International Journal of Biological Research, 5 (2) (2017) 48-58

International Journal of Biological Research

Website: www.sciencepubco.com/index.php/IJBR
doi: 10.14419/ijbr.v5i2.8479
Research paper

Attenuation of microbial induced deterioration of cellulose fibers by hornwort (*Ceratophyllum demersum* L.) methanolic extract

Ali Mohamed Omar [1], Taha Ayman Salah [2]*, Amal A. A. Mohamed [3], Mohamed G. Sheded [3]

[1] *Microbiology Department, Conservation center, Grand Egyptian museum, Egypt*
[2] *Conservation Department, Faculty of Archaeology, Aswan University, 81528, Egypt*
[3] *Department of Botany, Faculty of Science, Aswan University, Aswan 81528, Egypt*
**Corresponding author E-mail: aymansalahtaha82@yahoo.com*

Abstract

Plants are endowed by a variety of secondary metabolites which have potent antimicrobial activity to treat vulnerable subjects against microbial-induced damage. In this study, bacteria and fungi were isolated from an infected manuscript dated back to 8th century AH kept at Al-Azhar library in Cairo, Egypt. The material of that manuscript was made from cellulose fibers.

Three bacterial species, *Bacillus subtilis*, *Bacillus megatrium* and *Streptomyces sp* and five fungal species, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium citrinum*, *Alternaria alternata and Acremonium kilinase* were isolated.

The antibacterial and antifungal activities of methanolic extracts of stems and leaves of *Ceratophyllum demersum* were evaluated using agar well diffusion technique. The results showed remarkable inhibition in growth of isolated bacteria and fungi treated with the plant extracts.

In addition, treating of the modern Rakta papers with *Ceratophyllum demersum* extracts resulted in ameliorating physical and mechanical properties of the papers. Moreover, the resistance against microbial attack of Rakta papers was increased after treating with *Ceratophy llum demersum* extract.

Keywords: *Ceratophyllum demersum; Antimicrobial activity; Secondary metabolites; Cellulose fiber.*

1. Introduction

Bacteria and fungi cause serious destruction for manuscripts, books, paintings, and mummies. They penetrate into the materials and result in material loss due to enzymatic degradation (Szczepanowska et al., 2000; Kaarakainen et al., 2009).They accumulate in dust layers in libraries, collections and museums and play the most key role in their bio-deterioration (Pasquariello et al ., 2005). Almost all libraries are suffering from the bacterial and fungal deterioration (Prasad, 1995).

Book components include leather, textiles, paper and binding substances. All are prone to attack by microbes in humid conditions (Prasad, 1995). In paper industry, cellulose fibers and certain chemical compounds, *e.g*., alum and rosin are used for sizing of paper. These chemical compounds cause acidic effect and facilitate chemical deterioration of the paper after a period of time (Bokhare, 1997).

Studying the mechanisms of the microbiological contamination of historical materials has been extensively the main focus of laboratories concerned in the conservation of cultural heritage (Lord et al., 2002). Finding safe, cheap and eco-friendly materials is an important modern trend to control the microbial deterioration of books and manuscripts in libraries.

For a long period of time, plants have been a precious source of natural products for control of microbial attack (Shaik et al., 1994; Santos et al., 1995). Aquatic plants endow with an extensive set of benefits to human being and from one of the richest ecological units in Egypt (Malathy and Shaleesha, 2015). There are 25 families of aquatic plants subdivided into 45 genera and 87 species of flowering plants in the Nile River (El-Hadidi, 1971; Tackholm, 1974; Springuel and Murphy 1991; Shaltout et al., 1994; Zahran, 2009).Various bioactive compounds are produced by aquatic plants and they showed antimicrobial activities (Bhosale et al., 1999; Li and Hu, 2005; Abu Ziada et al., 2008; Fareed et al., 2008; Shin et al., 2010; Sridevi et al., 2010; Yi et al., 2012).

The antimicrobial properties of aquatic plants are owed to a variety of secondary metabolites such as alkaloids which have been found in the rooted floating leaved species, whereas submerged species contain both simple phenols and flavonoids (Bushmann and Ailstock, 2006; Majid et al., 2017).

In the present study, one dominant species of aquatic plants, *Ceratophyllum demersum* belonging to family *Ceratophyllaceae*, was used for screening its protective effect against deterioration of historical manuscripts caused by microbial contamination.

2. Material and methods

2.1. Collection and preparation of plant materials

Ceratophyllum demersum (hornwort) is a submerged aquatic perennial plant. The plant is a dominant species within the River Nile system. The plant is collected from sites located around Isis island (N: 24º 04.646'; E: 32º 52.701') and Saluga and Ghazal island (N: 24º 04.328'; E: 32º 52.279').

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One set of fresh biomass was authenticated according to Täckholm (1974) and voucher specimen was deposited at Herbarium of Botany Department, Faculty of Science, Aswan University. The number of voucher specimen is 11813.
Another set of plant biomass was washed to remove any debris, separated into stems and leaves, and left for air-drying. Using a laboratory blender, the dried tissues were ground to a fine powder to be prepared for further extraction process.

