts inhibited the heat induced hemolysis of HRBCs to varying degree (Table 3). The maximum inhibition was 69.5% by ethanolic extract of *Emericella nidulans*. The diclofenac standard drug recorded 74.3% of inhibition. This result came in harmony with those recorded by Govindappa et al. [48] who showed that the maximum inhibition by methanol extract of *Aspergillus niger* was 78.42% followed by *Penicillium* sp. (77.61%) and *Aspergillus alternata* (77.98%). Also they found that the aspirin standard drug showed 85.92%. Results of our findings confirmed the use of some endophytic fungi such as *Emericella nidulans*, *Pleospora tarda*, *Aspergillus versicolor*, *Penicillium aurantiogriseum*, *Penicillium funiculosum*, *Aspergillus awamori*, *Aspergillus niger*, *Aspergillus oryzae* and *Aspergillus fumigatus* as sources of anti-microbial and/or anti-inflammatory drugs. This biological activity could be returned to the presence of phytochemicals like alkaloids, phenols, flavonoids, saponins, and terpenes in the endophytes [14]. The levels of phenolic and flavonoid compounds were correlated with the anti-inflammatory activity of the extracts [52]. The correlation between presence of flavonoids and their membrane stabilizing ability was approved by Sankari et al. [53]. Moreover, the main action of the anti-inflammatory agent is the inhibition of the cyclooxygenase system which is responsible for the biosynthesis of prostaglandins. NSAIDS like prostaglandins acts by inhibiting the lysosomal enzymes or by stabilizing the lysosomal membrane. Since RBCs membranes are similar to the lysosomal membrane components, the prevention of hypotonicity-induced HRBCs lysis was taken as measure of anti-inflammatory activity of drugs. The indomethacin drugs as inhibitor of prostaglandins biosynthesis act either by inhibiting these lysosomal enzymes or by stabilizing the

lysosomal membrane [54, 55]. Thus secondary metabolites of endophytic fungi have a promising potential to be included in antimicrobial and anti-inflammatory drug discovery program.

AUTHORS' CONTRIBUTION

All authors contributed in design, execution the research plan point to point, and writing of the manuscript. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest.

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