Nanomed Res J 8(3):231-245, Summer 2023

RESEARCH ARTICLE

Gold and Silver Nanoparticles with Modified Chitosan /PVA : Synthesis, Study The Toxicity and Anticancer Activity

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ARTICLE INFO

Article History:
Received 15 Jan 2023
Accepted 27 Apr 2023
Published 01 May 2023

Keywords:
AuNPs
AgNPs
Nanocomposites
Molecular Docking
Toxicity
Modified Chitosan
Human Lung
Adenocarcinoma Cells

ABSTRACT

The new novel polymers nanocomposites based modified chitosan (CS) blending with polyvinyl alcohol (PVA) and coated gold or silver nanoparticles (AuNPs), (AgNPs) were synthesized from many sequence reactions as presented in (Scheme1, 2 and 3). By utilizing ^{1}H-NMR spectroscopy, FTIR, and Field Emission Scanning electron microscope , the synthesized compounds have been identified. Molecular docking is studied, where operations are used to predict the binding status of compounds with the enzyme and to calculate the free energy (ΔG) of the compounds prepared. Also, the antibacterial activity regarding the synthesized compounds against two resistant pathogenic bacteria (G+) *S. aureus* and *E. coli* (G-) was examined *in vitro* compare with standard antibiotic (Amoxicillin). The cytotoxic effect of novel polymers nanocomposites against Human Lung Adenocarcinoma Cells line (A549) using MTT assay was used to estimate and compare with normal cell line Rat Embryonic Fibroblasts (REF), the (modified chitosan/PVA/Au) exhibited very excellent inhibition rate. Finally, the Acute Toxicity Test of these nanocomposities were examined showed non-toxicity of these nanocomposities and histological examination of internal organs: liver, lung and kidney related to treated group showed no changes but similar those of control group.

How to cite this article
Abed Shlaka W., S. Saeed R. Gold and Silver Nanoparticles with Modified Chitosan /PVA : Synthesis , Study The Toxicity and Anticancer Activity. Nanomed Res J, 2023; 8(3): 231-245. DOI: 10.22034/nmrj.2023.03.002

INTRODUCTION

Chitosan (CS), is a natural polycationic linear polysaccharide that is derived from the chitin, is a copolymer made up of N-acetylglucosamine and glucosamine. Chitin is the second most abundantnatural polysaccharide after cellulose and serves as a structural element regarding the exoskeleton of crustaceans, insects (mostly crabs and shrimp), and fungi. Biocompatible, biodegradable, safe, non-toxic, and with biological properties including anticancer, antitumor, and antimicrobial properties, CS is employed as an anticoagulant in pharmaceutical products [2,3]. The presence of -OH and NH2 groups in CS's structure [4] makes it simple to modify into various derivatives and separates it from cellulose [5]. The reactivity of polymer depends on amino groups, which are responsible for fundamental behaviors and cationic properties [6]. New characteristics of the modified CS include solubility, biocompatibility, biological activity, and hydrophilicity. PVA, a synthetic polymer mostly made up of C-C bonds, is a semi-crystalline hydrophilic biodegradable polymer [9]. It is frequently used in the bio-medical disciplines due to its non-toxicity, water-solubility, bio-degradability, and bio-compatibility. Additionally, PVA has hydroxyl groups on its carbon backbone, which are a source of hydrogen groups. The formation of polymer composites is aided by these interactions between hydrogen bonding [10]. Blending of two polymers or more is a highly significant method for the improvement of commercial products' cost effectiveness [11, 12]. The CS films are brittle and unsuitable to be used in the dry state. CS could be potentially miscible with the PVA as a result of hydrogen bonds' formation [13,14]. In general, CS and PVA combination has advantageous effects on biological properties of blends. CS has been considered as a suitable

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capping agent for syntheses of the metal NPs as a result of amine as well as hydroxyl groups in its structure. Amine group has strong affinity toward the metal ions; as a result, it facilitates chitosan's binding to the metal [15]. One NP type which had lately gained much attention and in comparison, with other NPs, shows low level of toxicity is the nano-particulated gold [16,17]. The AuNPs had been majorly utilized in the applications of drug delivery, intra-cellular gene regulation, bio-imaging (as contrast agents), anticancer therapy (photo-thermal and photo-diagnostic therapy), and anti-inflammatory therapy because of their simple surface functionalization and chemical stability [18,19]. Additionally, recent research has shown that Au NPs have anti-microbial potential [20, 21]. Additionally, because they limit bacterial growth and have significant bactericidal activity, AgNPs have highly intriguing biological characteristics, ranging from broad-spectrum anti-bacterial actions to anti-viral and anti-cancer activities [22]. Therefore, this study attempted to synthesis Nanocomposities through blend of modified chitosan/ polyvinyl alcohol with gold (AuPNs) and Silver (AgNPs) to increase antibacterial activity and anti-cancer activity and decrease toxicity of nanocomposities.

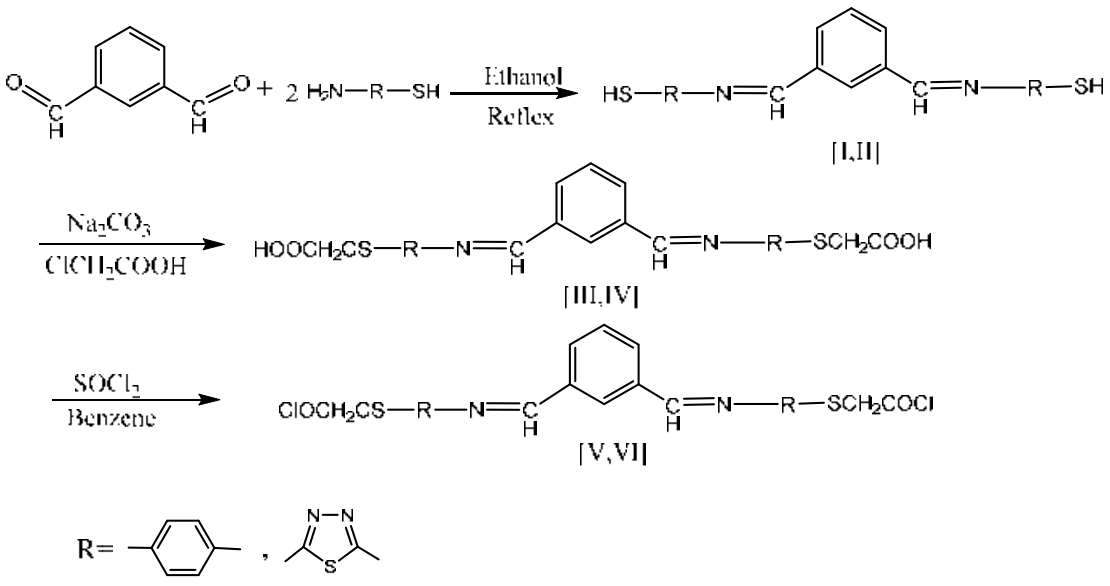
MATERIALS AND METHODS

Materials

Chitosan (M.Wt=100000), Polyvinyl alcohol ((M.Wt=72000) and Isophthalaldehyde, 4-amino benzene thiol have been provided from CDH, sodium carbonate and chloroacetic acid have been provided from BDH, liquid solvents were provided from SCR.

