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RESEARCH ARTICLE

Modification and Study Biological Activity of Chitosan with Compounds Containing Azo Group

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ABSTRACT

In the present research synthesis and study of biological activity a series of new polymers modified of chitosan with compounds containing azo group. Beginning diazonium salt produced from 3,3'-dimethyl-[1,1'-biphenyl]-4,4'-diamine reacted with concentrated HCl acid and sodium nitrite. The coupling reaction between diazonium salt with substituted aromatic aldehyde to produce Azo derivatives (1-6). Azo Schiff bases Chitosan (7-12) were synthesized by condensation of Chitosan with Azo derivatives (1-6) in ethanol with some drops of glacial acetic acid. The structural modifications of Chitosan ring (linked to a bioactive azo moiety) were expected to give new derivatives (7-12) with a diverse range of biological functions. These compounds' structures have been determined using FT-IR, ¹H-NMR spectroscopic and Field Emission Scanning Electron Microscopy studies. Additionally, two other kinds of bacteria: *Staphylococcus aureus* and *E. coli* were tested for possible antibacterial properties utilizing some new compounds. Modified Chitosan (7-10) showed high activity comparable to a penicillin (used as the reference antibiotic), Especially the modified polymer(7), which showed high inhibition against both types of bacteria. The anticancer activity of modified chitosan (7) against MCF-7 (human breast carcinoma cells) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to determine and compare with normal cells WRL-68(the human hepatic cell line). Polymer (7) exhibited a high cancer cell inhibition rate and less toxicity to normal cells.

Keywords: Azo compounds, Chitosan, Diazonium salt, FESEM study, MCF-7, Schiff bases**Introduction**

Azo compounds are very important organic compounds having a wide spectrum of biological activities. Diazonium coupling reactions are typical electrophilic aromatic substitutions in which the positively charged diazonium ion in the electrophile interact with the electron-rich ring of substituted benzaldehyde.^{1,2}

Schiff bases, having imine groups (C=N) are formed by nucleophile addition (condensation reaction of (NH₂) primary amines with carbonyl groups (C=O) of aldehydes or ketones. These compounds have been used for industrial purposes such as pigments, catalysts, liquid–liquid extraction, active

transport, intermediates in organic synthesis and as polymer stabilizers.^{3–6} Azo compounds, such as the azobisisobutyronitrile (AIBN) may be utilized as radical initiators in the polymerization of the alkenes for making plastics.⁷ The aromatic azo compounds have been utilized as acid-base indicators like themethyl orange, methyl red and Congo red.⁸ Chitosan is a natural biopolymer generated from the parent substance chitin, It is a semi-crystalline, linear, nontoxic, biocompatible, biodegradable, odorless, safe and antibacterial polymer.^{9,10} Due to its ability to produce bio functional materials, it has a wide range of biological activities including anti-diabetic, anti-oxidant, anti-bacterial activities, antimicrobial, antitumor and is used

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Table 1. Physical properties of compounds (1-6).

Com. No.	Nomenclature	Structural formula	M.P. °C	Yield%	Colour
1	3,3'-(3,3'-dimethyl biphenyl-4,4'-diyl) bis(diazene-2,1-diyl) bis(4-(dimethylamino) benzaldehyde)		63-65	79	Orange
2	5,5'-(3,3'-dimethyl biphenyl-4,4'-diyl) bis(diazene-2,1-diyl) bis(2-chloro-benzaldehyde)		265-267	76	Red Brown
3	3,3'-(3,3'-dimethyl-biphenyl-4,4'-diyl) bis (diazene-2,1-diyl)bis(4-hydroxy-benzaldehyde)		273-275	87	Pale red
4	3,3'-(3,3'-dimethyl biphenyl-4,4'-diyl)bis (diazene-2,1-diyl)bis(5-chloro-2-hydroxy benzaldehyde)		288-290	89	Brown
5	5,5'-(3,3'-dimethyl biphenyl-4,4'-diyl)bis (diazene-2,1-diyl)bis(2-hydroxy-3-methoxy benzaldehyde)		> 300	90	Red Brown
6	3,3'-(3,3'-dimethyl biphenyl-4,4'-diyl)bis (diazene-2,1-diyl)bis(4-ethoxy-benzaldehyde)		> 300	76	Dark orange

pharmaceutically as an anticoagulant agent.¹¹⁻¹³ It can also be helpful in several fields including textiles, environmental protection, water treatment, cosmetics and biotechnology. Due to the presence of -OH and NH₂ groups, which separate chitosan from cellulose, its structure is easily changed to produce various derivatives.¹⁴⁻¹⁶ The modified chitosan exhibits new properties like biological activity and biocompatibility.¹⁷⁻¹⁹ Because of all the above datum facts, This study is aimed at the achievement of the syntheses, characterization and study of the biological activities of some synthesized new polymers modified of chitosan based to Schiff bases containing azo groups, whose molecules include the three moieties, chitosan, imine group (-N=CH-) and azo groups together,²⁰ since the combination of all these biologically active moieties in one molecule may increase the likelihood of producing more potent newly-developed prodrugs with a wide range of diverse biological activities (antibacterial and anticancer), as an attempt to correlate

the biological results with their structural characteristics.

