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Extraction, Modification and Characterization of Cellulose NanoWhiskers as a Nanodelivery System for Doxourubicin

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Abstract

Doxorubicin represents the most powerful antineoplastic agent available in most of cancer treatment protocols. The use of Doxorubicin is associated with world wild problem which is cancer resistance. Therefore Cellulose Nanowhiskers (CNWs) as a novel nancarriers were extracted and incorporated chemically with Doxorubicin as an attempt to increase its intracellular concentration and reduced its resistance. CNWs were extracted from commercial cotton, structurally modified and linked with a biological linker in a way to be capable for incorporation with Doxorubicin (DOX). The nanosized particles had been characterized by Atomic force microscopy(AFM), Scanning electron microscopy (SEM) and X-ray diffraction (XRD).The synthesis of the target compounds (I-III) had been successfully achieved and the characterization and identification were took place using Fourier transform infrared spectroscopy (FT-IR) and Nuclear magnetic resonance (1H-NMR) spectroscopy.

Key words: Cellulose nanowhiskers(CNWs) , Doxorubicin , GABA, Nanoparticles

INTRODUCTION

In the past, treatment strategies diagnosed many pathological cases as incurable fatal diseases[1] but now days numerous medication approaches have been developed to treat such complicated cases, some of the newly introduced medications found to have serious side effects [2] and others were absolutely useless within the biological fluids i.e. cannot withstand the acidic media or enzymatic hydrolysis of the gastrointestinal tract(GIT) if they give orally, making them absolutely worthless in vivo [3].Thus many studies focused on introducing better medication with improved delivery and targeting abilities [4].

Within the previous few decades; a great attention focused on the nanotechnology science as a broad area for research and development [5]. Applying nanotechnology to medicine, nanoparticles are designed to alter or mimic biological processes [6]. Nanoparticles are solid, colloidal, with a size between 10nm - 1000 nm, in the medical field less than 200nm is desirable [7]. There is a huge increase in the medical applications of nanotechnology mainly due to the unique properties of the nanoparticles such as high surface area, hydrophilicity and long circulation time as compared with small molecules. It's very known that the efficiency of most of drug delivery systems is directly proportional to the particle size [except intravenous and solution preparations]. The small particle size of nanoparticles and high surface area increase their solubility, bioavailability, enhance the ability to cross blood brain barrier (BBB) and enhance the entrance to the pulmonary systems [8]. The characterization of the nanoparticles determine the size, shape and surface changes of the tested nanoparticles using different microscopic techniques including Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) [9].

Cellulose represents one of the most abundant renewable material on the planet, could be used in future as an oil-replacing based feed stocks. In the mid-19th century the first plastic materials were derived from cellulose as a cellulose nitrate. The need for natural raw materials increased from the Second World War when the supplies of raw materials were limited [10]. Cellulose produced by the condensation polymerization of glucose monomers in plants, bacteria and some animals (tunicates). In the plants, the photosynthesis process produces anhydroglucose units as along chains connected together by β-1,4-glycosidic linkages. In the native state cellulose presents as (cellulose-I) form as a natural crystalline compound , in which the chains aggregate by hydrogen bonding [11].

MATERIALS AND METHODS

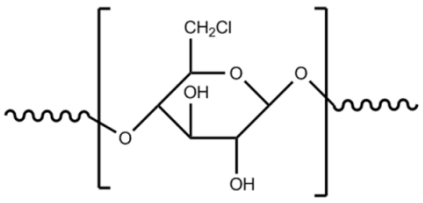
1. Chemicals and Instruments

All chemicals and solvents were of analar type and received from the commercial suppliers (Iraq, BDH-England, Himedia-India, Merck-Germany, Fluka AG-Switzerland, and Sigma-Aldrich, Germany). Doxorubicin was supplied by EBEWE pharma, Germany. IR bands were recorded using FT-IR Shimadzu (Japan), 1HNMR bands (solvent DMSOd6) were documented on 400 MHZ spectrometer (Bruker Avance III, Switzerland) with TMS as internal standard. The identification of compounds was done using a IR spectra were recorded on a FT-IR spectrophotometer Shimadzu as KBr disks in University of Al-Baghdad, at college of science.1HNMR bands were measured using Bruker 400 MHz (Avance III, Switzerland), in Moscow, Russia. Atomic force microscope (AA3000 angstrom Advanced Inc.-Germany) this type of analysis was done in University of Baghdad-Collage of Science- Department of Chemistry , Autoclave (Gallenkamp-England), Centrifuge (Hettich –Germany), Electronic Melting Point (Stuart-UK), florescent microscope (Bruker-Germany), Hot plate magnetic stirrer (Gallenkamp-England), Lyophilizer (Burker-Germany), Mechanical stirrer (Modex-China), Rotatory evaporator (Buchi-Switzerland), Scanning Electron Microscope (Zeiss-Germany) this microscopic technique performed in University of Baghdad- College of science – Department of Chemistry and X-ray Diffraction (Bruker-Germany) which also performed in University of Baghdad-College of science – Department of Chemistry. . The in vivo study of the loaded DOX was done in tissue culture unit/ Iraqi Center for Cancer and Medical Genetic Researches ICCMGR , Al-Mustansiriyah University.