Instrumentation

^{1}H-NMR spectra were carried out by: Ultra Shield 500MHz, Bruker, University of Tehran, Iran. FT-IR Spectra were registered on Shimadzu FT-IR-8400 s, which range between 400 cm^{-1} and 4000cm^{-1}. With DMSO serving as the solvent, TMS was used as the internal standard. Iranian university of Tehran performed SEM. Molecular docking investigations for compounds have been carried out with the use of the fully licensed CCDC genetic optimization for ligand docking (GOLD)Hermes 2021.2.0 (Build 327809) for visualizing protein, hydrogen bonding interactions, ligands, brief contacts, and bonds length computation. At the Central Environmental Laboratory of the University of Baghdad's College of Science, biological activity was conducted. The Department of Molecular and Medical Biotechnology at AL-Nahrain University's Biotechnology Research Center conducted anticancer screening. In the lab of the Center for Cancer Research and Medical Genetics/Mustansiriyah Univ., study acute toxicity test. Histological Changes in the University of Baghdad's College of Veterinary Medicine, Histology Unit Laboratory, and Percussion Diseases



Scheme 1. Synthesis of compounds[I- VI]

Synthesis methods

In order to obtain newly nanocomposities, we illustrated scheme 1, 2 and 3:

Synthesis of compounds [I,II]

Isophthalaldehyde (1.34g, 0.01mol) mixed with (2.5g, 0.02mol) 4-amino benzene thiol or (2.66g,0.02mol) 2amino-5- mercapto -1,3,4-thiadiazole, 20mL of ethanol with two glacial acetic acid drops, refluxing at 70°C for 18h. The reaction mix has been cooled, yellow precipitate filtration and recrystallized from ethanol and dried to yield 85%.,93%**[23,24]**.

Synthesis of compounds [III, IV]

A liquot (0.01mol) one of compounds [I,II] mixed with (0.04 mol) Na_2CO_3 in distilled water of (15mL), after that (0.02mol) of $ClCH_2COOH$ has been added. Then, the solution refluxed for 6h., then added conc. Furthermore, hydrochloric acid to reached out PH= 2. Filtered the result and washed by water and re-crystallized from C_2H_5OH **[25]**.

Synthesis of compounds[V,VI]

(0.01mol) of one of compounds [III, IV] mixed with thionyl chloride(0.02mol) in dry benzene (15mL) was refluxed for8 h., excess thionyl chloride and benzene have been outlying under the vacuum. **[26]**

The strategy for the synthesis of the *O,N* chitosan derivatives [VII,VIII] molecules has been shown in Scheme 2.

Synthesis of O,N chitosan derivatives [VII,VIII]

0.5 gm of the chitosan has been marinated in 50mL of the chloroform and pyridine (1:1) for 10h., one of compounds[V,VI] was added to them under water-ice bath, this mix has been stirred at 100^{0}C for 14h., cooled and poured to (25 mL) methanol, after cooled at 4^{0}C, and after that it has been filtered. The precipitate has been rinsed by methanol and dried at 50^{0}C. **[27].**

The reaction sequence that leads to formation of new modified CS/ PVA/ Nanocomposites is outlined in Scheme3

Synthesis of Polymer Blend [IX, X]

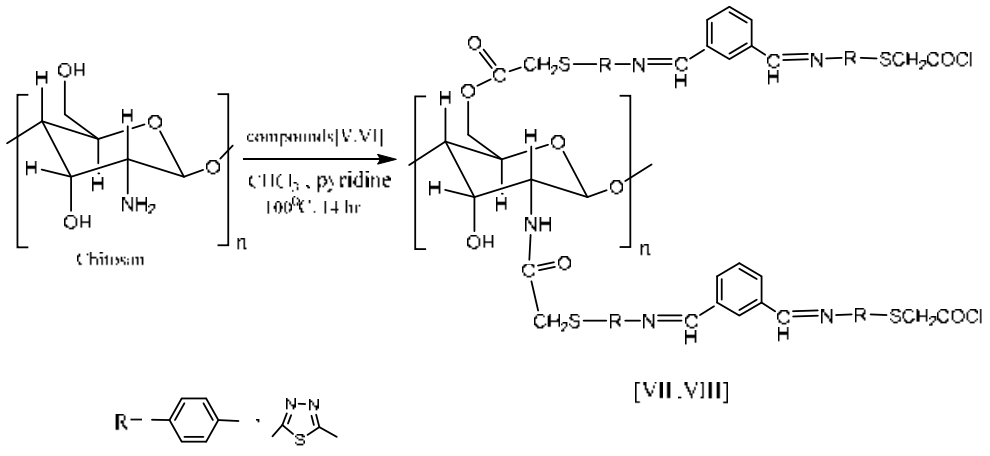
Solvent casting was used to create the polymer blend. The modified CS [VII, VIII] was dissolved in 2% aqueous acetic acid solution with stirring at room temperature to create the solutions regarding modified CS [VII, VIII]. In order to create 5 wt% solutions of polymer, PVA was dissolved in hot water (40 °C). After mixing the two polymer solutions, a homogeneous solution was created using a hot-plate stirrer for 60 minutes. The grafted Cs/PVA blends were created by combining modified Cs: PVA (5:5) in one ratio. **[28]**

Synthesis of Modified CS/ PVA /Nanocomposites [XI-XIV]