Materials and methods

All chemicals have been supplied from CDH, SCR and BDH. The FT-IR Spectra have been registered on Shimadzu FT-IR-8400 s, ranging between 400-4000 cm⁻¹, using the potassium bromide disk. Company: Ultra Shield 400 MHz, Bruker, University of Basrah, Iraq performed the ¹H-NMR spectra. With DMSO serving as the solvent, TMS has been used as the internal standard. Iranian University of Tehran performed FESEM. 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.

Table 2. FT-IR spectroscopy data of compounds (1-6).

Com. No.	(C-H) arom cm^{-1}	(C-H) aliph cm^{-1}	(C-H) aldehyde cm^{-1}	(C=O) cm^{-1}	(N=N) cm^{-1}	(C=C) cm^{-1}
(1)	3024	2912,2883	2819,2731	1662	1550,1535	1597,1575
(2)	3086	2912,2890	2800,2750	1674	1566,1530	1585,1570
(3)	3024	2954,2899	2810,2762	1647	1559,1539	1600,1580
(4)	3051	2908,2885	2828,2798	1664	1552,1533	1600,1580
(5)	3050	2920,2886	2822,2738	1681	1573,1537	1600,1565
(6)	3024	2920,2892	2870,2762	1660	1560,1525	1600,1575

Table 3. FT-IR data of modified chitosan (7-12).

Com. No.	(O-H) and (N-H) cm^{-1}	(C-H) aliph. cm^{-1}	(C=N) cm^{-1}	(C=C) cm^{-1}	(N=N) cm^{-1}
(7)	3402	2916, 2890	1648	1600,1589	1523
(8)	3410	2924,2887	1630	1604,1578	1550
(9)	3448	2943,2880	1641	1584,1500	1564
(10)	3411	2910,2895	1630	1597,1500	1556
(11)	3421	2924,2893	1635	1600,1587	1558
(12)	3414	2947,2896	1620	1602,1580	1554

Synthesis of Azo compounds (1-6)

3,3'-Dimethylbiphenyl-4,4'-diamine (2.12 g., 0.01 mole) has been dissolved in 2 mL of the 2N hydrochloric acid and 20 mL of the distilled water. This solution has been cold at 0–3°C in an ice-water bath. Sodium nitrite (1.38 g., 0.02 mole) has been dissolved in 10 mL of the distilled water and added dropwise to a cold solution while stirring. The mixture diazonium solution (is stable for a few minutes. The above cold diazonium solution was added slowly to a well stirred solution to 0.02 mole from different substituted benzaldehydes: (2.98 g.) of N,N-dimethylbenzaldehyde, or (2.81 g.) of 2-chlorobenzaldehyde, or (2.44 gm) of 4-hydroxybenzaldehyde, or (3.13 g.) of 5-chloro-2-hydroxybenzaldehyde, or (3.04 g.) of 2-hydroxy-3-methoxybenzaldehyde or (3 g.) of 4-Ethoxybenzaldehyde) in 20 mL of absolute ethanol and 5 mL of 10 % sodium hydroxide and the mixture was cooled to a temperature of 0–5°C with stirring for 2 hrs in order to obtain coupling agent. The progress of the reaction was monitored by TLC. When the reaction was completed, the orange to red compound result was precipitated, then filtered and recrystallized from absolute ethanol.^{21,22} Physical properties of compounds(1-6) are listed in Table 1.

Synthesis of Azo Schiff bases chitosan (7-12)

A mixture of ethanol (10 mL) and glacial acetic acid (5 mL) was added to chitosan (0.5 g.), which was then dissolved and stirred for 30 minutes at room temperature, then add one of azo derivatives(1-6) (0.01 mol). The mixture was heated by stirring for 24 hours in a water bath at a 60°C. The reaction mixture was cooled, and the residue produced

was filtered, washed with EtOH, dried at room temperature for 24 hours.²³

Biological activity

Antibacterial activity

Some of the synthesized compounds and modified polymers have been screened for antibacterial activities against (*Staphylococcus* and *Escherichia coli*) using cup-plate agar diffusion method.²⁴ Penicillin (50 $\mu\text{g/ml}$) was used as a standard drug for antibacterial activity. These sterilized agar media were poured into petri dishes and allowed to solidify. Some of the synthesized compounds (50 $\mu\text{g/ml}$) were placed serially in the cavities with the help of a micropipette and allowed to diffuse for 1 hr. DMSO was used as a solvent for all the compounds and as a control. These plates were incubated at 37°C for 24 hr for antibacterial activities. The zone of inhibition observed around the cups after respective incubation was measured in mm.