2.2. Extraction of plant material

A mass equal to 100 g of the fine powdered material from stems and leaves was soaked in methanol (100%), held with occasional shaking and left for overnight. The mixture was filtrated. Residue was retained to the flask, and another amount of methanol was added. The extraction procedure was repeated three times. The filtrated extracts were pooled together and dried under reduced pressure (~ 40 ºC) to obtain a semi-solid mass (the crude methanolic extract). The methanolic extracts of the plant was used for antimicrobial assays.

2.3. Isolation of microorganisms and growth media

The microbial contaminants were isolated from the infected manuscript "almutawil sharah talkhis almuftah fa almaeanaa walbayanlilqizwaynaa" dated back to the 8th century AH at Al-Azhar library in Cairo, Egypt. The public and special numbers of the manuscript are 84699 and 2977, respectively. (Fig.1).

Fig. 1: The bio-deterioration of the manuscript.

The bacterial and fungal stock cultures were incubated for 24 hours at 37 °C on nutrient agar and potato dextrose agar (PDA) medium (Microcare laboratory, Surat, India), respectively. The stock cultures were maintained at 4°C.

2.4. Antimicrobial activity

2.4.1. Determination of zone of inhibition method

Antibacterial and antifungal activities of plant extracts against the isolated microorganisms were tested using agar diffusion method. The bacterial and fungal cultures were incubated for 24 hours at 37 °C on nutrient agar and potato dextrose agar (PDA) medium, respectively. The zones of growth inhibition around the wells were measured after 18 to 24 hours of incubation at 37 °C for bacteria and 48 to 96 hours for fungi at 28 °C. The sensitivities of the microorganism species to the plant extracts were determined by the sizes of inhibition zones (including the diameter of well) on the agar surface around the wells. Values <15 millimeters were considered as not active against microorganisms. The controls were the solvents used for every extract.

2.4.2. Determination of minimal inhibitory concentration (MIC) of plant extracts

The sets of seven dilutions (25, 50, 100, 200, 300, 400, 500 ppm) of plant extracts were prepared. After 24 h at 37 °C bacteria and 48 to 96 hours for fungi at 28 °C, the MIC of each sample was determined by measuring the sizes of inhibitory zones (including the diameter of well) on the agar surface around the wells; values <15 mm were considered as not active against microorganisms.

2.5. Infection of modern paper with tested microorganisms

Rakta paper as a model for modern papers (cotton pulp) was used to determine the effect of tested microorganisms on the deterioration of these modern pieces, According to Technical Association of the Pulp and Paper Industry (TAPPI) T200 OS – 70 (the general company for paper industry Rakta – Alexandria). Pieces of Rakta paper were inoculated with the spore suspension of the tested microorganisms by spraying 5 ml of suspension containing $0.5x10^6$ cells or spores/ml. The infected pieces were incubated for 2 months at ambient temperature and 60-70% humidity. Physical and mechanical properties of paper pieces were determined before and after inoculation as described below.

2.6. Physical and mechanical properties

a) Visual properties. Changes in color and shape due to microbial inoculation were observed by naked eyes.
b) PH of Rakta paper was determined using a digital pH meter.
c) Structure of paper, tensile strength, burst resistance, tear resistance, brightness and darkness all were determined before and after inoculation of paper pieces.

2.7. Scanning electron microscopy

The morphology of the surface of the uninfected and infected samples was investigated to show any changes or damage to the fibers.

2.8. FTIR spectroscopy analysis

The changes of the molecular structure occurring in the modern paper before and after infection were determined by FTIR (Fourier Transform Infrared). In IR Lab at the conservation center in the Grand Egyptian Museum, changes in the shape and linkage of the structure of the tested paper was determined.

2.9. GC/MS analysis of the extracts

Analysis of chemical compositions of the studied extracts was carried out using GC/MS at Institute of Marine Sciences, Alexandria, Egypt. The chemical composition of the extracts was performed using Trace GC mass spectrometer IRM Calibration Status Inj Position ACQ Method. The column oven temperature was initially held at 120 ºC and then increased by 5 ºC/m into 200 ºC with holding 2 min then increased to 280 ºC (10 ºC /min). The injector and detector (MS transfer line) temperatures were kept at 250 ºC. Helium was used as a carrier gas at a constant flow rate of 1 ml min/1. The solvent delay was 2 min and diluted samples of 1 ml were injected automatically using Autosampler AS3000 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40e550 in full scan mode. The ion source and transfer line temperatures were set at 200 and 250 ºC, respectively. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 (Oberacher, 2011) and NIST 11 Mass Spectral (2011) databases.

3. Results and discussion

3.1. Microbial activity

Two gram positive bacteria, *Bacillus subtilus* and *Bacillus megatrium*, *Streptomyces sp*. and five fungal strains *Aspergillus niger*, *Aspergillus flavus*, *Penicillium citrinum*, *Alternaria alternate* and *Acremonium kilinase* were isolated from the infected manuscript.
Antibacterial and antifungal activities of extracts were assessed in terms of zone of inhibition of microorganism growth. The results

of the antibacterial and antifungal activities are shown in (Table 1, Fig. 2).