2. Chemical Synthesis

The procedures for synthesis of the nanocarrier-DOX complex were illustrated in Scheme (1).

2.1. Extraction of CNWs



cellulose nanowhisker
CNW (I)

In this method, about 60 g of commercial cotton was mixed with 1L of boiling hydrochloric acid (2.5 N HCl) solution and stirred for 45 min. at 450C to yield a thick suspension of hydrolyzed cotton wool .This process represents the hydrolysis step, which was stopped by the addition of excess amount of distilled water. To remove the acidic media, the suspension was subjected to centrifugation at 6000rpm for 10 min. until the turbidity of the supernatant appeared. The acidic supernatant was removed and the process was replicated. The resultant suspension containing the CMWs was dialyzed against tap water MWCO=12000-14000 until the medium became neutral. Then the neutral suspension was placed in ultrasonic bath for 10 min. and centrifuged again to collect the CNWs in form of cloudy supernatant, this process was continued to collect CNWs as much as possible until the supernatant became clear. The collected supernatant was lyophilized and collected as a white pure powder [12]. The structure of compound (I) was characterized by the appearance of the FT-IR characteristic absorption bands of OH stretching band at 3402 cm^{-1} as shown in figure (1) and 1H-NMR spectum shown a broad singlet for OH protons at 3.5 – 4.3 (δ, ppm) as shown in figure (2). The mechanism of this chemical extraction illustrated in scheme (2).

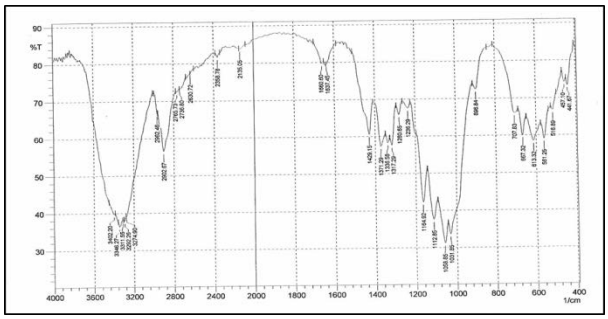


Figure (1): FT-IR spectrum of compound (I).

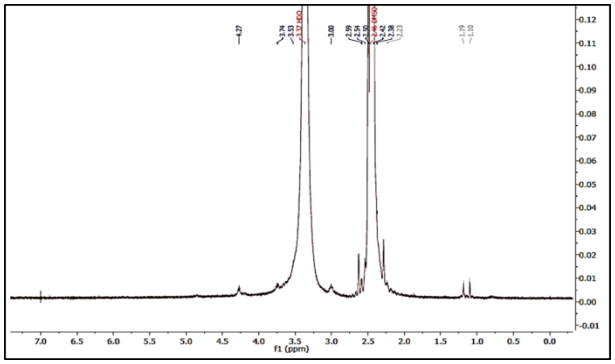
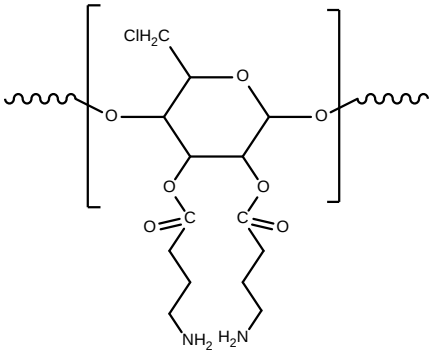


Figure (2): 1H-NMR spectrum of compound (I).

2.2. General Procedure for Synthesis of 3-(chloromethyl)-2-methoxy-5-methylhexane-1,6-diyl bis(4-aminobutanoate) hydrate [Compound (II)]:



This reaction performed in a 250 mL round bottom flask, in which 10 mL of dichloromethane containing (2.00 %, w/v) of CNWs, (10 equiv. per glucose unit) of Gamma aminobutyric acid (GABA) and (1.22 g, 1.00 mmol) of P-dimethyl aminopyridine (DMAP) were stirred in an ice bath to reach 0°C , during stirring (1.87 g, 8.00 mmol) of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and (0.50 mmol) of sulfo-N-hydroxysuccinimide (NHS) were added drop wise to the previous solution. After 5 min. the ice bath was removed and the reaction continued at room temperature with continuous stirring for 3 hrs.
While the reaction was performed, some of N-acyl urea were precipitated, which could be easily removed by Buchner. The resultant solution was washed two times with 10 mL of 0.5N HCl and again with 2 x 10 mL of 0.1N of sodium bicarbonate (NaHCO3). Some of N-acyl urea derivatives may precipitate again, which were removed by the filtration of the two layers. The organic layer was dried with magnesium sulfate (MgSO4) and the compound (II) was collected as a white powder [13]. This reaction involved the formation of ester linkage by Steglich esterification method .This method chosen instead of Fisher esterification procedure due to the need for acidic medium in the latter method, as CNWs are sensitive to further acidic hydrolysis. In addition to that Steglich method took place at room temperature.
Carboxylic acids in general are not reactive enough to undergo nucleophilic addition directly [14] , thus GABA was activated with EDC to be easily react with the alcoholic hydroxyl groups of the CNWs. DMAP used to reduce the side reactions which involve the formation of N –acyl urea which may interrupt the formation of the final product or lead to produce impure products [13,15-17] . The structure of compound (II) was identified by the appearance of the FT-IR characteristic absorption bands of υC=O stretching of ester at 1639 cm^{-1} and υC-O-C stretching of ester at1058 cm^{-1} as shown in figure (3) and the 1H-NMR spectrum of this compound shown in figure (4) .The mechanism of Steglich esterification method illustrated in scheme (3):

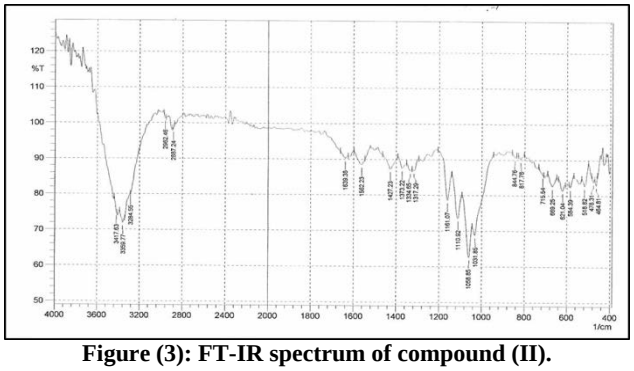


Figure (3): FT-IR spectrum of compound (II).

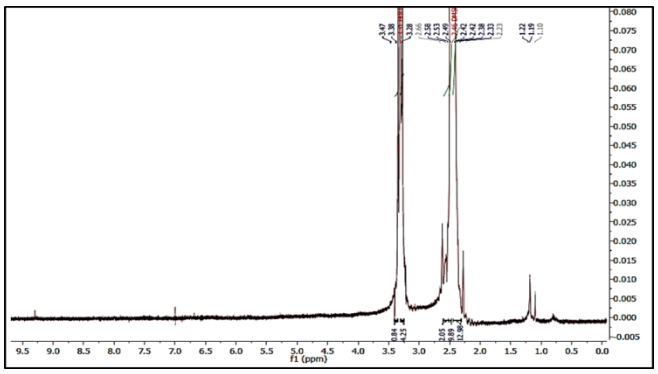
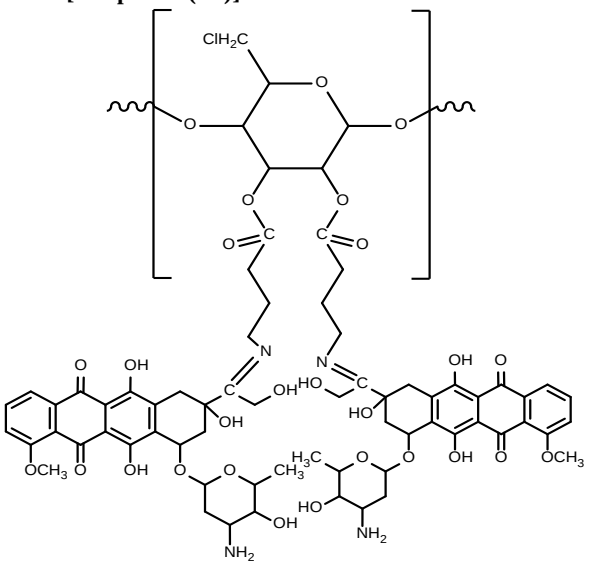


Figure (4): 1H-NMR spectrum of compound (II).

2.3. General Procedure for Synthesis of (E)-6-((4-((1-(4-((3-amino-4-hydroxy-5-methylcyclohexyl)oxy)-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-2-yl)-2-hydroxyethylidene) amino)butanoyl)oxy)-4-(chloromethyl)-5-methoxy-2-methylhexyl 4-(methyleneamino) butanoate compound with 10-((3-amino-4-hydroxy-5-methylcyclohexyl)oxy)-6,8,11-trihydroxy-8-(2-hydroxyethyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (1:1) hydrate [compound (III)]:



A 10 mL solution of DOX (5.5g, 10.18mmol) dissolved in benzene was added gradually to a solution of compound (II) (2.15g, 5.09mmol) in in 5mL benzene. The mixture was refluxed for 10 hr. until no water appeared and the solvent was removed by rotary evaporator. Recrystallization of the precipitate took place with ethyl acetate to obtain pure product [18].