Anticancer activity^{25,26}

The cytotoxic effect of modified chitosan (7) against MCF-7 (human breast carcinoma cells) was studied and compared with normal cell line WRL-68(the human hepatic cell line). The anti-proliferative activity of modified polymer (7) was tested by studying their ability to inhibit the proliferation of human breast carcinoma cells (MCF-7). The MTT test was used in 96-well plates to investigate the cytotoxic impact of polymer (7). Cells were treated with polymer (7) after 24 hours or when a confluent monolayer was established. After 24 hours of treatment, cell viability was determined by removing the medium, $\mu\text{l/well}$ solutions of MTT and incubating for 4 hr. at 37°C. The crystals in the wells were solubilized after

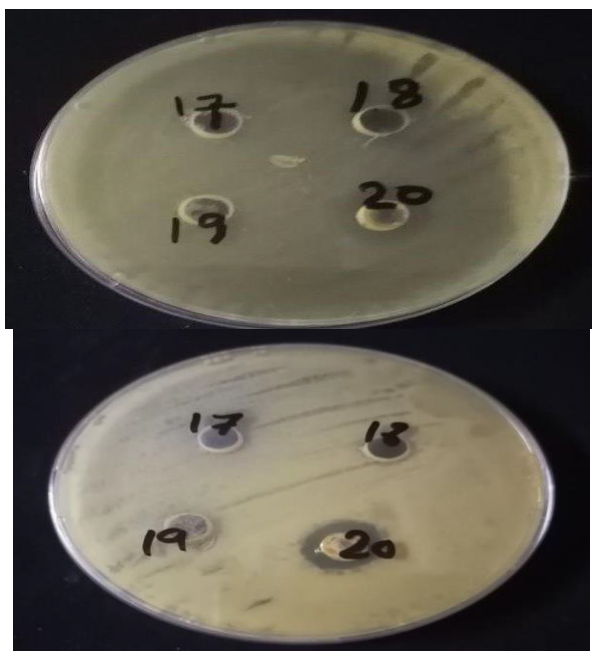


Fig. 1. Antibacterial activities of azo compounds (1-4).

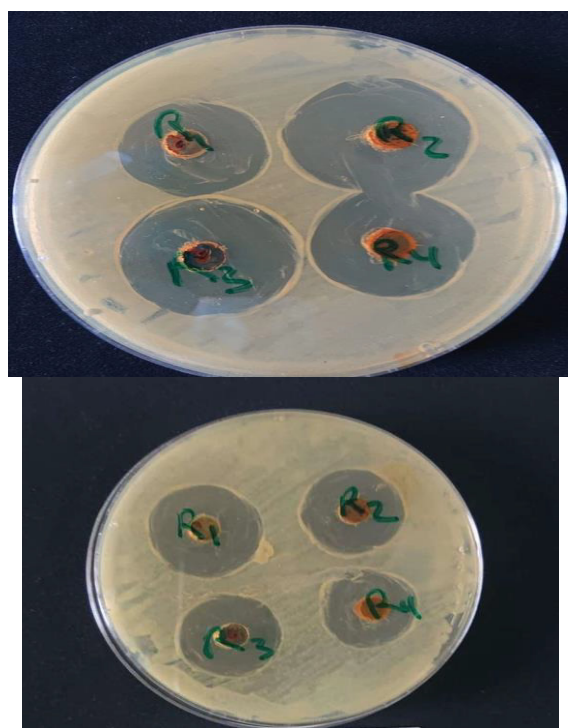


Fig. 2. Antibacterial activities of modified chitosan (7-10).

the MTT solution was removed by adding 200 mL of DMSO (Dimethyl Sulphoxide) and incubated at 37°C for 15 minutes while shaking with the use of a microplate reader, the absorbency was determined at 620 nm.

