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Article

Synthesis of Schiff Bases Based on Chitosan and Heterocyclic Moiety: Evaluation of Antimicrobial Activity

M. Ali,* Essam Nageh Sholkamy, Ahmed S. Alobaidi, Muhanna K. Al-Muhanna, and Assem Barakat

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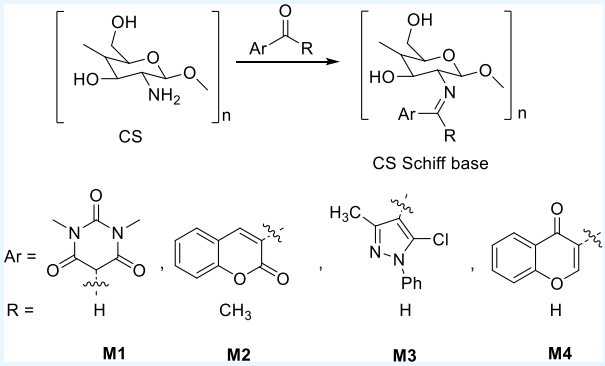
ABSTRACT: Schiff bases of chitosan (CS) were prepared by reaction of four different heterocyclic compounds, namely, 1,3-dimethyl-2,4,6-trioxohexahydropyrimidine-5-carbaldehyde (M1), 3-acetyl-2*H*-chromen-2-one (M2), 5-chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde (M3), and 4-oxo-4*H*-chromene-3-carbaldehyde (M4), with CS using thermal and ultrasound approaches. CS Schiff base formation was confirmed by using FT-IR, XRD, and TGA. Characteristic data show that amino groups in chitosan reacted with the functional group in the heterocyclic compound to form the Schiff base. CS Schiff bases show thermal stability more than pure CS. The antimicrobial activity of Schiff bases was tested against +ve Gram bacteria and −ve Gram bacteria. The result shows that Schiff bases prepared by temperature and ultrasound methods possess high antimicrobial activity against +ve Gram bacteria and −ve Gram bacteria; in comparison, Schiff bases produced by the ultrasound method have higher antimicrobial activity. The Schiff base (CSM4U), prepared by the ultrasound method by reaction of CS with 4-oxo-4*H*-chromene-3-carbaldehyde, exhibited higher antimicrobial activity than Gentamicin as an antibacterial agent. The inhibition range caused by CSM4U was between 19 and 27 mm. Moreover, CSM4U also acted as an antifungal agent, causing an inhibition zone of 21 mm for both *Candida albicans* and *Candida tropicalis*, which was higher than that of Terbinafine.



INTRODUCTION

Chitosan (CS) is a sustainable, naturally derived material with notable film-forming capabilities.[1] It also exhibits biocompatibility, biodegradability, and nontoxicity. Chitosan also occurs in naturally existing renewable resources.[1] Chitosan has three types of reactive groups, primary amine, primary hydroxyl, and secondary hydroxyl, located at C-2, C-3, and C-6, respectively.[2] Glucosamine's primary amine at C-2 is the most important group among the three reactive groups for chitosan's biological activity.[3,4] As a consequence of this, it has important applications in many different disciplines, including the biological sciences, agriculture, the development of functional foods, the treatment of wastewater, the preservation of the environment, biotechnology, and cosmetics.[5] Chitosan exhibits various advantageous biological properties, including antimicrobial and antitumor effects.[4,6] Chitosan, a biopolymer derived from chitin, undergoes a process called deacetylation to produce D-glucosamine. In our world, there are numerous organisms that possess the remarkable polysaccharide called chitin. Chitin composed of -(1−4)-linked *N*-acetyl-D-glucosamine units.[7] Chitosan is characterized by the presence of hydroxyl and amino functional groups along its polymer chain. The ability to modify chitosan chemically allows for customization of its physical and chemical properties for various applications. CS Schiff bases (−RC═*N*−) can easily be formed when amino groups in chitosan undergo reactions with aldehydes and ketones, which ultimately leads to the production of Schiff bases.[1] Extensive research has been conducted on the features of these substances, encompassing investigations into their physical and chemical attributes, as well as their biological functionalities. As a result, there is a substantial body of knowledge available on the properties and potential applications of CS Schiff bases.[8−10] Schiff bases, which are characterized by the presence of heterocyclic nitrogen, sulfur, and oxygen groups, are a unique category of compounds that have been shown to possess significant biological activity.[11] These compounds have been the subject of considerable scientific investigation due to their potential utility in the development of novel therapeutic agents for a variety of diseases and medical conditions. Given their diverse range of biological effects, the study of Schiff bases represents an important area of research[12,13] in the fields such as