Table 1: Inhibition zone (mm) of growth of microorganisms by methanolic plant extracts using agar diffusion method

Microorganism	Stem extract	Leaf extract
Bacillus subtilus	45	46
Bacillus megatrium	50	47
Streptomyces sp	36	40
Aspergillus niger	40	42
Aspergillus flavus	43	46
Penicillium citrinum	41	45
Alternaria alternate	38	42
Acremonium kilinase	35	47

Stem extract of *Ceratophyllum demersum* showed potent antimicrobial activity against all tested microorganisms, *Bacillus subtilus*, *Bacillus megatrium*, *Streptomyces sp*., fungal strains *Aspergillus niger, Aspergillus flavus, Penicillium citrinum, Alternaria alternate and Acremonium kilinase* of inhibitory zone values: 45, 50, 36, 40, 43, 41, 38 and 35 mm, respectively. On the other hand, leaf extract showed the highest antimicrobial activity against all tested microorganisms of inhibitory zone values: 46, 47, 40, 42, 46, 45, 42 and 47 mm for respective microorganisms (Table 1).

Evaluation of minimal inhibitory concentration (MIC) is a prevailing estimate for measuring the antimicrobial potency of a plant extract showing a minimal concentration to give apparent inhibition for growth of a microorganism (Saxena et al., 1994, Micheluz et al., 2015, Szczepanowska and Cavaliere, 2000). Tables 2 and 3 illustrate the MIC of *C. demarsum* for stem and leaf extracts, respectively. Evident inhibition zones were observed for *Bacillus subtilis, Bacillus megatrium, Aspergillus niger and Aspergillus flavus* of values equal to 20, 21, 20 and 19 mm, respectively for MIC of 50 ppm (stem extracts). The MIC was 100 ppm to inhibit *Streptomyces sp., Penicillium citrinum, Alternaria alternata and Acremonium kilinase* of values 17, 19, 18 and17, respectively (Table 2). Leaf extracts showed MIC at 50 ppm for most microorganisms, except, for *Streptomyces sp*. showed inhibition at 100 ppm (Table 3).

Fig. 2: Antimicrobial activity of stem extracts (Left) and leaf extracts (Right) of *Ceratophyllum demersum* on the isolated microorganisms.

Table 2: Inhibition zones (mm) for different concentrations (25-500 ppm) of stem extracts of *Ceratophyllum demersum*

	25	50	100	200	300	400	500
Bacillus subtilus	0.0	20	28	30	32	37	39
Bacillus megatrium	00	21	28	35	40	45	50
Streptomyces sp	0.0	0.0	17	20	25	29	32
Aspergillus niger	0.0	20	25	28	30	32	35
Aspergillus flavus	0.0	19	24	27	31	34	37
Penicillium citrinum	0.0	0.0	19	22	30	35	38
Alternaria alternate	0.0	0.0	18	21	25	30	34
Acremonium kilinase	0.0	0.0	17	20	22	26	30

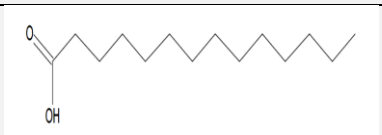
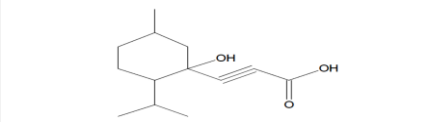
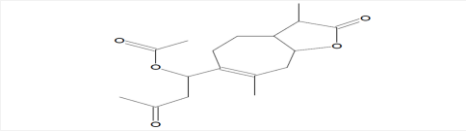
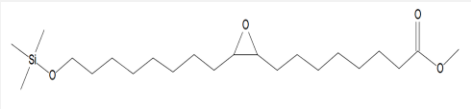
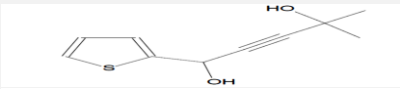
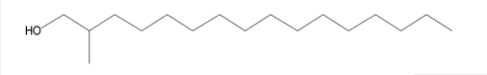
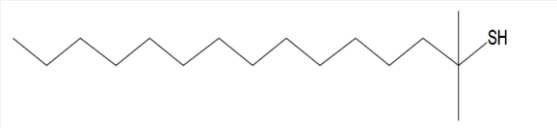
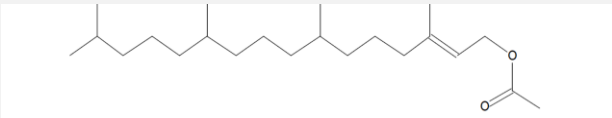
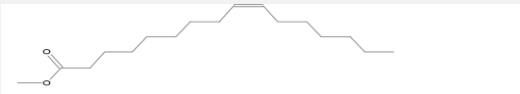
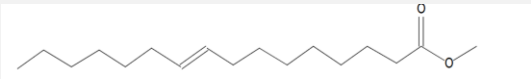
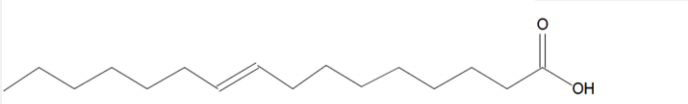
Table 3: Inhibition zones (mm) for different concentrations (25-500 Ppm) of leaf extracts of *Ceratophyllum demersum*

	25	50	100	200	300	400	500
Bacillus subtilus	0.0	21	27	29	31	36	38
Bacillus megatrium	0.0	21	27	30	32	38	40
Streptomyces sp.	0.0	20	30	37	39	42	45
Aspergillus niger	0.0	0.0	18	21	27	31	36
Aspergillus flavus	0.0	18	20	25	29	32	36
Penicillium citrinum	0.0	17	21	25	30	33	38
Alternaria alternate	0.0	17	21	25	29	32	40
Acremonium kilinase	0.0	18	20	23	27	32	37

3.2. Chemical composition of the extracts

GC/MS analyses of crud methanolic extracts from stems and leaves of *Ceratophyllum demersum* are shown in Tables 4 and 5. The analyses indicated presence of oleic acid, tetradecanoic acid, pentadecanoic acid, octadecanoic acid, 9-hexadecenoic acid, palmitoleic acid and linoleic acid ethyl ester (Fig.3 and 4).