This reaction represents a Schiff's base condensation reaction in which DOX dissolved in benzene and added drop wise to a solution of compound (II). The mixture was refluxed for 10 hr. to stimulate the initiation of the reaction. The reaction continued until no water appeared and the solvent was removed by rotary evaporator. This step represents the loading process of DOX with the prepared nanocarrier (II) which included the formation of imine compound (III).

The structure of compound (III) was identified by the disappearance of the FT-IR characteristic absorption bands of υN-H2 stretching of amine at 3417&3359 cm^{-1} and the appearance of υC=N stretching of imine at1519 cm^{-1} as shown in figure (5).The 1H-NMR spectrum of this compound shown in figure (6). The mechanism of this reaction illustrated in scheme (4).

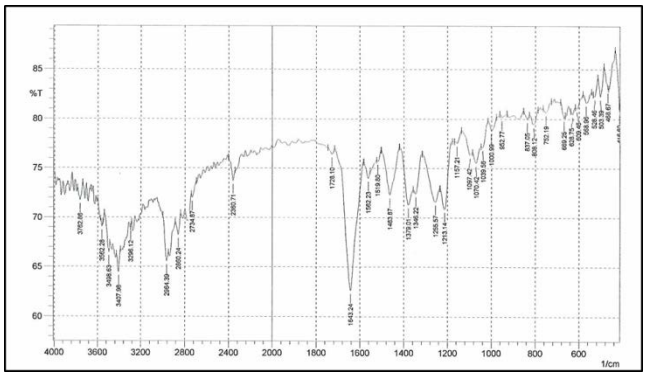


Figure (5): FT-IR spectrum of compound (III).

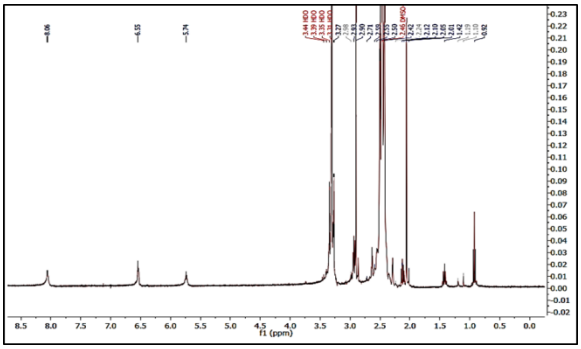
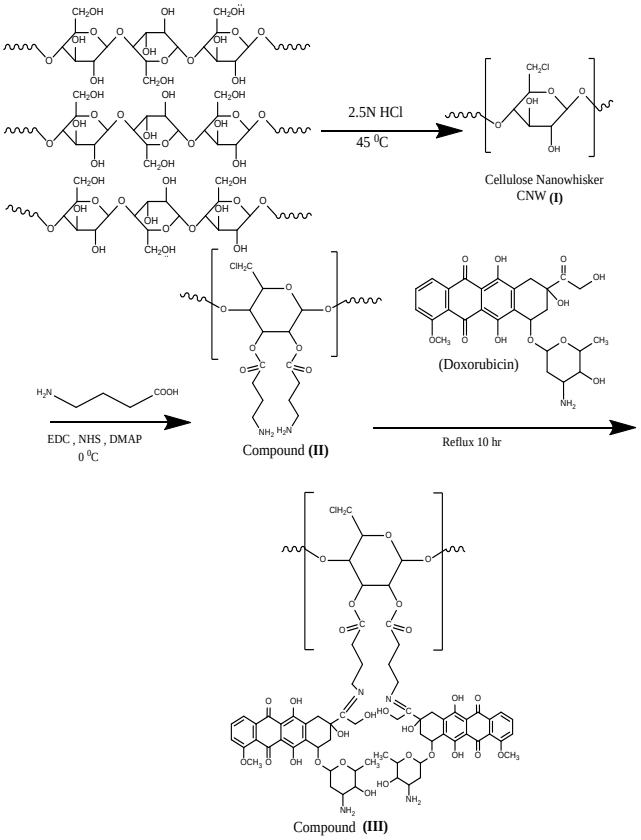
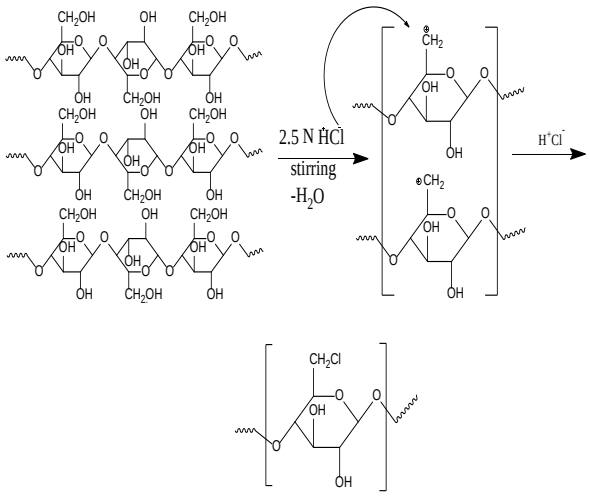