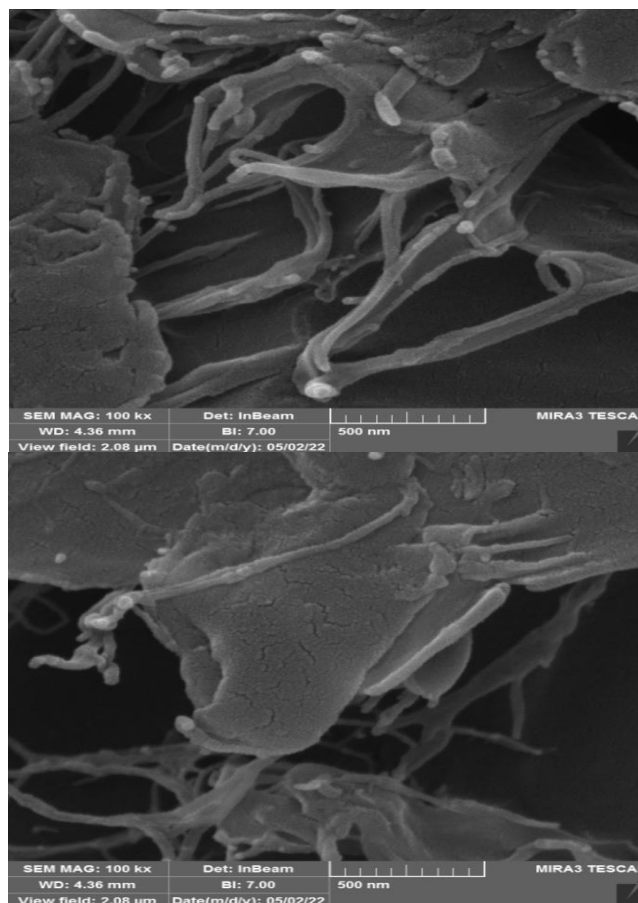


Fig. 3. FESEM of modified chitosan (7).

Results and discussion

Azo aldehyde derivatives(1-6), [Scheme 1](#) were obtained by coupling reaction between diazonium salt with two moles of substituted aldehydes. The new compounds (1-6) were identified by FTIR and ¹HNMR spectroscopy obtained for elegant compounds are listed in [Tables 2](#) and [4](#) respectively.

Azo Schiff bases chitosan (7-12) were produced from chitosan reaction with azo derivatives (1-6). The novel polymers modified of chitosan [Scheme 2](#), were identified by FTIR and ¹HNMR spectroscopy.²³ The FT-IR spectral data are given in [Table 3](#). ¹H-NMR spectra are listed in [Table 4](#).

These findings provide good support for the formation of the structure for polymers of chitosan.

Antibacterial activity²⁷⁻²⁹

Anti-bacterial activities of some of the synthesized compounds and modified chitosan (azo schiff bases chitosan) were observed (*in vitro*) against *E. coli* (G⁻) and (G⁺) *Staphylococcus aureus*, based upon agar diffusion approach. A standard drug (50 μg/mL of

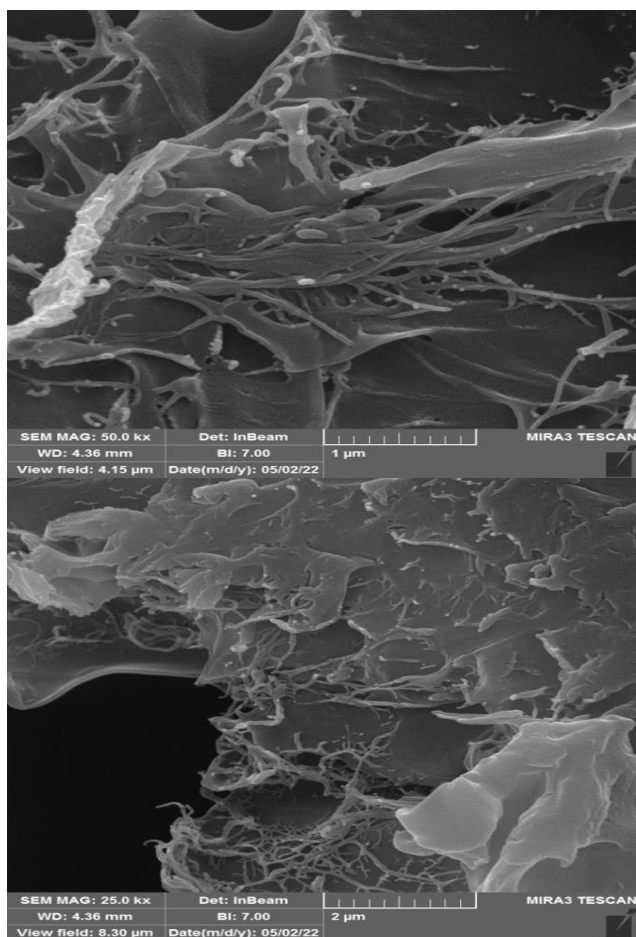


Fig. 4. FESEM of modified chitosan (12).

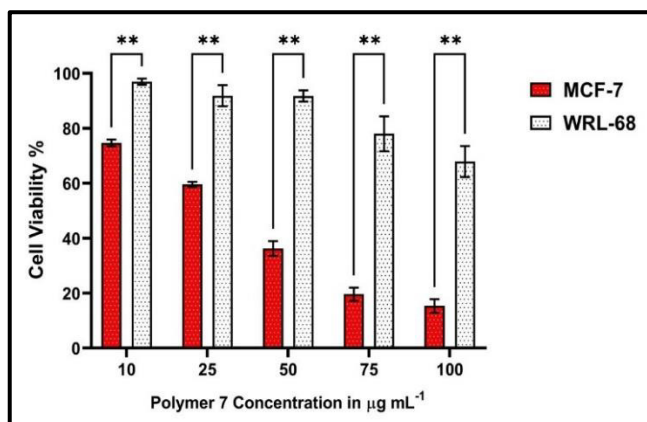


Fig. 5. Cell viability of polymer (7) on MCF-7 and compare with WRL-68.

