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Microbial deterioration of a 13 AH-century manuscript housed in Al-Azhar library in Egypt: A case study

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Abstract

Four saprophytic bacterial and fungal species attacked the manuscript that dated back to 1251 AH at Al-Azhar library in Cairo, Egypt were isolated. Based on the partial 16S rRNA gene sequence, the isolated bacteria were *Acinetobacter indicus* and *Exiguobacterium aurantiacum*. The fungal species were *Stachybotrys chartarum* and *Aspergillus flavus*. *Acacia nilotica* fruit extraction exhibited considerable antimicrobial activity at different concentrations against the isolated bacteria and fungi.

Key words: Saprophytic, manuscript, *Acacia nilotica*, antimicrobial.

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1. Introduction

Libraries keep all sources of human knowledge and cultural heritage [1], [15], [40]. Bio deterioration of ancient materials stored in libraries is a great problem over a world [8], [33], [64]. Industrial materials of paper including wood, cellulose, proteins and chemical additives represent a nutrition sources for saprophytic bacteria and fungi [7], [21], [28], [31], [38], [39], [52], [74]. In addition to ecological factors, bacteria and fungi cause degradation and discoloration of books and manuscripts stored in libraries [34], [52], [76]. Various proteolytic bacteria e.g. *Bacillus sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, *Virgibacillus sp.* and *Micromonospora sp.* were isolated from manuscripts and books [36]. Discoloration and purple spots of paper related to infection by alkaliphilic bacteria and various species of *Actinobacteria* [56], [58], [69]. Fungi represent important causative agents of deterioration of library materials [12], [37], [46], [53], [57], [62], [65], [67]. Chemical disinfectants, ultra violet and gamma radiation used for controlling microbial contamination in libraries [19], [27], [52]. The disadvantages of these methods are paper ageing, discoloration and cancer [10], [27], [30], [53], [59], [60]. Therefore, the present study was designed to identify bacteria and fungi invading the historical 13 AH-century manuscripts and searching for simple, cheap, non-toxic and eco-friendly control against causative microorganisms. This study will introduce an effective strategy for the protection of books and manuscripts in libraries from microbial attack.

2. Material and methods

2.1. History of the manuscript

The manuscript "Resala fe alfaraeid" dated back to 1251 AH at Al-Azhar library in Cairo, Egypt. Its Public number 85193 and special number 924. Its length is 16 cm^2 and width 11 cm^2 (Fig. 1).

Fig.1. the manuscript under investigation

2.2. Collection of samples

Sterile cotton swabs were wiped on the damaged area. Small fragments of paper (2-3 mm^2 width) were also collected from the margins of pages during restoration process to minimize invasive action on the manuscript [57].

2.3. Microbial analysis

The present work was performed in aseptic area under laminar-flow cabinet. Cotton swabs were inoculated directly on nutrient agar, Sabouraud-glucose agar and Czapek's agar plates. Multi-point inoculum method was also used to reduce the air-borne contaminants [24], In this method small fragments of paper were washed in sterile water and divided into 25 sub-samples that were inoculated directly on nutritive agar plates. Plates were incubated at 37 º C for 5 days for bacteria and at 28 º C for 7 days for fungi. The distinct colonies were picked up and purified using streaking method. Isolates were preserved at 4 ºC for identification and further studies.

2.4. Identification of isolates

The bacterial isolates were identified based on Gram's staining, morphological characteristics as well as the partial 16S rRNA gene sequence. The partial 16S rRNA was amplified using the universal primers 785 F and 907 R [73]. The reaction mixture of PCR contained 0.2 µM of each primer, 0.2 mM of dNTPs, 1X PCR buffer and 0.1 U of Taq polymerase. DNA was denatured for 5 min at 94 ºC, primer annealing was at 55 o C for 1 min and strand extension was at 72 o C for 2 min. PCR products were separated using 1.5 % agarose gel. After that, the PCR products were sent to Macrogen, Inc., Korea (http://www.macrogen.com/eng/) for sequencing. The obtained 16S rRNA sequences were compared and aligned with the sequences deposited in the NCBI GenBank database using the BLAST tool (Blastn) that opened from the URL (www.ncbi.nlm.nih.gov).