Table 4: Chemical composition of the methanolic crud extracts from *Ceratophyllum demersum stems*

RT	Compoundname	Chemical structure
77.4%	Tetradecanoic acid ,	
5.66%	Pentadecanoic acid	
5.19%	Ppropiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-	
6.66%	Dihydroxanthin	
4.79%	Octadecanoic acid, 9,10-epoxy-18-(trimethylsiloxy)-, methyl ester, cis-	
7.88%	2-Pentyne-1,4-diol, 4-methyl-1-(2-thienyl)-	
11.5%	1-Hexadecanol, 2-methyl-	
11.5%;	cis-13-Eicosenoic acid	
9.75%	tert-Hexadecanethiol	
6.15%	Phytol, acetate	
26.9%	9-Hexadecenoic acid, methyl ester, (Z)-	
17.2%	Methyl hexadec-9-enoate	
40.9%;	Palmitoleic acid	

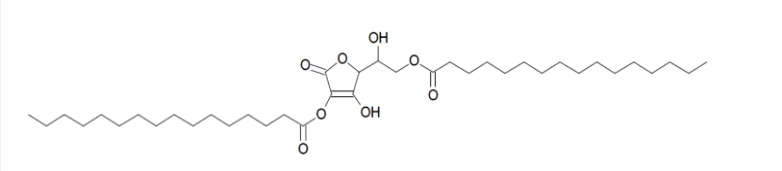
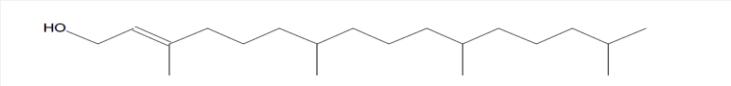
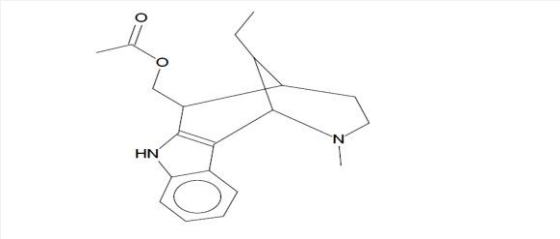
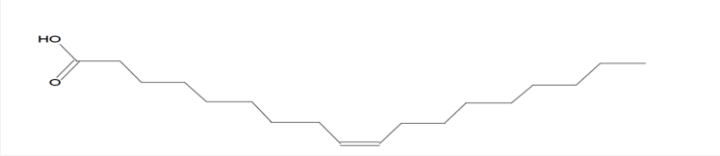
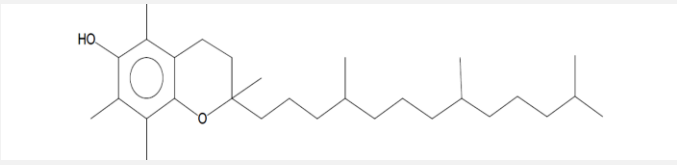
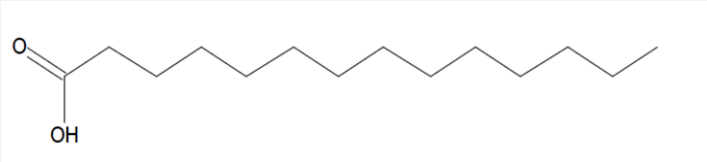