Penicillin) has been utilized for comparison with synthesized azo compounds and modified chitosan. The results showed that modified chitosan (7-10) has a higher effectiveness than azo compounds (1-4). The reason for the increased effectiveness is

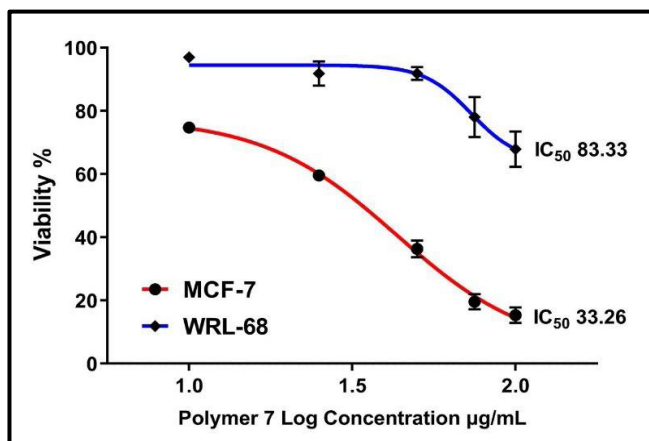


Fig. 6. IC₅₀ of polymer (7) on MCF-7 and compare with WRL-68.

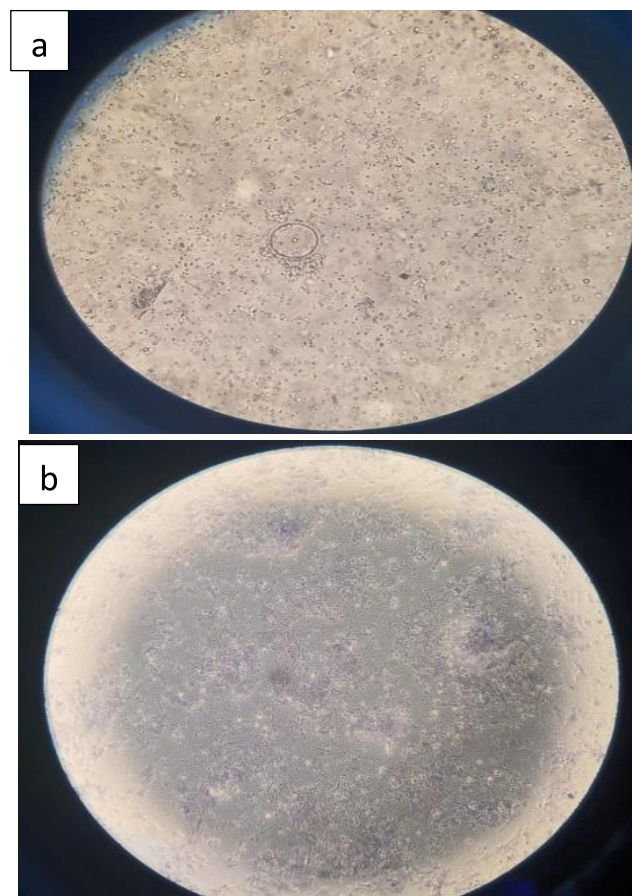
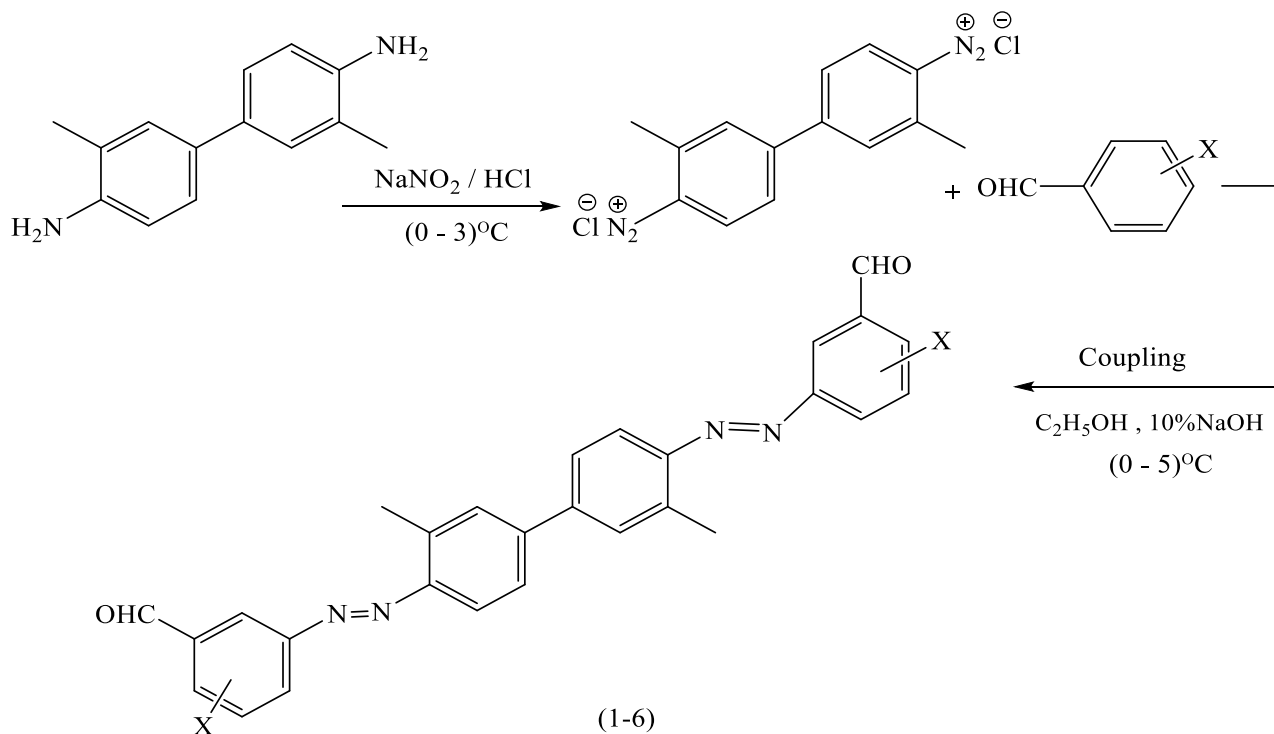