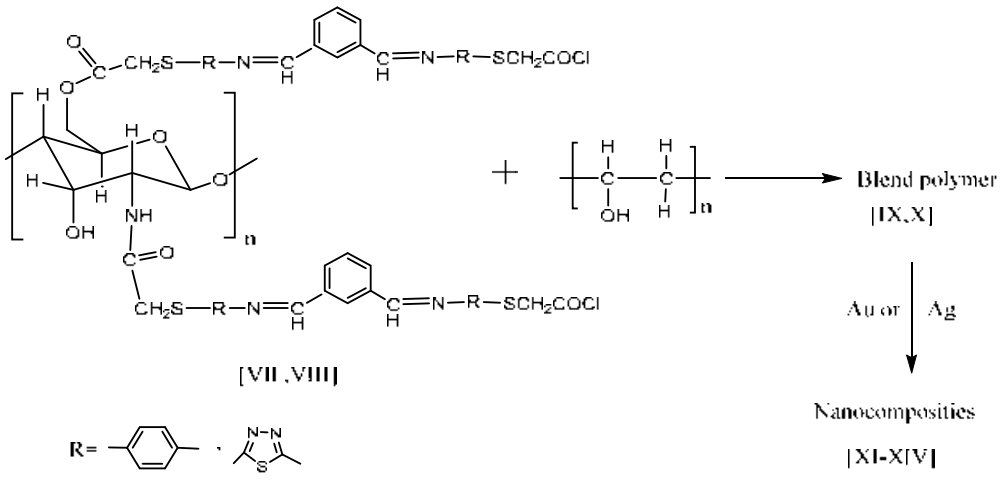
With the use of hotplate stirrer for three hours, 100 mg of dried Modified CS/PVA [IX, X] was dissolved in 50 mL of a 250 mg/L solution of AgNPs or AuNPs to bond the Ag and Au nanometal in the blend matrix. **[29]**

RESULTS AND DISCUSSION

The synthesis of new derivatives starting from bis Schiff bases is evinced in Scheme (1). Compounds [I,II] were synthesized through the



Scheme 2. Synthesis of modified chitosan [VII,VIII]



Scheme 3. Synthesis of blend polymer and nanocomposites [XI-XIV]

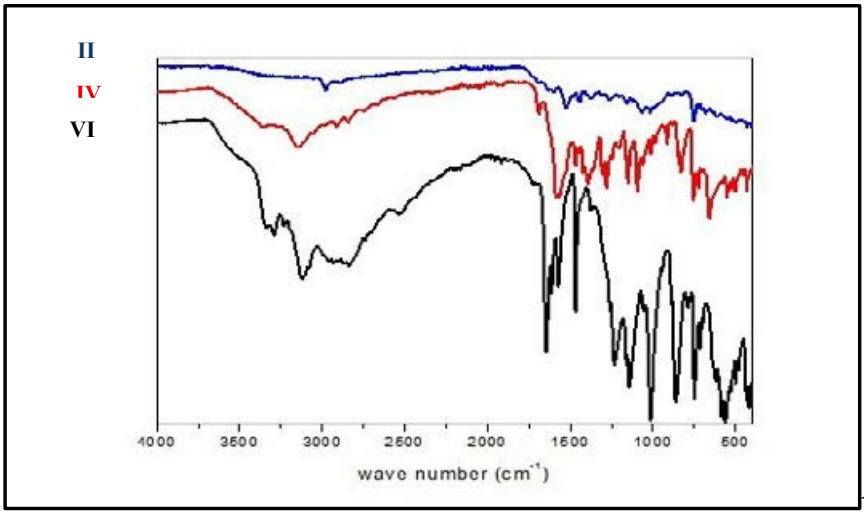


Fig. 1. FT-IR of [II], [IV] and [VI]

Table 1. FT-IR spectroscopy data of compounds [I-VI]

Comp. No.	(S-H) cm^{-1}	(O-H)cm^{-1}	=C-H arom.	(C=O) carboxlic.	(C=O)-Cl	(C=N)	(C=N) of thiadiazole	(C-S)	(C=C)
[I]	2541	-	3061	-	-	1626	-	696	1589
[II]	2540	-	3081	-	-	1647	1635	670	1586
[III]	-	3400-2400	3058	1996	-	1620	-	680	1595
[IV]	-	3400-2400	3050	1693	-	1635	1607	672	1559
[V]	-	-	3044	-	1732	1633	-	681	1601
[VI]	-	-	3044	-	1709	1635	1626	664	1572

reaction of 4-amino benzene thiol or 2-amino-5-mercapto-1,3,4- thiadiazole with isophthalaldehyde in ethanol under reflux for 18h. FTIR of compound [II] had shown appearance bands at (2540), (1647,1635) and (1532,1586) cm^{-1} because of the SH group, (C=N) exocyclic and (C=N) endocyclic, respectively. Compounds [III,IV] were prepared in basic media, by the reaction compounds [I,II] with chloroacetic acid in distilled water. FTIR spectrum of compound **[IV]** establishes a bands at 3400-

2400cm^{-1} for hydroxyl group and 1693cm^{-1} for carboxylic group. ^{1}H-NMR had exhibited a broad singlet signal with a chemical shift at δ 13.17 ppm as a result of the two protons of carboxylic protons, additional signal at δ10.18 ppm due to the presence of two protons for CH-N, multiple peaks appeared at δ(7.11-8.69)ppm for aromatic protons and singlet signal at δ3.70 for four protons for S-CH_2. The condensation reaction of synthesized compounds[III,IV] with $SOCl_2$ using a dry benzene as solvent resulted derivatives [V,VI] with a good yield. FTIR of the compound **[VI],** interpreted disappear the band at (3400-2400)cm^{-1} as a result of (OH) group of the carboxylic acid and appearance of the band at 1709cm^{-1} due to the acyl chloride.

O,N- chitosan derivatives [VII,VIII]were prepared through the reaction between[V,VI] with the chitosan in the pyridine and tri-chloromethane as solvent. FT-IR of the polymer **[VIII]** had illustrated a large peak at 3363cm^{-1} is related to N-H and O-H stretching from intra-and intermolecular hydrogen bonding of the molecules of the chitosan and appearance of new band at 1735, 1684cm^{-1} as a result of carbonyl group of the ester and amide groups, respectively. ^{1}HNMR spectrum of polymer [VII] elucidated singlet signal with a chemical shift region at δ 8.99 ppm due to the presence of protons of NHC=O group, singlet signals at δ (8.24-8.60)ppm for OH groups of chitosan, a multiple signals at δ(7.19ppm-7.96ppm) for aromatic protons, a sharp signal at δ7.88ppm for proton CH-N, singlet signal at δ(5.31-5.94)ppm for eight proton of SCH_2 groups, Also,the characteristic region at δ (0.82-1.21ppm) corresponded to the non-anomeric proton (H-1, H-3, H-4, H-5and H-6) of CS. ^{1}HNMR spectrum of compound [VIII] elucidated singlet signal with a chemical shift region at δ 10.11 ppm due to the presence of protons of NHC=O group, singlet