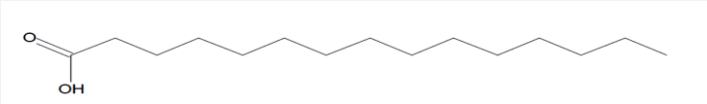
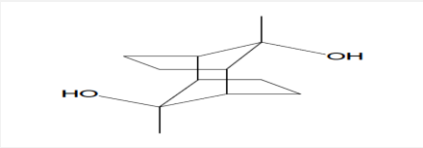
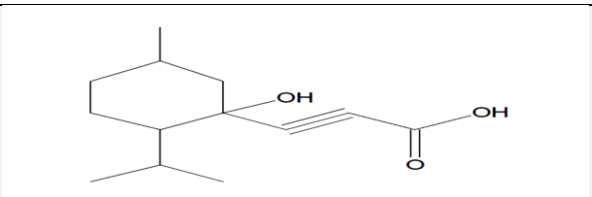
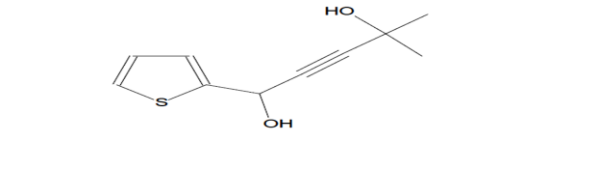
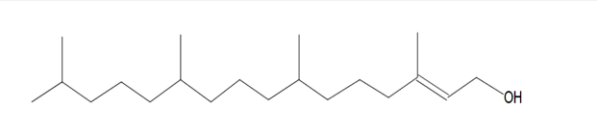
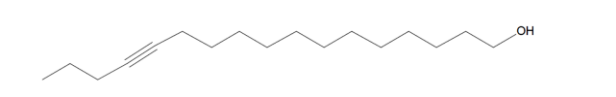
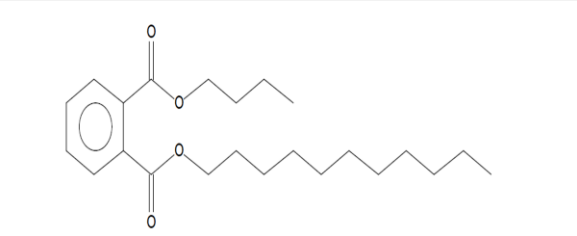
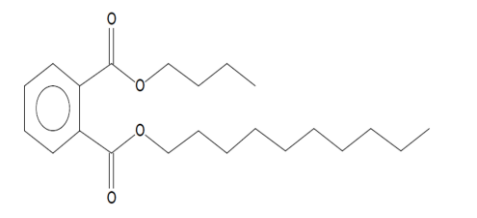
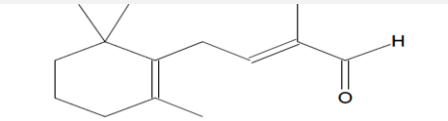
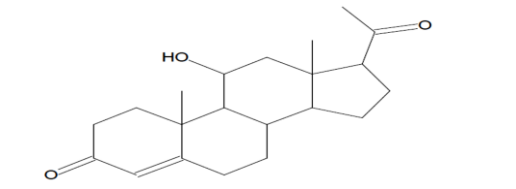
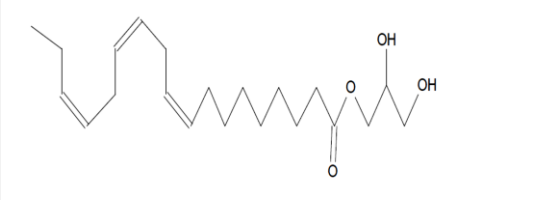
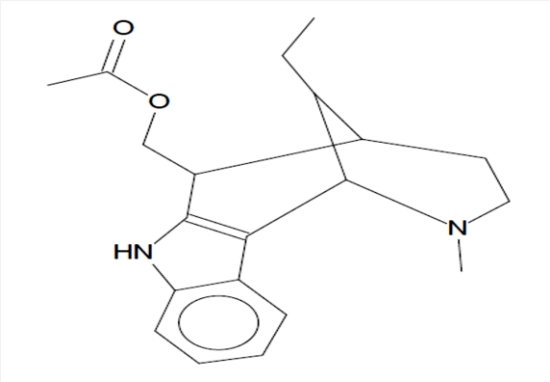
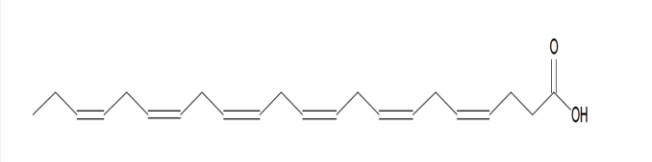
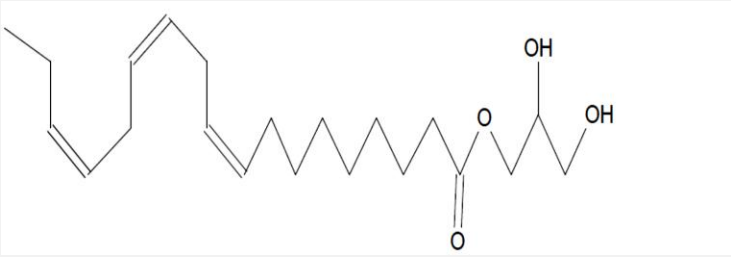
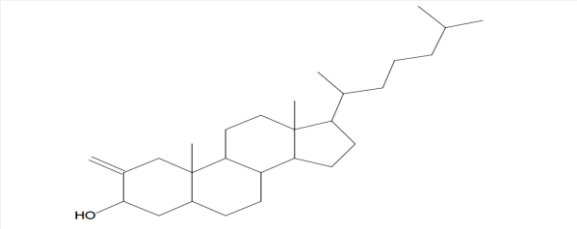
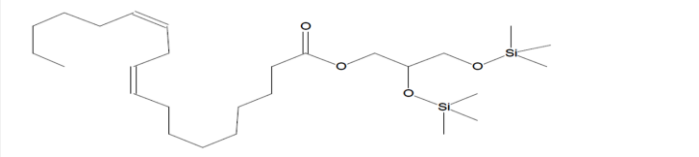
8.95%	l-(+)-Ascorbic acid 2,6-dihexadecanoate	
7.46%	Linoleic acid ethyl ester	
36.7%	Phytol	
37.1%	Dasycarpidan-1-methanol, acetate (ester)	
6.88%	Oleic Acid	
56.0%	Vitamin E	