Fig. 7. (a) Image of the MCF-7 well before staining (b) Image of the MCF-7 well after staining.

the presence of chitosan (biopolymer), which has biological activity against bacteria because it contains amino groups with positive charges that bind with the negative charges present on the surface of the bacterial cell, which causes a change in its properties. Such as the permeability of its cell membrane and



X = 4-N(CH₃)₂, 2-Cl, 4-OH, 5-Cl-2-OH, 2-OH-3-OCH₃, 4-OCH₂CH₃

Scheme 1. Synthesis of compounds (1-6).

Table 4. ¹H-NMR spectral data (ppm) for some compounds.

Com. No.	Spectral signals in ¹ H-NMR (δ, ppm) (in DMSO-d ₆)
(1)	2.18 (s, 6H, CH ₃), 3.46 (s, 12H, N(CH ₃) ₂), 6.77-7.69 (m, 12H, Ar-H), 9.67 (s, 2H, CHO).
(2)	3.83(s, 6H, CH ₃), 7.05-7.83 (m, 12H, Ar-H), 8.64(s, 2H, CHO).
(6)	1.31-1.37(t, 6H, OCH ₂ CH ₃), 4.12-4.17 (q, 4H, OCH ₂ CH ₃), 3.35 (s, 6H, CH ₃), 7.10-7.87 (m, 12H, ArH), 9.86 (s, 2H, CHO)

Table 5. Zone of inhibition (in mm) of some synthesized azo compounds (1-4) and modified chitosan (7-10).

Com. No.	<i>Escheria. coli</i>	<i>Staphylococcus aureus</i>
Penicillin	22	22
(1)	15	16
(2)	10	9
(3)	9	9
(4)	10	12
(7)	26	30
(8)	23	24
(9)	25	21
(10)	25	29

an imbalance in osmosis processes, which results in preventing the growth of bacteria, Figs. 1 and 2. All of the compounds and their anti-bacterial activities have been listed in Table 5.

Table 6. The inhibition of cells growth of polymer (7) μL/well.