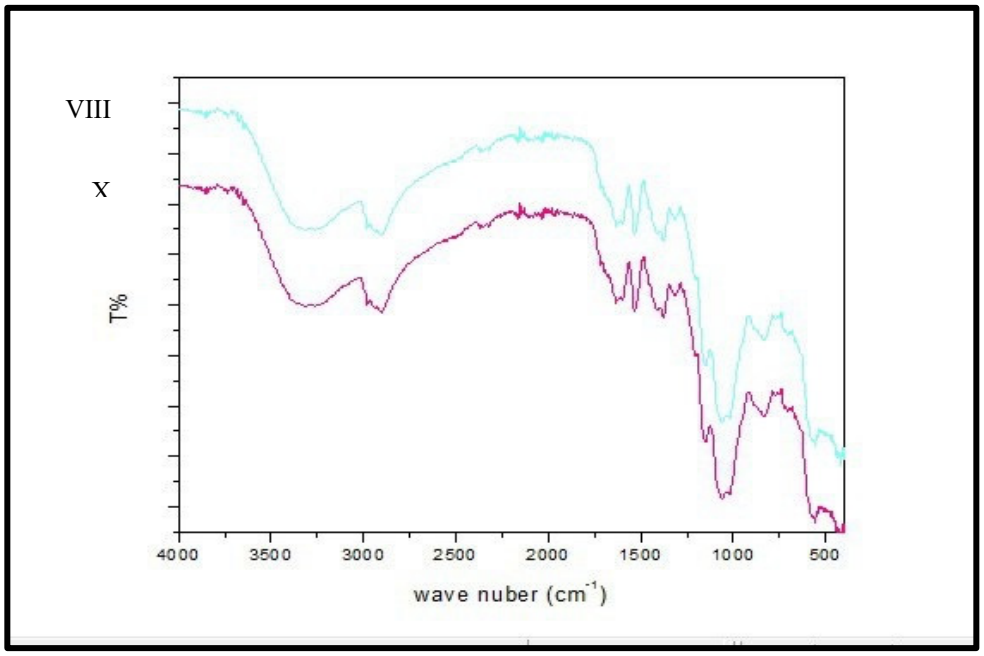


Fig. 2. FT-IR of [VIII] and [X]

Table 2. FT-IR data of polymers[VII- X]

Com. No.	υ (O-H) and (N-H)	υ (C-H) aliph. cm^{-1}	υ (C=O) ester. cm^{-1}	υ (C=O) amide cm^{-1}	υ (C=C) cm^{-1}	υ (-CH_2-O-CO) cm^{-1}	υ (C-O-C) cm^{-1}
[VII]	3363	2914-2844	1733	1683	1567	1280	1015
[VIII]	3266	2938-2906	1735	1684	1598	1273	1020
[IX]	3272	2938-2919	1733	1647	1600	1243	1067
[X]	3282	2934-2911	1715	1646	1590	1280	1067

signals at δ (8.86-9.99)ppm for OH groups of chitosan, a multiple signals at δ(7.09-7.98ppm) for aromatic protons, a sharp signal at δ7.88 ppm for proton CH-N,singlet signal at (δ5.45-5.54)ppm for eight proton of SCH_2 groups and the characteristic region at δ (1.02-1.89)ppm corresponded to non-anomeric proton (H-1, H-3, H-4, H-5and H-6) of CS **[30].** *O,N*-Chitosan derivative (Modified chitosan) Blended with PVA to prepared blend polymer research of the properties of the obtained blends have exhibited a good miscibility level between PVA and CS which had been shown by the results of the FT-IR of compound **[X],** band broadening in 2400cm^{-1} -3600cm^{-1} range due to strong inter-molecular hydrogen bonding which exists between (NH_2) of CS and (OH) of the PVA, 1636cm^{-1} due to (C=N) and 1715cm^{-1}which means C=O ester group**[31]** and for other polymers, band values have been listed in Table2.

Field Emission Scanning electron microscope studies (FESEM)

For Modified CS [VII] Fig. (3), Modified CS blend with PVA [IX] Fig. (4), and Modified CS/ PVA/AuNPs[XI] Fig. 5, the surface morphology varies. The surface topography regarding the composite membrane is changed by the addition of PVA, which significantly affects cell spreading. The homogeneous distribution of AuNPs on the matrix surface was observed in the FESEM micrograph used to detect their existence. The Modified CS [VII] particles range in size from (72-111) nm on average. When AuNPs are present, the average particle size is between 1 and 1.4 nm, whereas the average particle size of modified CS blend with PVA [IX] is ranged between (40-45) nm for presence of PVA. It was observed that the AuNPs are with homogenous distributions on the surface of the matrix. Nanocomposite

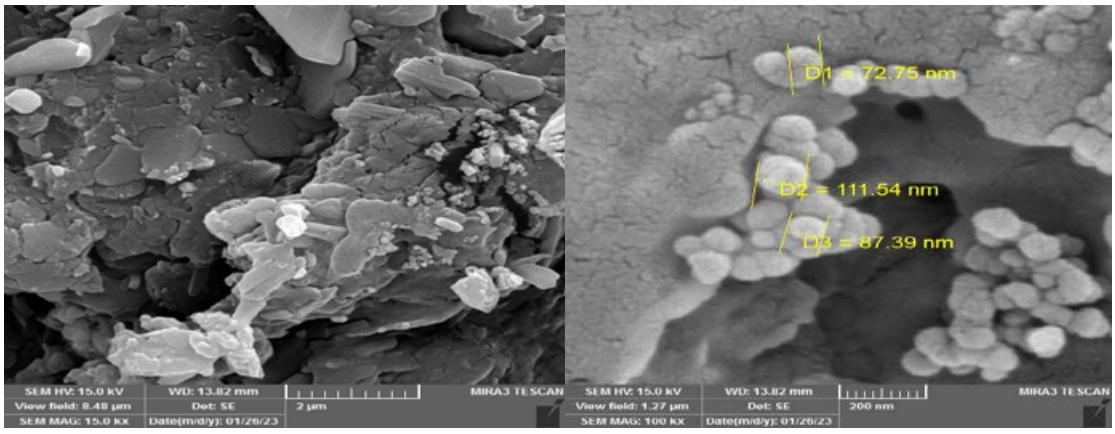


Fig. 3. FESEM of modified chitosan[VII]