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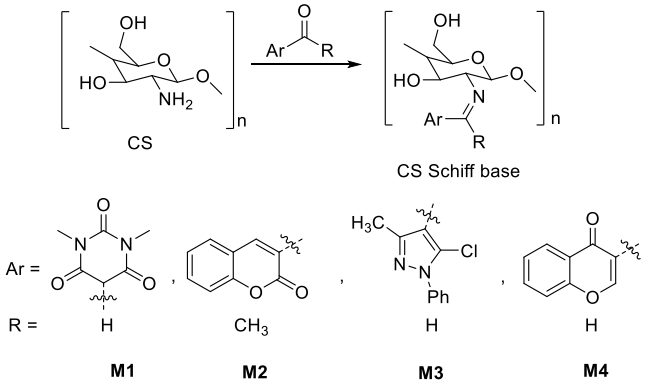
antibacterial activity,[12] antioxidant,[14] anticancer,[15] antitumor,[16] and antifungal.[17] Furthermore, the incorporation of Schiff bases into the CS has the potential to enhance its ability to interact with metallic ions through complexation. The coordination of Schiff bases with metal ions has the potential to improve their biological properties and offers the prospect of employing them as heterogeneous catalysts.[18,19] Schiff bases of chitosan are important parts of various self-healing hydrogels. They are getting a lot of attention for their potential to be used as drug delivery systems to fight against hepatocellular carcinoma.[20,21] Heterocyclic compounds are a specific type of cyclic compound that contain atoms from multiple elements in their ring structure. These organic compounds are extensively studied in various subfields of chemistry because of their significant importance.[22] Heterocyclic systems with different structures display various biological activities because of diversities in their molecular structures.[23] Heterocyclic compounds that incorporate nitrogen and oxygen atoms have attracted significant attention within the academic community due to its unique antimicrobial activity,[24] antifungal,[23] and anticancer[25] properties such as pyrazolos derivative and barbituric acid derivative. According to literature, Schiff bases are prepared by the reaction between the amino group in CS and the functional group in the carbonyl derivative by the thermal method. However, a comparison study between two approaches like thermal and ultrasound for achieving the CS. Schiff bases not reported so far to the best of our knowledge. Based on the factors mentioned above, in this text, we studied the synthesis of CS. Schiff bases proceeded via two approaches: thermal or ultrasound protocol. The final compounds were characterized and examined against antimicrobial reactivity, including +ve Gram bacteria, namely, *Salmonella aureus* ATTC 29213 and *Escherichia faecalis* ATTC 29212, and −ve Gram bacteria, namely, *Escherichia coli* ATTC 25922 and *Salmonella typhimurium* ATTC 14028 and in the case of fungus against *Candida* (Scheme 1).

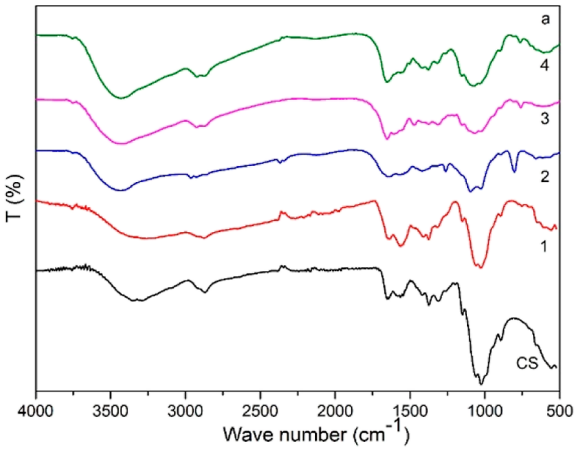
Scheme 1. Synthesis of CS Schiff Base



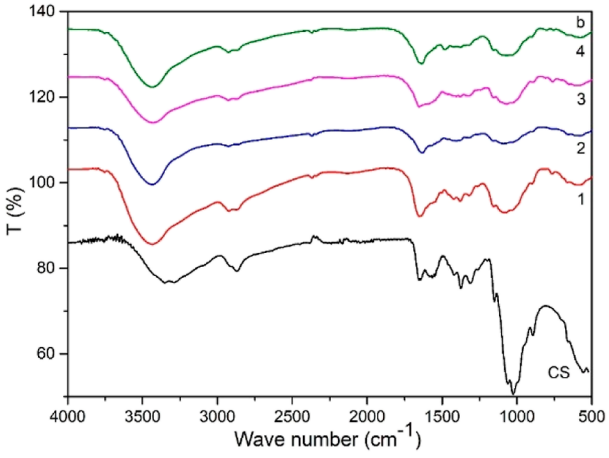
RESULTS AND DISCUSSION

Chitosan Schiff Base Characterization. Initially, we successfully synthesized four different CS Schiff bases by reaction of CS with four different heterocyclic compounds, namely, 1,3-dimethyl-2,4,6-trioxohexahydropyrimidine-5-carbaldehyde (M1), 3-acetyl-2*H*-chromen-2-one (M2), 5-chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde (M3), and 4-oxo-4*H*-chromene-3-carbaldehyde (M4), using thermal and ultrasound methods. The CC Schiff bases were investigated using FT-IR, XRD, and TGA.