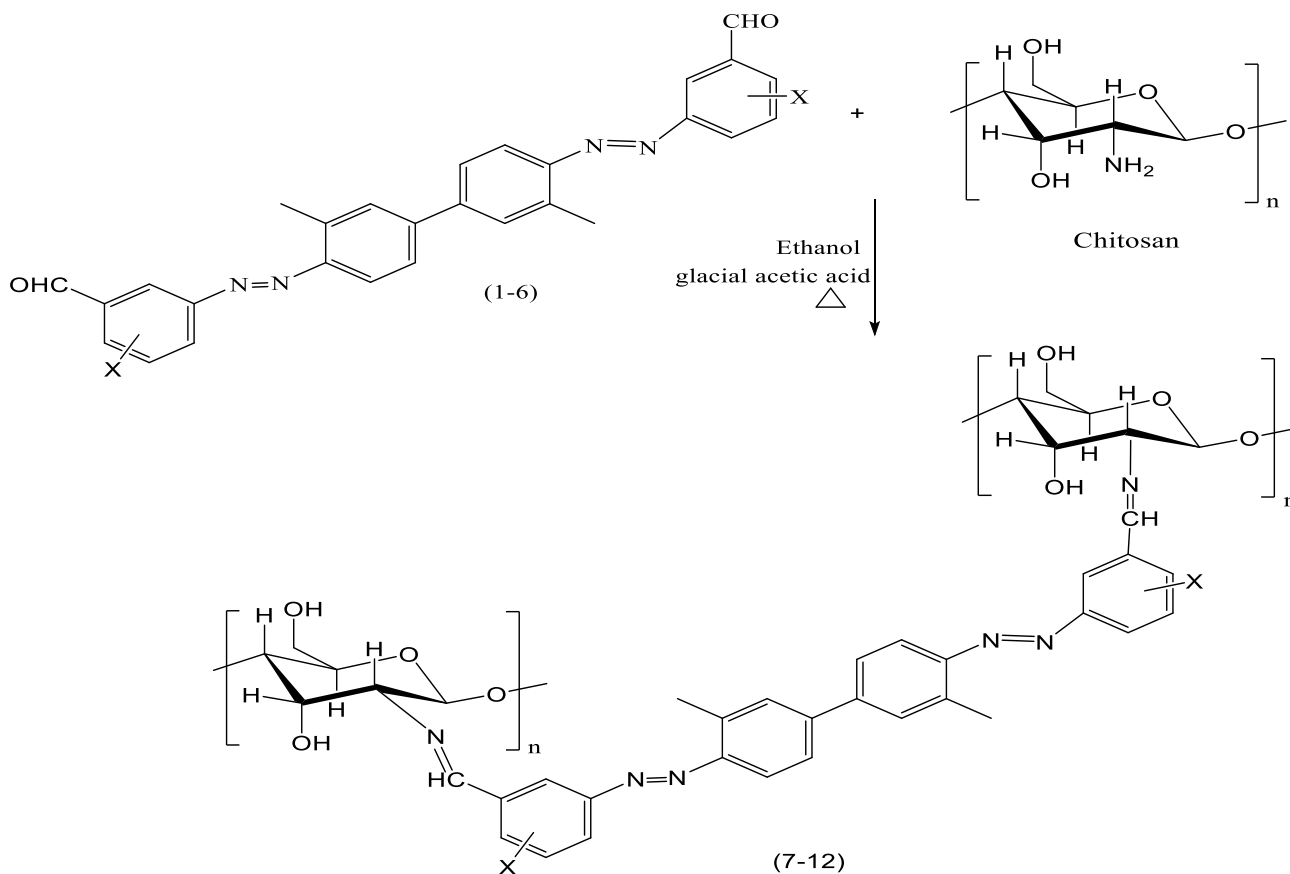
Concentration μg mL ⁻¹	Inhibition of cells growth for MCF-7
10	25.31
25	40.45
50	63.72
75	80.40
100	84.65

Table 7. Differences between different treatments at concentration levels within MCF-7 and WRL cell line.

Conc. μg/MI	MCF-7		WRL-68	
	Mean Viability%	SD	Mean Viability%	SD
10	74.69	1.26	96.95	1.14
25	59.56	0.94	91.85	3.85
50	36.28	2.67	91.80	2.01
75	19.60	2.43	78.03	6.36
100	15.35	2.47	67.91	5.61

FESEM studies³⁰⁻³⁴

FESEM micrographs have been utilized to study changes in surface morphology for prepared polymers(7) and (12). Figs. 3 and 4 shows the modified chitosan's surface morphology. In FESEM images, it can be noticed increasing average size of the pores in comparison with chitosan pore size. Variations of



X = 4-N(CH₃)₂, 2-Cl, 4-OH, 5-Cl-2-OH, 2-OH-3-OCH₃, 4-OCH₂CH₃

Scheme 2. Synthesis of Azo Schiff bases chitosan (7-12).

Table 8. Differences between MCF-7 and WRL with respect to treatments.

multiple comparisons test MCF-7 - WRL-68	Below threshold	Summary	Adjusted P Value
10	Yes	**	<0.0001
25	Yes	**	<0.0001
50	Yes	**	<0.0001
75	Yes	**	<0.0001
100	Yes	**	<0.0001

Table 9. IC₅₀ of polymer (7).

Cell Line	IC ₅₀ μg mL ⁻¹
MCF-7	33.26
WRL-68	83.33

surface morphology result from new bonds in the polymer that have been prepared.

Anticancer activity³⁵⁻³⁸

The results demonstrated the ability of prepared modified chitosan to destroy and kill cancer cells as shown in Figs. 5 to 7. Table 6 shows the inhibition

ratio of the modified polymer(7) equal is 84.65 while Table 7 shows cell viability% is 15.35. Table 8 show the activity of polymer(7) against cancer cells is dependent on concentration, the inhibition rate at the concentration (10, 25, 50, 75, 100) μg ml equal (25.31, 40.45, 63.72 80.40, 84.65) respectively and IC₅₀ of polymer(7) in Table 9 equal 33.26 with MCF-7 and 83.33 with WRL-68.