Fungal isolates were identified microscopically on the basis of their morphologically characteristics according to the keys of Barnett and Hunter [11] and Moubasher [48].

2.5. Microbial cellulolytic activity

Cellulase activity of the bacterial isolates was detected according to according to Dingle et al. [20]. Colonies were streaked on carboxymethyl cellulose (CMC) agar plates composed of carboxymethyl cellulose, 10 g; $NaNO_3$, 3 g; K_2HPO_4, 5 g; $MgSO_4.7H_2O$, 5 g, Agar, 15 g and distilled water 1000 ml. pH was adjusted to 7.2. After incubation for 72 h, plates were flooded with Schulze's solution (Chlor-zinc-iodide) which consists of 1 % iodide and 3 % zinc chloride. Clear zones appeared around bacterial growth indicating CMC hydrolysis.

Cellulases production by fungal isolates was also determined following the method of Teather and Wood [71]. In the center of the CMC assay plates, 7 mm discs of 6 days old cultures of the tested fungi were inoculated. Plates were incubated at 25 °C for 7 days. After incubation, plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature, then plates were counterstained using 1 M Na Cl. Clear zones were appeared around fungal growth indicating cellulolytic activity.

2.6. Antimicrobial activities of the crude extract of *Acacia nilotica* fruits

2.6.1. Plant material and extraction procedure

Acacia nilotica also known as gum Arabic, Egyptian thorn in Egypt, scanted tree in English, thorn in mimosa in Australia and commonly known as "*Bagaruwa*" among the Hausas, is a species of vachellia and a genus of Acacia. It is a thorny Acacia found in different parts of the world [66]. Fruits of *Acacia nilotica* were collected from Aswan city, Egypt. Fruits were identified at the Department of Botany, Faculty of Science, Aswan University, Egypt. The fruits were washed under running tap water and air dried for 24 h. Methanolic extract was prepared from the fruits according to Alanis et al. [3]. Fruits were grounded into a fine powder by an electric blender. 50 grams of the powder was extracted with 150 ml of 99 % methanol in a conical flask, flask was shaken intermittently for 72 h at room temperature. It was then filtered through Whatman filter paper No. 1 and the solvent was evaporated at room temperature. Then methanol extract was stored at 4 °C in airtight bottles until further use.

2.6.2. Preparation of test samples

Samples used for antimicrobial assay were prepared according to NCCLS [5]. For antibacterial assay 2 g of the solid extract was dissolved in 2 ml of 99 % methanol to obtain a stock solution of 1000 mg/ml. Concentrations of 500, 250, 125, and 62.5 mg/ml were prepared using serial doubling dilution. For antifungal assay, the stock solution and diluting concentrations were prepared by the same method, but Dimethyl Sulphoxide (DMSO) was used instead of methanol.

2.6.3. Preparation of standard inoculums

The bacterial inoculum was prepared according to Mc Farland [42] and NCCLS [49]. The turbidity of 24 h old broth cultures of the bacterial isolates were adjusted to 10^8 CFU/ml. using 0.5 Mc Farland standards.

The fungal inoculum was prepared following the method described by NCCLS [50]. Suspensions of 3 day old cultures were adjusted with UV-Visible spectrophotometer at 530 nm (OD ≈ 0.09- 0.11) to obtain standardized preparations containing approximately 10^6 spores/ml.

2.6.4. Antibacterial activity test

Antibacterial activity of methanolic extract of *Acacia nilotica* fruits was detected using the disc diffusion method [9], [17]. Discs of Whatman filter paper No. 1 (6 mm) were prepared and sterilized by autoclaving. 10 µl of each extract concentration was loaded on discs to obtain the final concentrations 5, 2.5, 1.25 and 0.6 mg/disc respectively. 100 µl of the standardized bacterial suspension (containing 10^8 CFU/ml) was spread uniformly on the surface of Mueller Hinton agar (Oxoid) using sterile cotton swabs. The discs were dispensed onto the surface of the inoculated agar plates. The positive control was chloramphenicol (30µg/disc) and the negative control was discs impregnated with methanol. Plates were incubated at 37 ºC for 24 h. Duplicates were made. The antibacterial

activity was expressed as the mean diameter of inhibition zones in millimeter.