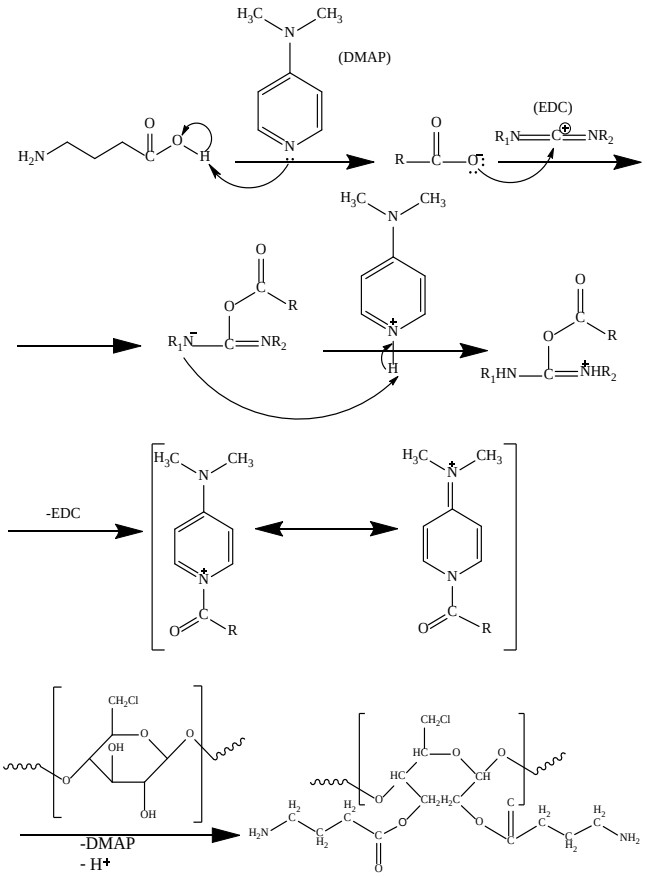
Figure (6): 1H-NMR spectrum of compound (III).



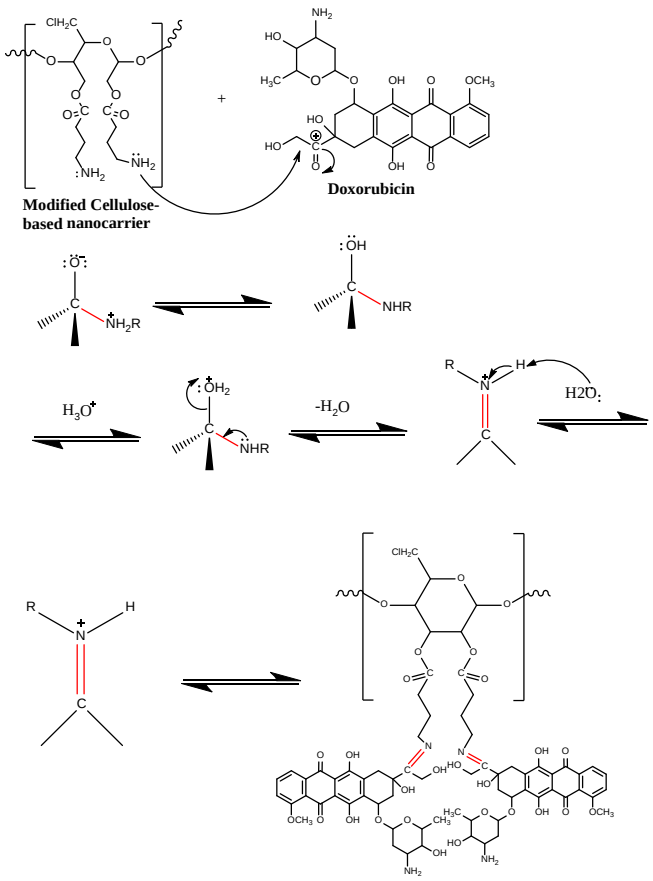
Scheme (1) : General Procedure for the Synthesis of Nanocarrier-DOX complex.



Scheme (2): Mechanism of synthesis of compound I [19].