Chitosan or its derivatives selectively penetrate tumor cells and exhibit anti proliferative activities via antiangiogenic, immunoenhancing, antioxidant defense, apoptosis and enzymatic regulation possibly because azo molecules are involved in the inhibition of DNA, RNA and protein synthesis, as well as hindering carcinogenesis. In addition, the presence of (N=N) in the azo molecular structure is accountable for the interaction with the active site of the target protein.

Conclusion

In this study, synthesis, characterization and study of antibacterial/anticancer activities of some new

modified polymers containing chitosan and azo group. FESEM studies showed the changes in the surface morphology of the synthesized polymers due to the new bonds between Chitosan and azo compounds. Results have shown that polymers had a greater diameter of the growth inhibition zone, polymer (7) had shown quite good inhibition towards the *Staphylococcus aureus* and *E. coli*. Anti-bacterial characteristics of the chitosan are associated with its poly-cationic character and the modified chitosan (7-10) has a higher effectiveness than azo compounds (1-4) due to the presence of the chitosan (biopolymer). The protonated functional groups of the chitosan interact with the negative-charged membranes of the cells of the micro-organisms, which cause damage. Finally, studied the anticancer activity of modified chitosan (7) against MCF-7 (human breast carcinoma cells) and compare with normal cells WRL-68 (the human hepatic cell line). Polymer (7) exhibited high Inhibition rate and less toxicity with $IC_{50} = 33.26$ on MCF-7 and 83.33 on WRL-68 cancer cell lines.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given permission for re-publication attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at the University of Baghdad.
- No animal studies are present in the manuscript.
- No potentially identified images or data are present in the manuscript.

Authors' contribution statement

R. S. S. has designed the work plan, analyzed the results, wrote the article and reviewed the article. H. A. H., D. F. H. and M. S. A. participated in the practical part and conducted the analyzes.

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تحويل ودراسة النشاط الحيوي للكيوسان مع مركبات تحتوي على مجموعة الأزو

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الخلاصة

في البحث الحالي تم تحضير ودراسة النشاط الحيوي لسلسلة من البوليمرات الجديدة المحورة من الكيوسان مع مركبات تحتوي على مجموعة الأزو. في البداية تم تحضير ملح الديازونيوم من تفاعل 3,3'-dimethyl-[1,1'-biphenyl]-4,4'-diamine مع حامض الهيدروكلوريك المركز و نترتيت الصوديوم. ثم تفاعل الازدواج بين ملح الديازونيوم مع الديهايدات اروماتية معوضة لإنتاج مشتقات الازو (1-6). ازو شف بيس كيتوسان(7-12) والتي حضرت من تفاعل الكيوسان مع مشتقات الازو (1-6) في مذيب الايثانول مع قطرات من حامض الخليك الثلجي. التحويلات الهيكلية في موقع المجموعة الأمينية لخلقة الكيوسان (المرتبطة بمجموعة الازو النشطة بايولوجيا) كان من المتوقع أن يعطي مشتقات جديدة(7-12) ذات مجموعة واسعة من الأنشطة البيولوجية. تم استخدام تحليلات FT-IR, ¹H-NMR الطيفية والمسح الضوئي بالمجهر الإلكتروني لمسح الانبعاثات الميدانية لتوضيح هيكل هذه المركبات علاوة على ذلك، تم فحص بعض المركبات الجديدة المحضرة والكيوسان المحور للأنشطة المحتملة المضادة للبكتيريا ضد نوعين من البكتيريا: البكتيريا السالبة E.coli والبكتيريا الموجبة Staphylococcus aureus. أظهرت كل هذه البوليمرات المحورة المستهدفة نشاطاً عالياً مقارنة بالبنسلين (المستخدم كمضاد حيوي مرجعي). وخصوصاً البوليمر المحور رقم (7) الذي أظهر الذي أظهر تثبيطاً عالياً ضد كلا النوعين من البكتيريا Staphylococcus aureus و E.coli تم دراسة النشاط المضاد للسرطان للكيوسان المحور (7) ضد خط خلايا سرطان الثدي البشري (MCF-7) باستخدام تقنية 3-(4,5-ثنائي ميثيل ثيازول-2-يل)-2,5-بروميد ثنائي فينيل تيترازوليوم (MTT) ومقارنته مع خط الخلايا الطبيعية (خط الخلايا الكبدية البشرية WRL-68) حيث أظهر البوليمر (7) تثبيطاً عالياً للخلايا السرطانية وأقل سمية للخلايا الطبيعية.

الكلمات المفتاحية: مركبات الازو، كيتوسان، ملح الدايازونيوم، دراسة المجهر الإلكتروني لمسح الانبعاثات الميدانية، خط خلايا سرطان الثدي البشري، قواعد شف.