2.6.5. Antifungal activity test

Agar well diffusion method was used to detect the antifungal activity of the extract of *Acacia nilotica* fruits [17]. Plates containing 20 ml of Czapek's agar were uniformly inoculated with the test fungus by using sterile cotton swab. Wells of 7 mm diameter were made in the medium using sterile cork borer. 50 µl of each extract concentration was dropped into each labeled well. 50 µl of 1mg/ml fluconazole was used as positive control and 50 µl of DMSO was used as negative control. Plates were kept in the refrigerator for 1 h to allow the extracts to diffuse into the agar. Plates were then incubated at 28 ºC for 72 h. Antifungal activity was determined by measuring the diameter of inhibition zones in millimeter.

3. Results and discussion

3.1. Microbial analysis

Based on colony morphology and Gram's staining, two different isolates were selected and then sub-cultured onto a fresh nutrient agar medium for 24 h at 37 °C by the streaking method.

Blast analysis of the sequences of the present isolates with 16S rRNA gene sequences in the NCBI GenBank database, showed similarity with *Acinetobacter indicus* (96 %) and *Exiguobacterium aurantiacum* (99 %). The partial 16S rRNA gene sequences of isolates were deposited to NCBI Gen Bank under the accession numbers KX998197 and KX998198 respectively. (Fig.2-3)

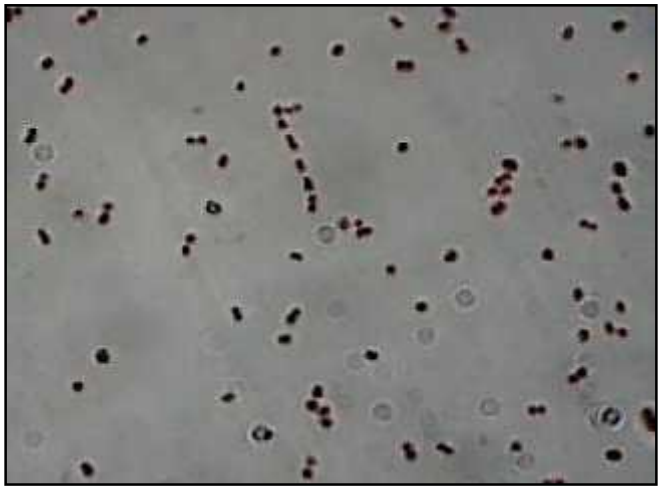

Fig.2. *Acinetobacter indicus* (1000 X)

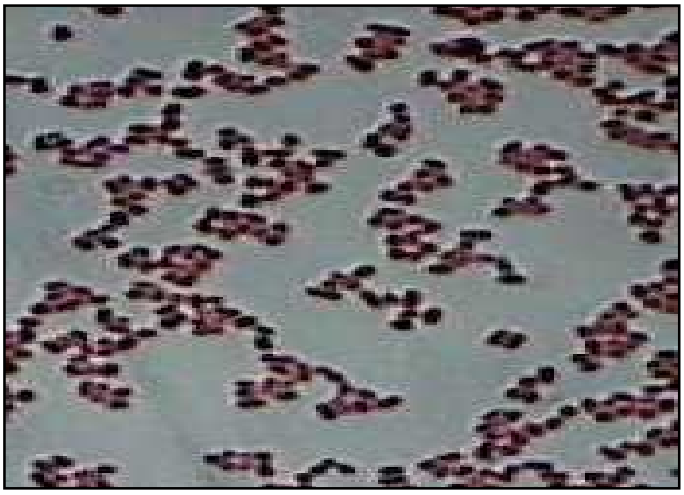

Fig.3. *Exiguobacterium aurantiacum* (1000 X)

Several species of bacteria belong to *Pseudomonas, Cellulomonas, Cellvibrio, Myxobacteria, Actinomycetes, Streptomyces, Nocardia, Massilia timonae, Lysobacter dokdonensis, Bacillus sp., Microbacterium sp., Curtobacterium sp., Virgibacillus* and *Brevibacterium pityocampae* were isolated from paper and documents [4], [25], [29], [32], [36], [55], [72].