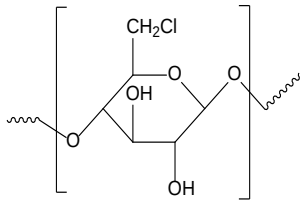
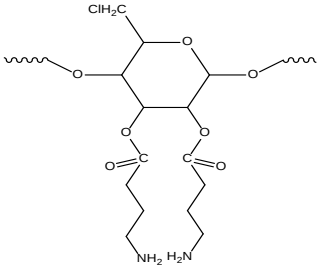


Scheme (3): Mechanism of synthesis of compound II [15-17].



Scheme (4): Mechanism of synthesis of compound III [14].

Table (1): Characteristic FT-IR Absorption Bands of the Synthesized Compounds

Compound	*Band (cm^{-1})*	*Interpretations*
 I	3402	OH stretching band
	2962	CH asymmetric stretching of CH2
	2906	CH symmetric stretching of CH2
	1429	C-C stretching
	1058	C-O stretching
	667	C-Cl
 II	3417&3359	NH2 stretching bands of amine
	2962	CH asymmetric stretching of CH2
	2887	CH symmetric stretching of CH2
	1639	C=O stretching of ester
	1427	C-C stretching of CH2-CH2
	1334	C-N stretching of C-NH2
	1058	C-O stretching of O-CH2
	669	C-Cl

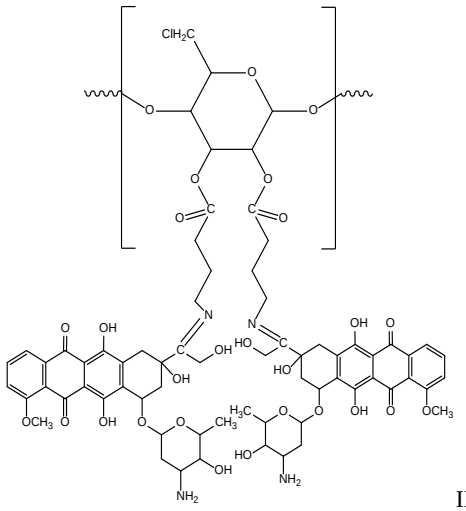
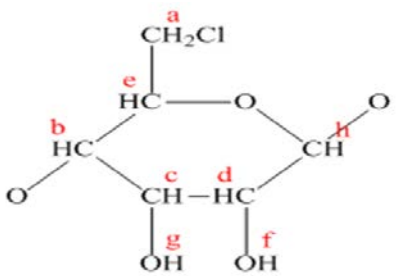
Compound	*Band (cm^{-1})*	*Interpretations*
 III	3562	OH stretching
	3498 & 3407	NH2 stretching band of amine
	3150	=C-H stretching of aromatic
	2964	-C-H asymmetric stretching of CH2
	2860	-C-H symmetric stretching of CH2
	1643	C=O stretching of ester
	1562	C=C stretching of aromatic
	1519	C=N stretching
	1463	C-C stretching of aliphatic
	1070	C-O stretching of O-CH
	669	C-Cl

Table (2): Table (2): 1H-NMR Data and their Interpretation of Compound (I).



Group	*Chemical shift ppm*	*No. of H*	*Interpretation*
a	1.1	2	Doublet for CH2Cl protons
b	1.2	1	Triplet for CH proton
c	2.4-3.5	1	Triplet for CH proton
d	2.4-3.5	1	Triplet for CH proton
e	2.4-3.5	1	Quartet for CH proton
f	3-3.53	1	Doublet for CH proton
g	3.7	1	Broad Singlet for OH proton
h	4.27	1	Broad Singlet for OH proton

RESULTS AND DISCUSSION

The desired compounds (I-III) were successfully prepared and characterized, the methods of characterization are:

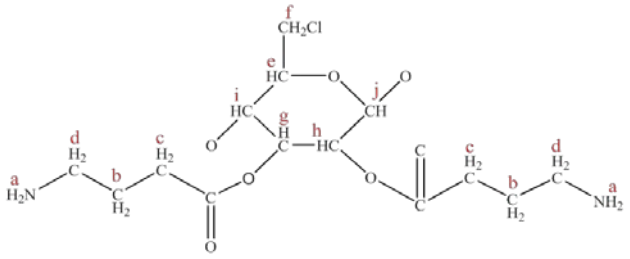
1- Infrared Spectroscopy:

The FT-IR spectra of the synthesized compounds showed the characteristic absorption bands by which the functional groups were identified. The values of the interesting bands of these spectra have been discussed according to the references books [14, 20] and are summarized in Table (1).

2- Interpretation of the Results of 1H-NMR Spectra:

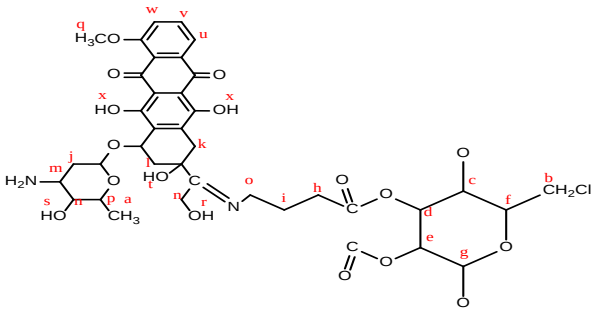
The1H-NMR analysis was used to identify the synthesized compounds. The spectra were recorded in DMSO solvent. The values of characteristic chemical shifts have been discussed according to the references books [14, 20] and summarized in Tables (2-4).

