

STUDY OF THE EFFECT OF EXTRACT *SALIX MUCRONATA THUNB* LEAVES AS A BACTERIAL ANTAGONIST ON SOME BACTERIA ISOLATED FROM THE ARCHAEOLOGICAL MANUSCRIPTS

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Abstract

This paper focused on studying the bacterial deterioration of the manuscript at Al-Azhar library in Cairo, Egypt. The partial 16S rRNA gene sequence analysis indicated that the two bacterial isolates AN1 and AN2 were *Acinetobacter indicus* and *Exiguobacterium aurantiacum* respectively. The present study investigated the antibacterial activity of methanolic extract of *Salix mucronata* Thunb leaves against the two isolates. It is shown that the extract at different concentrations exhibited considerable effects on the growth of the two isolates. So, we suggested that *Salix mucronata* Thunb leaves extract can be used as a natural growth control to reducing the degradable effect of these bacterial causative agents that attack books and manuscripts kept in libraries.

Keywords: Antibacterial; Deterioration, Library; Manuscript; *Salix mucronata* Thunb

Introduction

Biodeterioration of older library materials represents a greater problem over a world [1, 2]. The ingredients which used in paper industry such as wood, cellulose, proteins and chemical additives are suitable substrates for microbial growth [3-13].

Environmental factors such as heat, light, humidity and dust as well as biological factors such as bacteria, fungi and insects cause damage and discoloration of books and manuscripts stored in libraries [5, 10, 14, 15]. A variety of bacterial species which have the ability to secrete hydrolytic enzymes such as *Bacillus sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, *Virgibacillus sp.*, and *Micromonospora sp.* Play an important role in the damage of manuscripts and books [16]. Furthermore, some alkaliphilic bacteria and various Species of *Actinobacteria* cause damage and purple spots, discoloration of paper [17-19].

Microbial contamination on books and manuscripts were controlled using different methods such as ultraviolet and gamma radiations as well as some chemical disinfectants [5, 20].

Although, these methods have many disadvantages such as paper ageing and discoloration as well as cause cancer [20-25]. Therefore, the present study was designed to identify bacteria invading the historical 13 AH-century (XIX AD Century) manuscript and searching for simple, cheap, non-toxic and eco-friendly natural control against causative isolates. This study will introduce an effective strategy for the protection of books and manuscripts in libraries from bacterial attack.

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Material and methods

Sampling

The deterioration of the manuscript was observed under both natural and UV light. Samples were obtained by rubbing the damaged areas using sterile cotton swabs. Small fragments of infected paper (2-3mm² widths) were also collected from the margins of pages during the restoration process (Fig. 1) To minimize invasive action on the manuscript [26].



Fig. 1. Showing purple-colored spots throughout the pages

Isolation of bacteria from samples

The present work was performed in the aseptic area under laminar-flow cabinet. Cotton swabs were inoculated directly onto agar plates. Paper fragments were washed in sterile water and divided into 25 sub-samples that were inoculated directly onto nutrient agar plates, this method was used to avoid airborne contaminants [27, 28]. Plates were incubated at 37°C for 48h. Based on morphological characteristics; colonies were picked up, sub-cultured on agar plates and purified using streaking method. The purified colonies were preserved at 4°C for identification and further studies.

Molecular identification

The partial 16S rRNA was amplified using the universal primers 785 F and 907 R [29]. The reaction mixture of PCR contained 0.2µM of each primer, 0.2mm of dNTPs, 1X PCR buffer and 0.1U of Taq polymerase. DNA was denatured for 5min at 94°C, primer annealing was at 55°C for 1min and strand extension was at 72°C for 2min. 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.

Analysis of 16S rRNA 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) [30]. The sequences with maximum percent identity were then selected for multiple sequence alignment to construct the phylogenetic trees using CLUSTALW2 tool that opened from the URL (www.ebi.ac.uk) [31].

Cellulolytic activity of bacterial isolates

The Cellulase activity of the bacterial isolates was detected according to [32]. Colonies were streaked on carboxymethyl cellulose (CMC) agar plates composed of carboxymethyl

Cellulose, 10g; NaNO₃, 3g; K₂HPO₄, 5g; MgSO₄·7H₂O, 5g; Agar, 15g and distilled water 1000mL. pH was adjusted to 7.2. After incubation for 48h, 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.

The antibacterial activity of the crude extract of Salix mucronata Thunb leaves

Plant material and extraction procedure

Fresh leaves of *Salix mucronata* Thunb were collected from Aswan city, Egypt. The Plant was identified in the Department of Botany, Faculty of Science, Aswan University, Egypt. The Leaves were washed under running tap water and air dried at room temperature. Methanolic extract was prepared according to [33]. The Leaves were ground into a fine powder by an electric blender. 50 grams of the powder were extracted with 150mL of 99% methanol in a conical flask, flask was shaken intermittently for 72h 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.