Two fungal species *Stachybotrys chartarum* and *Aspergillus flavus* were also isolated. Previously different species belong to *Penicillium, Mucor, Phoma,*

Cladosporium, Aspergillus, Stachybotrys, Alternaria, Botrytis, Chaetomium, Chromelosporium, Epicoccum, Phlebiopsys and *Toxicocladosporium* were isolated from ancient paper and documents by many researches [36], [43], [44], [45], [47], [61], [68], [70] , [76] . (Fig. 4 A & B and Fig. 5 A & B).

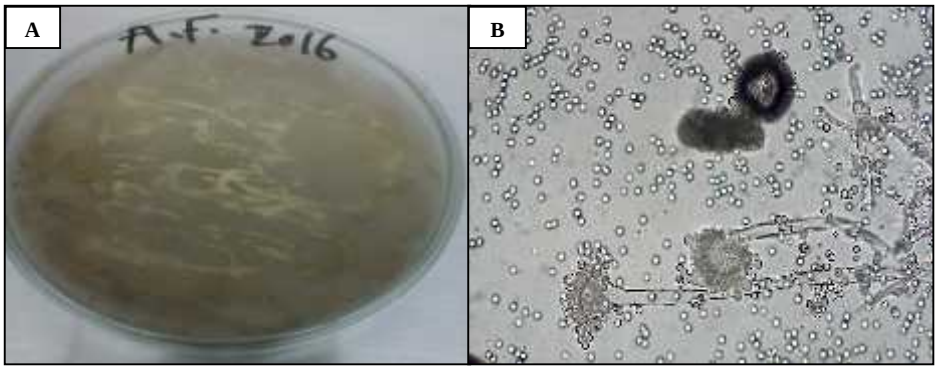

Fig. 4: A: pure culture of *Aspergillus flavus* on Czapek's agar
B: *Aspergillus flavus* under light microscope (400 X)

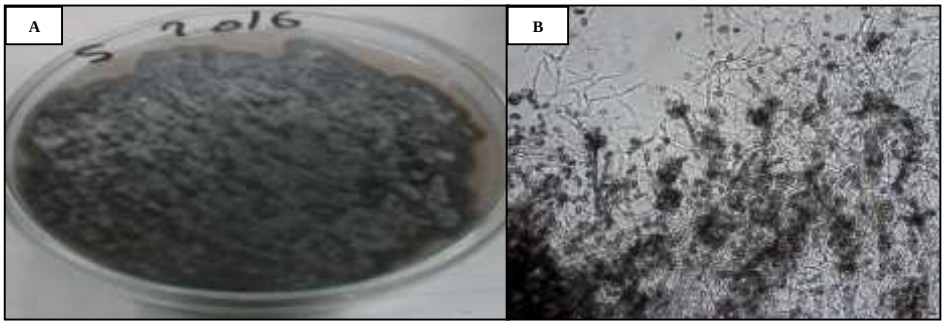

Fig. 5: A: Pure culture of *Stachybotrys chartarum* on Czapek's agar
B: *Stachybotrys chartarum* under light microscope (400 X)

3.2. Microbial cellulolytic activity

Cellulolytic activity of *Acinetobacter indicus* and *Exiguobacterium aurantiacum* was determined by streaking on CMC agar plates. It was found that the two tested bacteria exhibited strongly cellulolytic activities which indicated their effective role in the deterioration process of the manuscript by decomposing cellulose in paper and binding textiles. Cellulases secretion was previously reported for various genera of bacteria which have been isolated from papers (Fig. 6 and 7) [2], [4], [25], [55], [63], [75].

Fig. 6 . Cellulase activity of *Acinetobacter indicus*

Fig.7. Cellulase activity of *Exiguobacterium aurantiacum*

Cellulase activity of the fungal isolates was qualitatively determined using CMC agar plates. Interestingly, both *Stachybotrys chartarum* and *Aspergillus flavus* exhibited strong ability to produce cellulases. Many cellulolytic fungi such as *Alternaria sp*., *Aspergillus sp*., *Fusarium sp*., *Humicola grisea*, *Myrothecìlim verrucaria*, *Penicillium sp*., *Stachybotrys sp*., *Stemphylium sp*., *Trichoderma sp*., *Ulocladium sp*. and *Chaetomium sp*. were isolated from books and documents [22], [34], [35].