Table (3): Table (3): 1H-NMR Data and their Interpretation of Compound (II).



Group	*Chemical shift ppm*	*No. of H*	*Interpretation*
a	1.53	2	Singlet for NH2 protons
b	2.14	2	Multiplet for CH2 protons
c	2.23	2	Triplet for CH2 protons
d	2.3	2	Triplet for CH2 protons
e	2.39	1	Quartet for CH proton
f	2.42	2	Doublet for CH2Cl protons
g	2.5	1	Triplet for CH2 protons
h	2.58	1	Triplet for CH2 protons
i	2.67	1	Triplet for CH proton
j	2.71	1	Doublet for CH proton

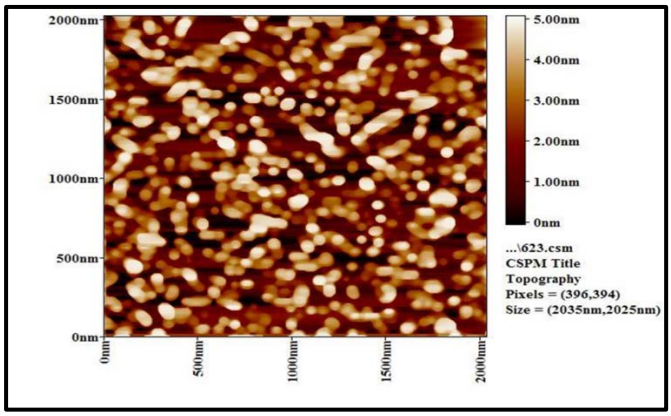
Table (4): Table (4): 1H-NMR Data and their Interpretation of Compound (III).



Group	*Chemical shift (ppm)*	*No. of H*	*Interpretation*
a	0.92	3	Doublet for CH_3 protons
b	1.1	2	Doublet for CH_2Cl protons
c	1.2	1	Triplet for CH proton
d	1.4-2.5	1	Triplet for CH proton
e	1.4-2.5	1	Triplet for CH proton
f	1.4-2.5	1	Multiplet for CH proton
g	2.6	1	Doublet for CH proton
h	1.5-2.4	2	Triplet for CH_2 protons
i	1.5-2.4	2	Multiplet for CH_2 protons
j	1.5-2.4	2	Triplet for CH_2 protons
k	1.5-2.4	2	Singlet for CH_2 protons
l	3	1	Doublet for CH_2-O
m	3.1	1	Quartet for CH-NH_2
n	3.1	1	Quartet for CH-OH
o	3.3	2	Triplet for CH_2=N
p	3.4	1	Multiplet for CH proton
q	3.7	3	Singlet for CH_3
r	3-3.8	1	Broad singlet for OH proton
s	3-3.8	1	Broad singlet for OH proton
t	3-3.8	1	Broad singlet for OH proton
u	3.8-6.7	1	Doublet for aromatic proton
v	3.8-6.7	1	Triplet for aromatic proton
w	3.8-6.7	1	Doublet for aromatic proton
x	8	1	Broad singlet for phenolic proton

3- Interpretations of the AFM Results:

The AFM analysis determines the average grain size as shown in Table (5). Figure (7) showed the AFM images of the CNWs's typical surface (in three and two dimensions) and the granularity cumulating distribution for CNWs. The average diameter of CNWs particles is found to be 84.25 nm.



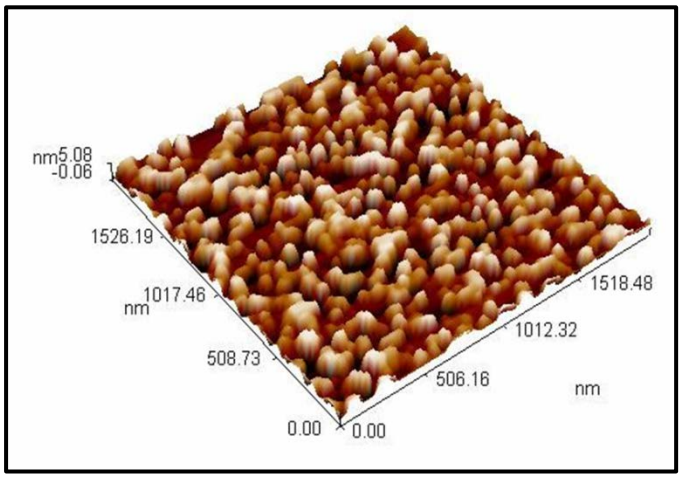


Figure (7): AFM images describing the granularity of CNWs in three and two dimensions.