Table 5: Chemical composition of the methanolic crud extracts from *Ceratophyllum demersum* leaves

RT	Compoundname	Chemical structure
79.8%	Tetradecanoic acid	
5.10%	Pentadecanoic acid	
7.59%	Isopropyl palmitate	
17.9%	9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	
13.0%	Ppropiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-	

		
6.31%	2-Pentyne-1,4-diol, 4-methyl-1-(2-thienyl)-	
26.1%;	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	
8.56%	13-Heptadecyn-1-ol	
8.89%	Phthalic acid, butyl tetradecyl ester	
3.52%	1,2-Benzenedicarboxylic acid, butyl decyl ester	
17.6%	2-Butenal, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	
6.04%	Pregn-4-ene-3,20-dione, 11-hydroxy-, (11α)-	
15.0%	Methyl hexadec-9-enoate	
	Palmitoleic acid	
36.3%		

6.60%	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	
5.83%	9,12-Octadecadienoyl chloride, (Z,Z)-	
20.0%	Dasycarpidan-1-methanol, acetate (ester)	
5.42%	Doconexent	
20.7%	1-Heptatriacotanol	
24.5%	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	
4.76%	Cholestan-3-ol, 2-methylene-, (3β,5α)-	
5.15%	1-Monolinoleoylglycerol trimethylsilyl ether	

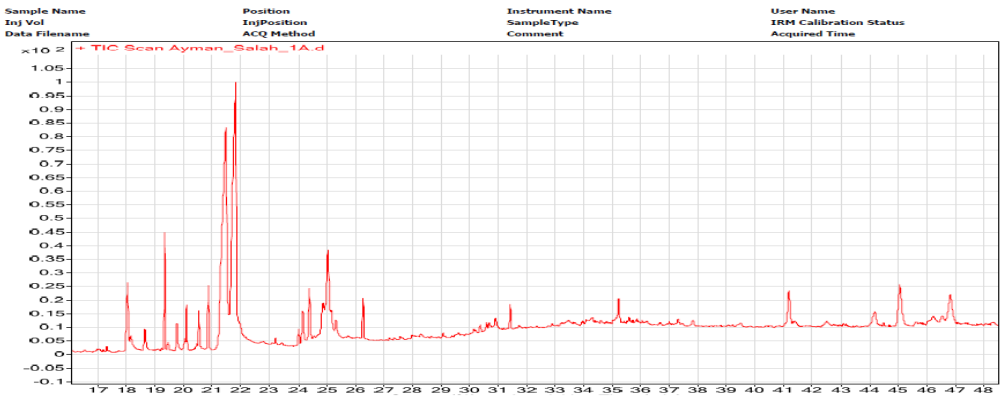


Fig. 3: GC-mass spectrum of methanolic crud extract of *Ceratophyllum demersum stems*.

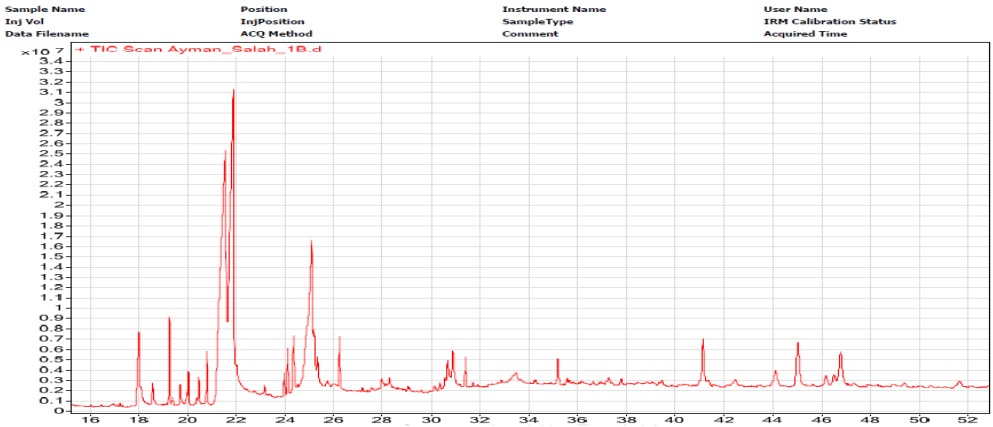


Fig. 4: GC-mass spectrum of methanolic crud extract of *Ceratophyllum demersum* leaves.

3.3. Physical and mechanical properties of the infected paper

Clean pieces of RaKta paper were inoculated with the previously mentioned microorganisms. Inoculated pieces were incubated for 2 months at ambient temperature and under 60-70% humidity. Physical properties of specimen were determined before and after infection.

Changes in physical, mechanical properties of papers are illustrated in Table 6. Black spots were observed on the infected pieces of paper. The pH of the paper was decreased as a result of acid production which ultimately leaded to altering and weakening of the paper. In addition, many molds contain colored substances were found. In addition, it was found that prior treating of the paper with *Ceratophyllum demersum* extract decreased the effect of microbes on physical properties.