3.3. Antimicrobial activities of the crude extract of *Acacia nilotica* fruits

3.3.1. Antibacterial activity test

The methanolic crude extract from *Acacia nilotica* fruits was clearly inhibited the bacterial growth at all the tested concentrations (Table 1) and (Fig. 8 & 9). This correlates with the findings of previous researchers who reported that the extracts from *Acacia nilotica* fruits contain polyphenolic compounds and volatile oils which cause inhibition of a wide range of microorganisms [13], [16], [23].

Table 1

Antibacterial activity of *Acacia nilotica* fruits extract at different concentrations against the growth of *Acinetobacter indicus* and *Exiguobacterium aurantiacum*

Extraction concentration (mg/disc)	Inhibition zone (mm)	
	Acinetobacter indicus	*Exiguobacterium aurantiacum*
5	26	20
2.5	22	16
1.25	19	14
0.6	15	10
chloramphenicol (30µg/disc)	26	25

Fig.8. Effect of *A. nilotica* fruits extract on extract on *Exiguobacterium aurantiacum*

Fig.9. Effect of *A. nilotica* fruits *Acinetobacter indicus*

3.3.2. Antifungal activity test

Results in table (2), Fig. (10 and 11) showed that the *Acacia nilotica* fruits extract exhibited strong antifungal activity at various concentrations ranging from 500 to 62.5 mg/ml. Many researchers reported that *A. nilotica* fruits can be used as antifungal drugs due to its high activity [41]. Chemical analysis of *A. nilotica* fruit extract exhibited that it contains highly effective compounds such as phenolic compounds, tannins, saponins and flavonoids which have a wide spectrum of antimicrobial activity [6], [18], [41].

Table 2

Antifungal activity of *Acacia nilotica* fruits extract at different concentrations against the growth of *Stachybotrys chartarum* and *Aspergillus flavus*

Extraction concentration (mg/ml)	Inhibition zone (mm)	
	Stachybotrys chartarum	*Aspergillus flavus*
500	25	35
250	22	32
125	20	28
62.5	14	20
Fluconazole (1mg/ml)	25	19

Fig.10. Effect of *A. nilotica* fruits extract on *Stachybotrys chartarum*

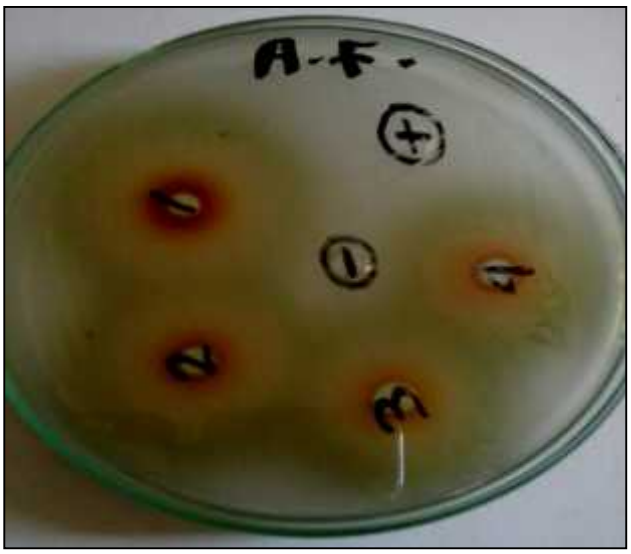

Fig.11. Effect of *A. nilotica* fruits extract on *Aspergillus flavus*

4. Conclusions

The microbial analysis of the infected manuscript "Resala fe alfaraeid" which had kept in Al-Azhar library at Cairo, Egypt was carried out. Two bacterial and two fungal species were isolated which were identified as *Acinetobacter indicus*, *Exiguobacterium aurantiacum*, *Stachybotrys chartarum* and *Aspergillus flavus* respectively. Interestingly, the methanolic crude extract of *Acacia nilotica* fruits was used as non- toxic, cheap and eco- friendly control against the causative microbial isolates.

Conflicts of interest

The authors declare that there is no conflict of interest.

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