4- Interpretations of the Results of SEM pictures:

The pictures shown in figure (8) represented the SEM images of CNWs starting from 1mm measurement to 5µm measurement. The images confirmed the cylindrical shape of the CNWs with multiple agglomeration areas, due to the hydrogen bonding attraction between multiple hydroxyl groups those had not been hydrolyzed by acidic hydrolysis.

5- Interpretations of the Results of X-RD Spectra:

The XRD pattern showed in figure (9) represents the crystalline structure of the prepared nanoparticle .The results showed strong diffraction peaks at 2θ of (23.5°) indicates that the nanoparticles mainly consists of crystalline structure of the CNWs.

Table (5): Granularity Cumulating Distribution and Average Diameter of CNWs.

Avg. Diameter:84.25 nm	**<=10% Diameter:70.00 nm**
<=50% Diameter:80.00 nm	**<=90% Diameter:100.00 nm**

Diameter(nm)<	Volume(%)	Cumulation(%)	Diameter(nm)<	Volume(%)	Cumulation(%)	Diameter(nm)<	Volume(%)	Cumulation(%)
70.00	2.56	2.56	85.00	14.74	56.41	100.00	7.05	89.74
75.00	19.23	21.79	90.00	17.31	73.72	105.00	5.77	95.51
80.00	19.87	41.67	95.00	8.97	82.69	110.00	4.49	100.00

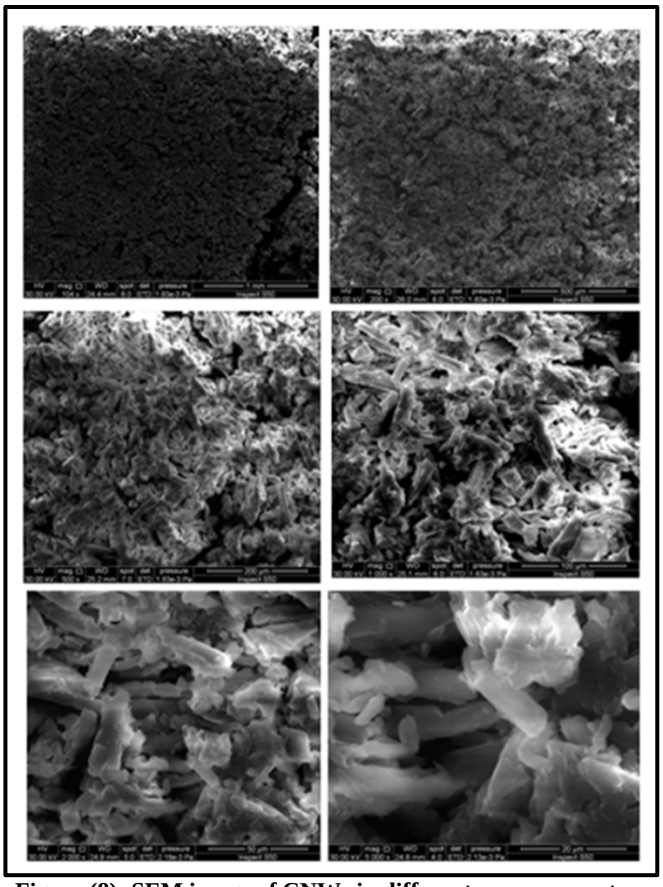


Figure (8): SEM image of CNWs in different measurements.

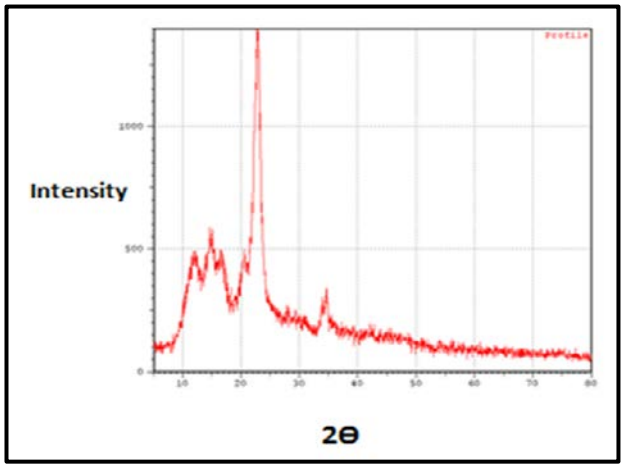


Figure (9): XRD pattern of CNWs.

CONCLUSION

1- The synthesis of the nano-sized carrier starting from commercial cotton has been successfully achieved, the size of the nanocarrier was successfully confirmed by AFM, SEM and XRD.
2- Characterization and identification of the synthesized compounds were confirmed by determination of physical properties, FT-IR spectroscopy and 1HNMRspectra.

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