Table 6: Changes in Physical and Mechanical Properties of Paper as Function of Microbial Infection

Properties	Before microbial infection	After microbial infection	Plant extract+microbial infection	Plant extract only
Appearance	White	Black spots	Blackish spot	White
Texture	Smooth	Weakness	Smooth	Smooth
PH	6	5	5.8	6.3
Tensile strength	3.4	2.8	3.1	3.5
Burst resistance	26	24.7	25.6	27.3
Tear resistance	131	128.3	129.2	132.7
Brightness	65.3	63.5	64.2	66.3
Darkness	85.3	83.4	84.8	86.7

3.4. SEM microscopy observation

Fungal detrimental changes reduce the quality of a material and make it less functional in utilization terms. Similar results were reported by Najietal, (2014) who stated that a pack of extracellular hydrolytic enzymes excreted by fungi is responsible for the formation of acidic products that cause chemical alteration of the material under attack. (Figs. 5, 6, 7, 8).

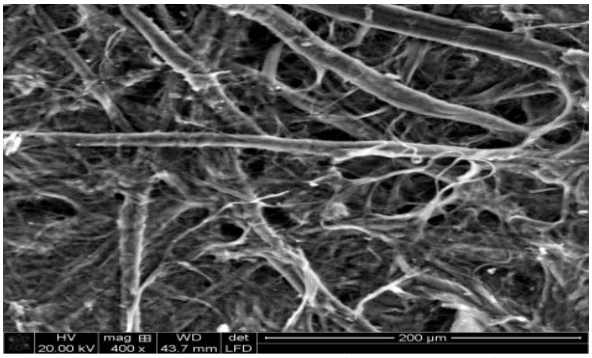


Fig. 5: Scanning electron micrograph of modern magnification 400X; Scale Bar = 200 microns.

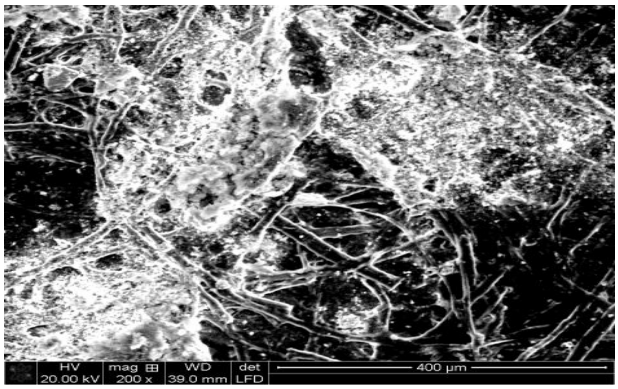


Fig .6: Scanning electron micrograph of modern infected paper sample show that fungal growth on the surface. Magnification 400X, scale bar = 200 microns.

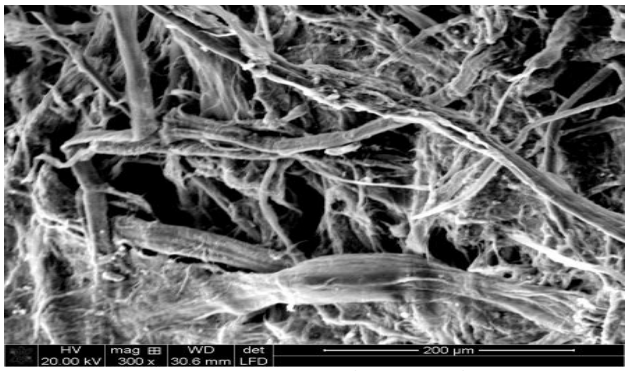


Fig .7: Scanning electron micrograph of modern infected paper sample treatment with plant extract. Magnification 400X, scale bar = 200 microns.

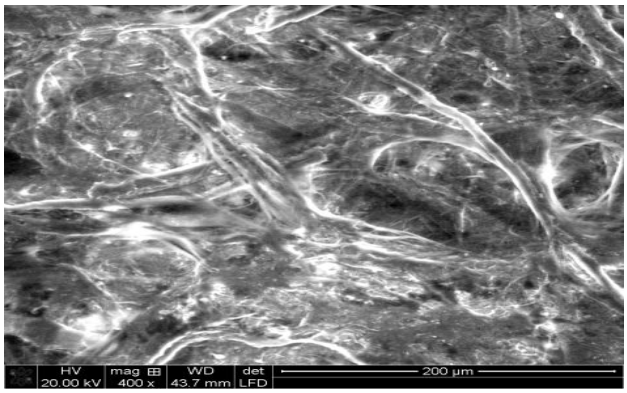


Fig .8: Scanning electron micrograph of modern paper treatment with plant extract without infection. Magnification 400X, scale bar = 200 microns.

3.5. FTIR analysis

The functional groups of different combinationin different paper fibers can be identified by means of FTIR (Chow, 1971; Ajuong and Breese, 1998; Nuopponen et al., 2003; Aluong and Redington, 2004; Nuopponen, 2005).