Preparation of test samples

Samples used for antibacterial assay were prepared according to [34]. 2g of the solid extract was dissolved in 2mL of 99% methanol to obtain a stock solution of 1000mg/mL. Concentrations of 500, 250, 125, and 62.5mg/mL were prepared using serial doubling dilution.

Inoculums preparation

The bacterial inoculum was prepared according to [35, 34]. To obtain approximately 10⁸CFU/mL, the turbidity of 24 hold broth culture was adjusted with sterile saline to obtain turbidity optically equivalent to that of the 0.5 *McFarland standards*.

Antibacterial susceptibility test

The effect of methanolic extract of *S. mucronata* leaves on the growth of the present bacterial isolates was detected using the disc diffusion method [36, 37]. Six millimeter discs of Whatman filter paper No. 1 were prepared and sterilized by autoclaving. 10μL of each extract concentration was loaded with discs to obtain the final concentrations of 5.0, 2.5, 1.25 and 0.6mg/disc respectively. 100μL of the standardized bacterial suspension (containing 10⁸ CFU/ml) was spread uniformly on the surface of Mueller Hinton agar (Oxoid) using sterile cotton swabs. Then, 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°C for 24h. Tests were performed in duplicates. The antibacterial activity was expressed as the mean diameter of inhibition zones in millimeter.

Results and discussion

Appearance of stains

The first examination of the manuscript under both natural and UV light appeared to display typical symptoms of biodeterioration. Purple colored spots were distributed throughout the papers, suggesting that these spots were caused by migration of microorganisms through the pages (Fig. 2).

Isolation and identification of bacteria

Based on the morphological features and Gram's staining, two different colonies were selected and then sub-cultured onto the nutrient agar medium for 24h at 37°C by streaking method. Isolate AN1 was gram negative, ovoid cells, colonies are white to creamy colored, circular, small (0.5-1.0mm in diameter), convex and entire. Isolate AN2 was gram positive, ovoid cells, colonies on being circular, large (2-3mm in diameter), orange, slightly convex with irregular margins [38].



Fig. 2. Showing the deterioration of the manuscript

Analysis of the partial 16S rRNA sequencing

Blast analysis of the obtained sequences with 16S rRNA gene sequences in the NCBI GenBank database, showed 96 and 99% similarity with *Acinetobacter indicus* and *Exiguobacterium aurantiacum* respectively. The partial 16S rRNA gene sequences of isolating AN1 and AN2 were deposited to NCBI GenBank under the accession numbers KX998197 and KX998198 respectively. The phylogenetic trees were constructed using CLUSTALW2 (Figs. 3 and 4).

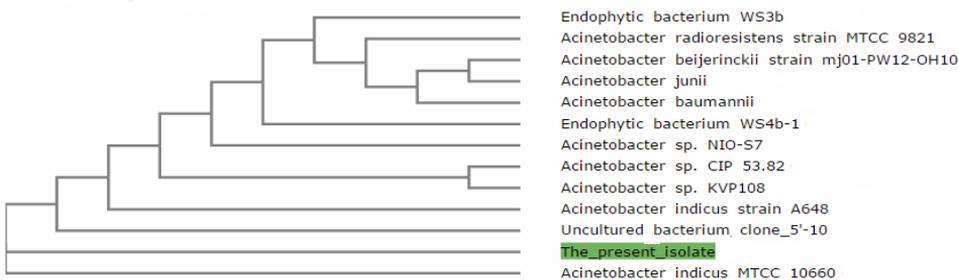


Fig. 3. Phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship of the present isolate AN1 with the other members of the genus *Acinetobacter*

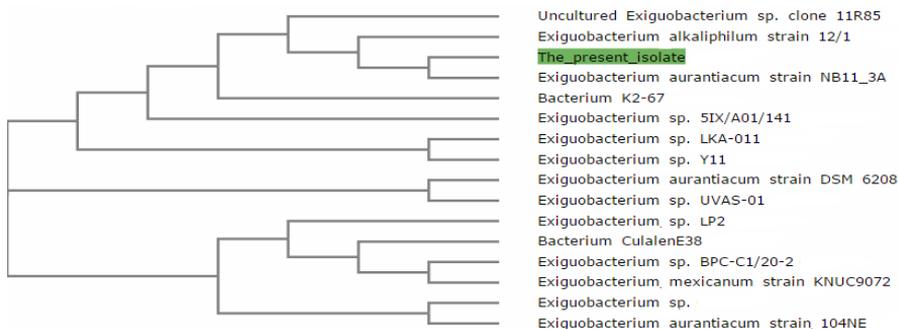


Fig. 4. Phylogenetic trees based on partial 16S rRNA gene sequences showing the relationship of the present isolate AN2 with the other members of the genus *Exiguobacterium*

Cellulolytic activity of bacterial isolates

Cellulolytic activity of isolated AN1 and AN2 was determined by streaking on CMC agar plates. It was found that the two isolates 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 [39 - 41].