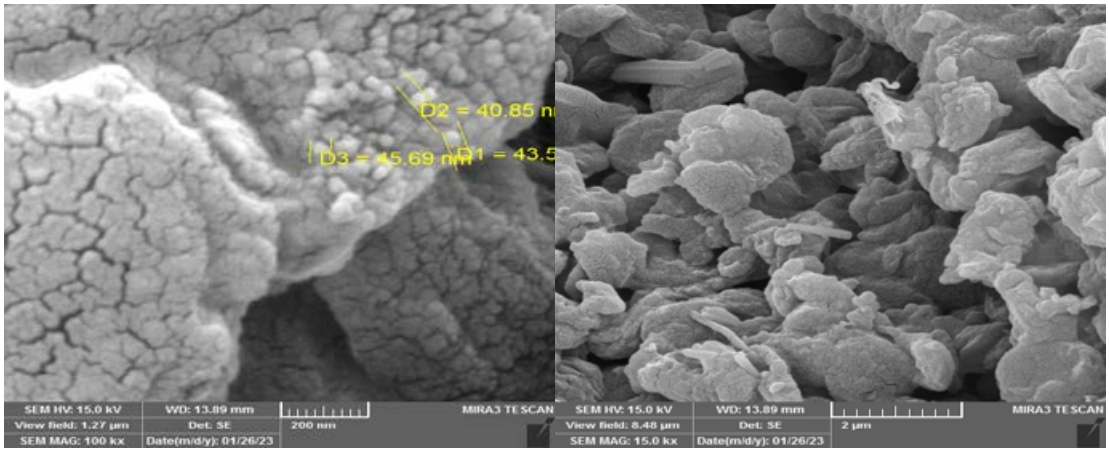


Fig. 4. FESEM of modified chitosan/PVA [IX]

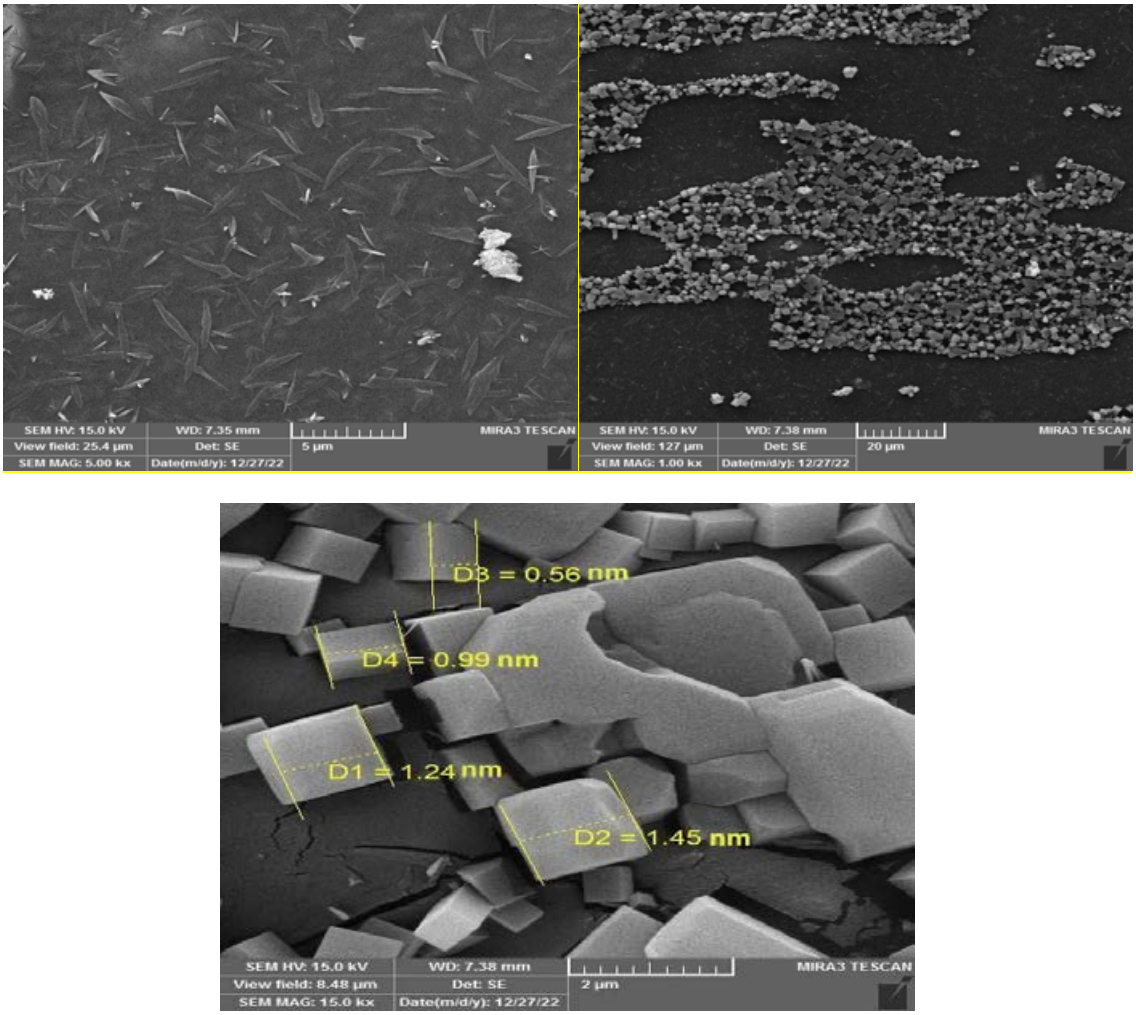


Fig. 5. FESEM of Nanocomposities (modified chitosan/PVA/Au) [XI]

film's particles were discovered to have a nearly spherical shape. Yet, certain NP agglomerations have been present in figures, and surface has been rather rough [32,33]. In CS/PVA, homogenous AuNPs distribution and robust NP attachment to the polymer were observed. Human cells did not exhibit any detectable cytotoxic effects when exposed to CS/PVA/Au in the size range of 1-1.4 nm. Another potential explanation for diminished toxic effects or possibly complete toxicity exclusion is the polymeric coating on the NP surface.

Molecular docking Study

For predicting the binding status regarding compounds with the enzyme, compute the free energy (ΔG) of compounds generated with the enzyme (Dihydrofolate reductase (DHFR), and examine molecular similarity, operations are utilized in compound [VI]. 2w9h: DHFR is the enzyme that converts 5,6-di-hydrofolate to 5,6,7,8-tetra-hydrofolate in a NADPH-dependent manner. Tetrahydrofolate represents a crucial cofactor in syntheses of purines, thymidylate, methionine, as well as other important metabolites. The DHFR was the focus of numerous studies that used antimicrobial, anti-cancer, and anti-bacterial drugs to target the enzyme because of its importance in numerous cellular processes. Methotrexate, which is used to treat cancer, and di-aminopyrimidines (DAPs), such as tri-methoprim (TMP), which are used to treat bacterial infections, are two drugs that are clinically utilized and target DHFR. Over three decades of clinical use of DHFR DAP inhibitors led to a high rate of resistance to