Data recorded in Figs. (9,10,11,12) showing chemical changes inside the paper structure resulted from degradation of large organic compounds by the action of extra-cellular enzymes secreted by deteriorative fungal species which converting these compounds into smaller ones and disappearance of different characteristic chemical groups, the following bands were obtained:

1) Change in the shape of (N=H) stretch linkage are typical protein bands assigned to the amide functions of the peptide groups at wave number area (1560-1640 cm^{-1})
2) Change in the shape of (C=O) Easter stretch linkage movement at wave number area (1100-1300 cm^{-1})
3) Change in the shape of (C-H) bending linkage movement at wave number area (860-900 cm^{-1})

After treatment of infected paper with the plant extract we found change in functional groups and disappearance of other chemical groups, the following bands were obtained:

1) Change in the shape of (C=C) bending linkage movement at wave number area (1636-1508 cm^{-1})
2) Change in the shape of (C-X) bending linkage movement at wave number area (400-500 cm^{-1})
3) Change in the shape of (C-H) bending linkage movement at wave number area (2720-2960 cm^{-1})

After treatment of uninfected paper when compared with the control:

1) Change in the shape of (C-H) bending linkage movement at wave number area (2720-2960 cm^{-1})
2) Change in the shape of (C=O) Easter stretch linkage movement at wave number area (1100-1300 cm^{-1})
3) Change in the shape of (C-X) bending linkage movement at wave number area (400-500 cm^{-1})

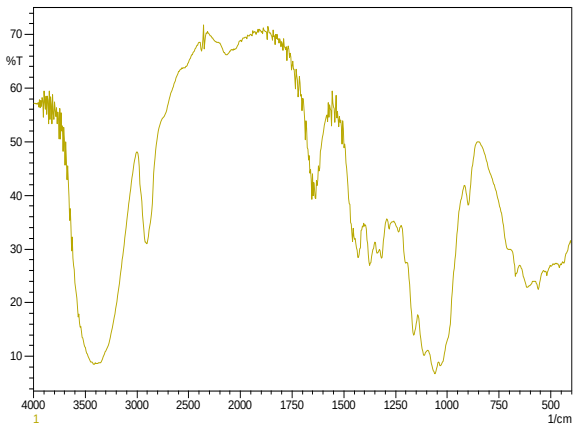


Fig. 9: Analysis of paper fragment by FTIR before infection (Control).

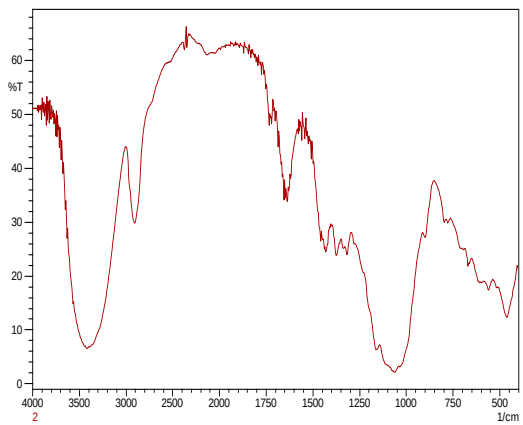


Fig. 10: Analysis of paper fragment by FTIR after infection by tested microorganisims.

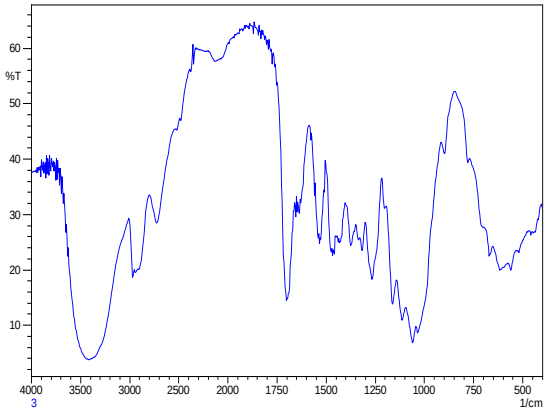


Fig. 11: Analysis of paper fragment by FTIR after infection and treatment with plant extract.

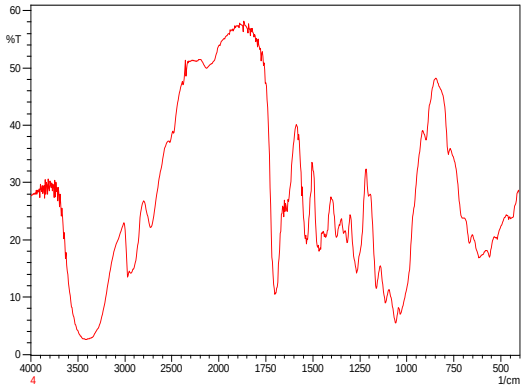


Fig. 12: Analysis of paper fragment by FTIR treatment with plant extract without infection.

4. Conclusions

The crud methanolic extracts of stems and leaves of *Ceratophyllum demersum* have a great potential as antimicrobial compounds against microorganisms which cause damage to stuffs of libraries. Thus, they can be used in the treatment of infected paper. Treatment of infected paper with the methanolic extracts of shoots and roots of *Ceratophyllum dermersum* at MIC 100 ppm showed no changes in physical, morphological and mechanical properties of the infected paper as compared with the control.

Acknowledgment

The authors of this paper are thankful to the Microbiology laboratory in conservation center in the Grand Egyptian Museum for helping and providing necessary facilities for this research work, Grateful thanks to the staff members of Al-Azhar library in Cairo for their kind assistance and cooperation for samples collection.

Conflicts of interest

The authors declare that there is no conflict of interest.

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