Chemical composition of the extracts

GC-MS analysis of methanolic extract of *Salix mucronata* Thunb shown in (Fig. 5) Revealed that the main chemical constituent was the organic compound, Octadecanoic acid, ascorbic acid, dichloromethane, n-butanol, α -pinene, α -terpinene, (5,4'-dihydroxy-3'-methoxystilbene-3- β -D-glucoside, Hexadecaneperoxoic acid(2-phenyl-1,3-dioxolan-4I-Propanone).

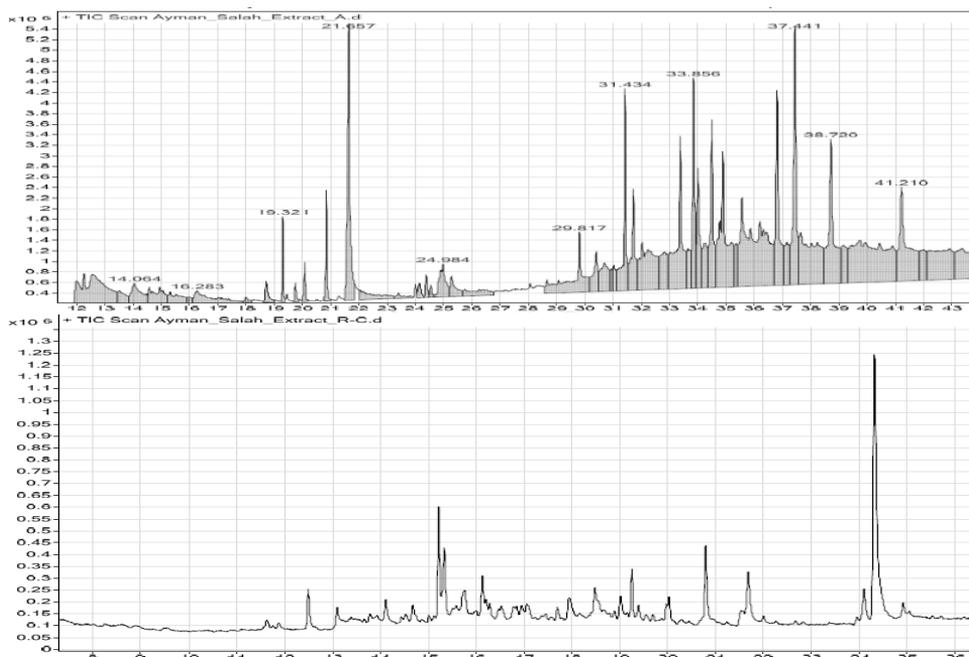


Fig. 5. Showing the GC-MS analysis of methanolic extract of *Salix mucronata* Thunb

Antibacterial activity of the crude extract of *S. mucronata* leaves

Salix genus (Family Salicaceae) includes about 400 species which have a great medicinal importance [42]. *Salix mucronata* Thunb. (Syn. *Salix safsaf* or *Salix subserrata*) exist in abundance along the Nile River in Egypt. Many researches reported that *Salix* species contain many phytochemical components such as natural aspirin, flavonoids, terpenoids, lignans and phenolic acids [42, 43]. Up to now, the present study is the first one focused on the antibacterial effect of *S. mucronata* leaves extract. It is interestingly observed that the extract at its different tested concentrations was effective against the growth of the two isolates Table 1. The inhibition effect of the extract may be related to the presence of phenolic compounds, tannins, saponins and flavonoids [42], where it has previously been reported that, plants which are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, saponins and flavonoids have been found *in vitro* to have antimicrobial properties [44]. So that, the leaf extract of *S. mucronata* can be used as a safe and cheap natural control agent to protect books and manuscripts kept in the libraries against bacterial attack.

Table 1. Antibacterial activity of methanolic crude extract of *S. mucronata* leaves

Extract concentration (Mg/disc)	Inhibition zone (mm) Mean \pm SD	
	Isolate AN1	Isolate AN2
5.0	24.8 \pm 0.7071	20.8 \pm 0.7071
2.5	22.7 \pm 1.4142	16.8 \pm 0.7071
1.25	19.8 \pm 0.7071	15.5 \pm 2.1213
0.6	15.7 \pm 1.4142	11.7 \pm 1.4142
Chloramphenicol (30 μ g/disc)	25.8 \pm 0.7071	24.8 \pm 0.7071

Conclusions

The bacterial analysis of the damaged manuscript which has kept in Al-Azhar library in Cairo, Egypt was carried out. Based on the partial 16S rRNA gene sequences, two bacterial isolates identified as *A. indicus*, *E. aurantiacum* were detected. Interestingly, the study showed that the methanolic crude extract of *S. mucronata* leaves can be used instead of hazardous chemical controls as non-toxic, cheap and eco-friendly natural control against the causative bacterial isolates for protecting manuscripts kept in libraries.

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