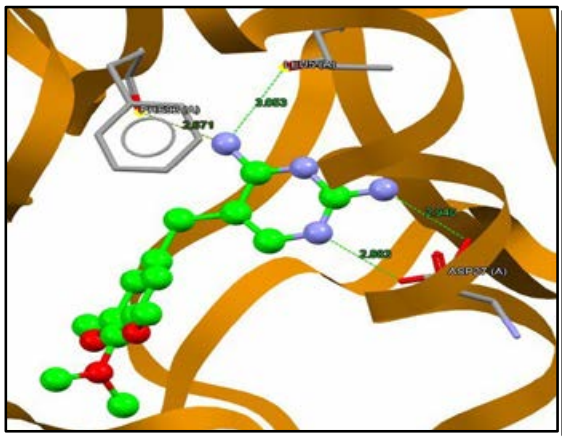


Fig. 6. Molecular docking of DHFR

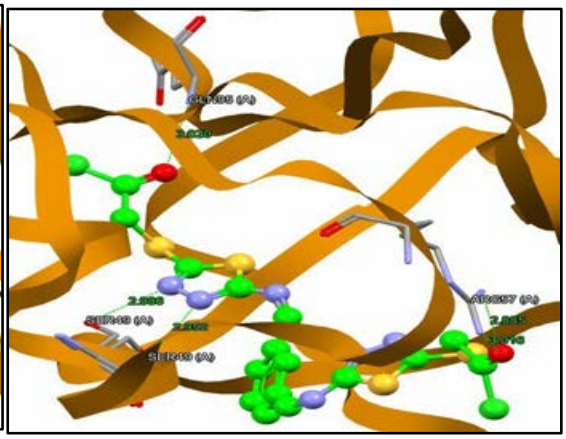


Fig. 7. Molecular docking of [VI]

Table 3. Molecular docking of compound[VI]

Docking study					
Compounds	*Binding Energy (PLP Fitness) Kcal/Mol*	*No. of Amino Acids Included in H-bonding*	*Amino Acids Included in H-bonding*	*no. of bonding*	*power of bonding*
2W9H	96.17	4	LEU 5	1	3.053
			PHE 92	1	2.671
			ASP 27	2	2.940 2.883
			GLN 95	1	3.020
[VI]	98.44	5	SER 49	2	2.986 2.952
			AGR 57	2	3.016 2.885

such drugs. Methicillin-resistant Staphylococcus aureus (MRSA), the cause of several serious community-acquired and nosocomial infections, as well as other gram-positive organisms, may develop resistance to the DAPs through chromosomal gene mutation or acquisition of another DHFR known as the "S1 DHFR." It is crucial to comprehend the molecular causes of DAP resistance in order to create novel treatments for health risks like MRSA. In this investigation, we will present crystal structure of S. aureus wild-type chromosomal DHFR in association with the NADPH and TMP. We discovered the structures of TMP-resistant S-1 DHFR, apo, and exogenous structures. Thermodynamic and structural studies had indicated important molecular variations between those two enzymes, and this had led to a marked decrease in DAP's affinity for the S-1 DHFR. Due to these variations in the binding affinity of the, S. aureus strains expressing the S-1

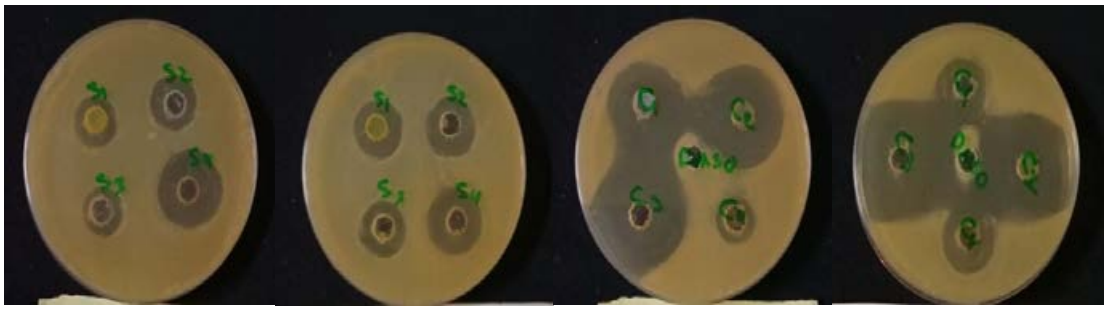


Fig. 8. Antibacterial activities of some polymers and nanocomposites

Table 4. Anti-bacterial screening data of some synthesized polymers and nanocomposites

Comp.No.	*E .coli*	*S. aureus*
Amoxicillin	17	23
[VII]	14	15
[VIII]	17	17
[IX]	23	14
[X]	18	26
[XI]	38	37
[XII]	37	35
[XIII]	22	32
[XIV]	28	23

DHFR have exhibited less antibacterial activity. The produced chemical [VI] demonstrated higher efficacy than the comparator compound. [34]

Antibacterial Activity

The modified CS, modified CS/PVA, and modified CS/PVA containing AuNPs or AgNPs have all been investigated for their antibacterial properties against the pathogenic bacteria E. coli (G-) and S. aureus (G+). The findings of the antibacterial activity are shown in Table 4 and Fig. 8, where the ternary mix of modified CS and PVA with Au or Ag nanoparticles showed good antibacterial activity comparable to that of the antibiotic amoxicillin. Due to their excellent cell affinity and precise delivery at the infection site, the AuNPs have demonstrated very good antibacterial activities against E. coli through the absorption of light and conversion of it into heat. This facilitates the damage and inhibition to the microbial pathogens. A good antibacterial property of Ag silver nanocomposite also lends itself to biological applications. Ag+, which is strongly bound to electron donor groups like nitrogen, oxygen, or sulfur in microbial cell walls, is what gives silver its antibacterial effect. The ability of AgNPs to adhere to and infiltrate through bacterial cell walls, as well as their creation of free radicals, which can harm cells and pierce their membranes, all have an adverse effect on bacteria [35-40].

Anticancer activity

Preparation of cell lines for cytotoxicity assay [41] with the use of the cultured cells (96wells) in micro titer plate. Absorbance has been measured at (620nm) on micro plate reader. Calculated cell growth inhibition rate granted to equations[42]:

$$\text{Inhibition rate} = \frac{mean\,of\,control - mean\,of\,treatment}{mean\,of\,control} \times 100$$

Furthermore, the anticancer activity of various concentrations of some polymers and nanocomposites was investigated against A549 (Human Lung Adenocarcinoma Cells) and Rat Embryonic Fibroblasts(REF) revealing a good activity, which had no effect on the growth of normal Rat Embryonic Fibroblasts. All polymer nanocomposites [XIII,XIV] exhibit good inhibition than the polymer blend [X]. It is necessary to additionally inspect know mechanism through which nano and heterocyclic unit act to give

Table 5. Rate Inhibition of blend polymer and nanocomposities

Comp.No.	Inhibition rate of Concentration12.5 µg mL^{-1}	Inhibition rate of Concentration25 µg Ml^{-1}	Inhibition rate of Concentration50 µg mL^{-1}	Inhibition rate of Concentration 100 µg mL^{-1}
M6=[X]	25.12	37.31	54.12	55.11
M2=[XIII]	91.65	92.65	92.75	93.46
M4=[XIV]	81.03	82.01	85.32	88.45

Table 6. IC50 of blend polymer and naocomposites

Compound	IC_{50} in µg/Ml	
	A549	REF
M6(polymer blend) = [X]	57.50	75.43
M2(M6+AuNPs) =[XIII]	29.96	74.17
M4(M6+AgNPs) = [XIV]	30.66	83.92

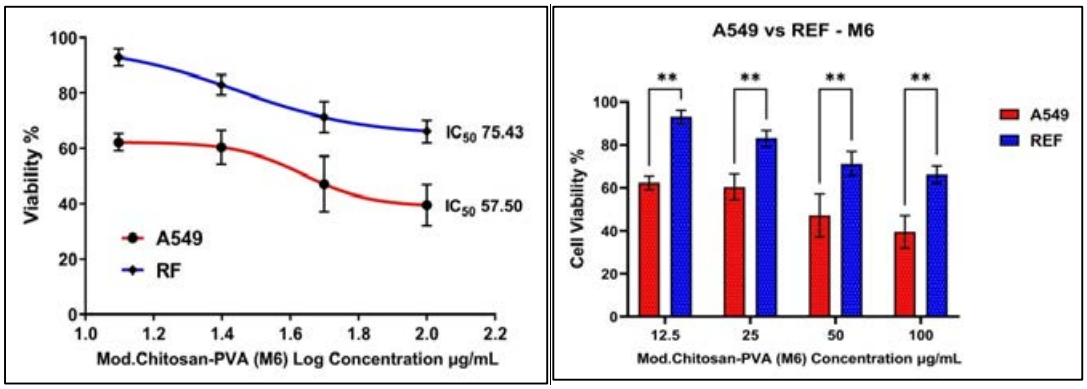


Fig. 9. IC50 and cell viability of M6=(modified chitosan /PVA)on A549 and compare with REF

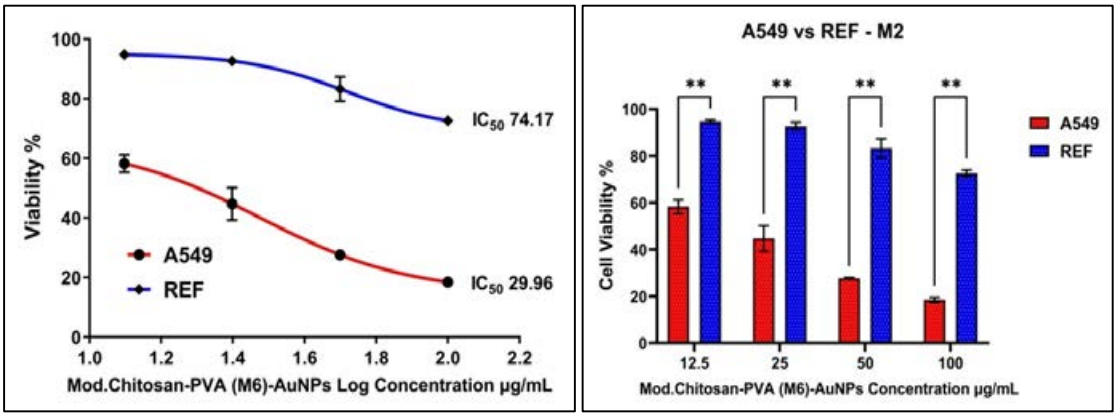


Fig10.IC50 and cell viability of M2= modified chitosan /PVA/AuNPs on A549and compare with REF

potent cytotoxic effect which might get chitosan derivatives an interest for being promise anti-cancer drugs. AuNPs could lead to the induction of thee cytotoxicity via ROS, generating damages to the cellular components via intra-cellular oxidative stress, thereby resulting in the increase of the

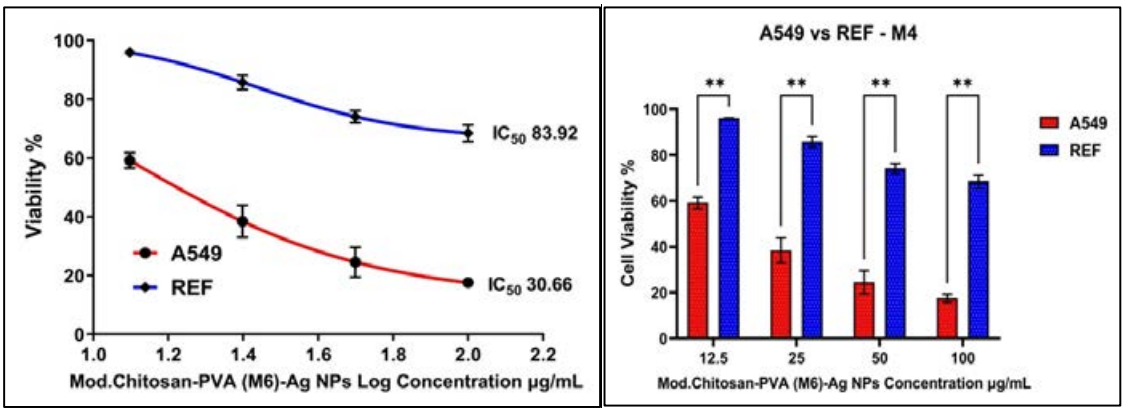
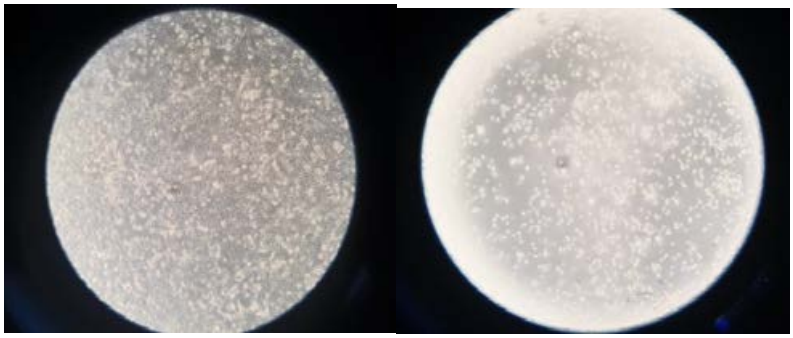
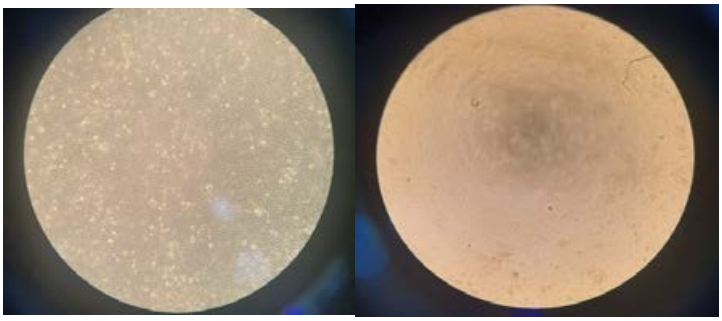


Fig.11. IC50 and cell viability of M4= modified chitosan /PVA /AgNPs on A549and compare with REF



A:Image of well for (A549) before Staining B:Image of well for (A549) after Staining



C:Image of well for (REF) before Staining D:Image of well for (REF) after Staining

E: Image of plate before Staining F: Image of plate after Staining

Fig 12. Image of mice during injection and through necropsy

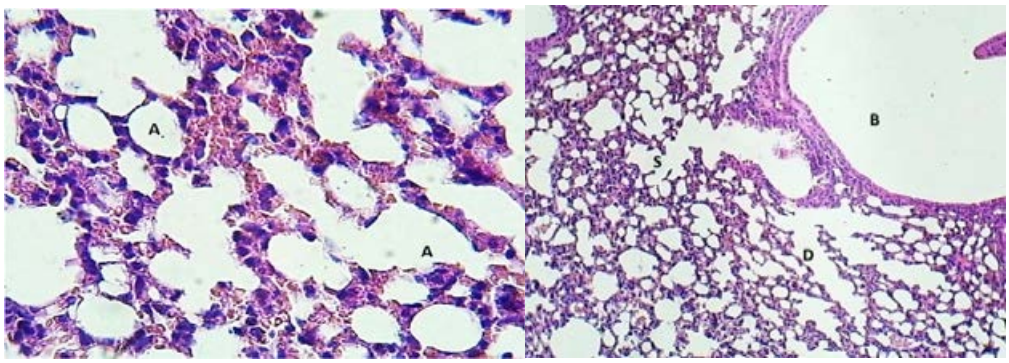


Fig. 13. section of lung (control) shows: normal alveoli (A).
(B), alveolar duct (D) & alveolar sac (S).H&E stain 40x H&E stain 400x.

generation of ROS and oxidation of the glutathione could be a basis of Au NPs' anti-cancer activities. Moreover, Ag NPs could increase ROS levels, to destroy cancer cells. Bio-synthesized metal NPs, especially silver and gold NPs as well as their conjugates with the bio-polymers have massive potentials in different areas of the science because of their broad range of the applications, which include the bio-medical applications[43,44].

Acute Toxicity Test

This study has been conducted in the laboratory of the Centre for Cancer Research and Medical Genetics to estimate the acute toxicity of some synthesized polymers nanocomposites [XI-XIV], using the Lorke-written method.

The study included (25) laboratory mice of the type Albino mice, three months old, average weights (22-26) gm and they were all males, these animals were placed in plastic cages with Metal lids covered with fine sawdust and supplied with water by plastic bottles, equipped with food. Mice have been fasted for 18h with free access to water and food before test. The compounds have been dissolved in the distilled water then treated through injection (5g/kg and 10g/kg). The treatment group and the control group were compared with doses of the injection and the study showed after 14 days: no mortality with 5g/kg and 10g/kg body weight doses, no contrasts in mice weight daily that have been measured between group control and treated groups, no modification in the behaviors of the

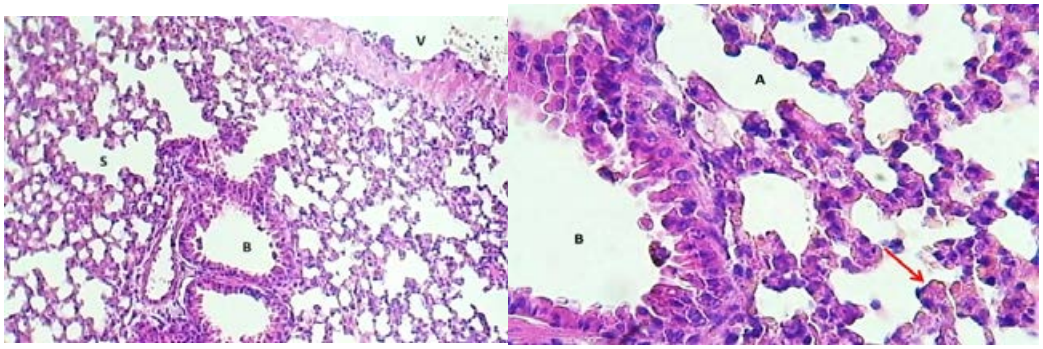


Fig.14. section of lung (nanocomposites) shows: normal bronchus (B), Fig.15:section of lung (nanocomposites) shows:normal, normal alveolus (A) sac (S), & septum (Arrow). H&E stain bronchus (B) normal alveolar sac (S), & pulmonary 100x vessel (V). H&E stain 100

mice has been carried out and no toxicity symptoms were reported. In addition to that, some of the mice have been sacrificed with cervical dislocation and liver, kidneys heart and lungs have been weighed. The visual evaluation of mice organs had shown normal appearance. These results indicated that polymer nanocomposites has low toxicity towards both investigated organisms[45,46]

Histological Study

Histological examination of liver, lung and kidney related to treated group showed no changes but similar those of control group.

CONCLUSION

The aim of the research was to create nanocomposities by a series of reactions that began by the formation schiff base. The antibacterial activity of most synthesized compounds were tested *in vitro*. The modified chitosan/PVA/Au exhibited very excellent antimicrobial activities comparable with Ampicillin as standard antibiotic,cancer cell line(A549) using MTT assay was used to estimate the cytotoxic effect of different concentrations of the created nanocomposities and compare with normal cell line (REF), the (modified chitosan/ PVA/Au) exhibited very excellent Inhibition rate. Finally, study Toxicity Test for these nanocomposities, where it showed non-toxicity of these nanocomposities, however, more studies are also needed.

ACKNOWLEDGEMEN

The research is part of the McS requirements for researcher Widad Abed Shlaka.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

AUTHORS' CONTRIBUTIONS

All authors contributed toward data analysis, drafting and revising the paper and agreed to be responsible for all the aspects of this work.

CONFLICT OF INTEREST

We have no conflicts of interest to disclose.

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