Studies of FT-IR Spectra. Figure 1a shows CS (medium molecular weight) and the Schiff bases (CSM1T, CSM2T, CSM3T, and CSM4T). Figure 1b shows CS (medium molecular weight) and the Schiff bases (CSM1U, CSM2U, CSM3U, and CSM4U). CS spectra showed the absorption band at 3325−3283 cm^{-1} (amine −N−H symmetrical stretching) and 2926 cm^{-1} (O−H asymmetric stretching). At 1641 cm^{-1}, the presence of *N*-acetyl groups is confirmed. The presence of CH_2 bending and CH_3 symmetrical stretching is confirmed by the presence of the band at 1416 and 1372 cm^{-1}, respectively. The peaks at 1156 cm^{-1} [(1−4) glycoside bridge], 893 cm^{-1} (C−O−C antisymmetric bending), and 1020 cm^{-1} (C−O stretching skeletal vibrations) are characterized to the saccharide structure of chitosan.[26] Figure 1a shows Schiff bases FT-IR spectra of CSM1T, CSM2T,



FT-IR of CS, (1) CSM1T, (2) CSM2T, (3) CSM3T& (4) CSM4T



FT-IR of CS, (1) CSM1U, (2) CSM2U, (3) CSM3U& (4) CSM4U

Figure 1. (a) FT-IR of CS, (1) CSM1T, (2) CSM2T, (3) CSM3T, and (4) CSM4T. (b) FT-IR of CS, (1) CSM1U, (2) CSM2U, (3) CSM3U, and (4) CSM4U.

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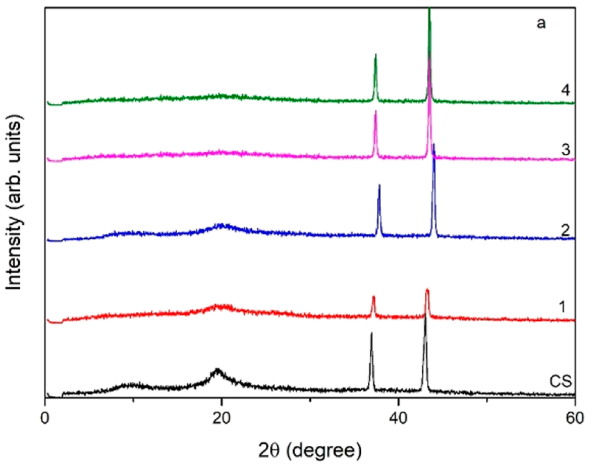
CSM3T, and CSM4T, which show the presence of the absorption peak at 3344 to 3457 cm^{-1} corresponding to the −OH gp stretching vibration. This is due to the moiety withdrawing effect of the heterocyclic compound on the chain of CS. FT-IR of Schiff bases (CSM1T, CSM2T, CSM3T, and CSM4T) show absorption of a new band at 1639 cm^{-1} related to C═N imines' characteristic vibration.[27] Disappearance of the characteristic band at 1597 cm^{-1} in all prepared Schiff bases indicates that the $-NH_2$ group was decreased, which confirms the reaction of amine groups in CS with aldehyde group in heterocyclic compounds (M1, 2, 3, and 4), forming Schiff base. At 3325 cm^{-1}, the broad band corresponding to amine −N−H symmetrical stretching shifted to a higher frequency. Figure 1b represents Schiff bases prepared by the ultrasound method, showing that disappearance of the characteristic band at 1597 cm^{-1} in all prepared Schiff bases in (CSM1U, CSM2U, CSM3U, and CSM4U) indicates the formation of Schiff bases.

XRD Studies. Figure 2a shows XRD diffraction patterns of CS and the Schiff bases (CSM1T, CSM2T, CSM3T, and CSM4T). Figure 2b shows the XRD pattern of CS (medium molecular weight) and the Schiff bases (CSM1U, CSM2U, CSM3U, and CSM4U). The CS pattern shows the characteristic peak at $2\theta = 10.1°$ and 19.5°, which corresponds to (020) and (110) crystalline lattice planes, respectively. For the prepared Schiff bases (CSM1T, CSM2T, CSM3T, and CSM4T) and (CSM1U, CSM2U, CSM3U, and CSM4U), the peak at $2\theta = 10.1°$ disappeared. The peak at 19.5° became wider and weaker than CS. This indicates that the prepared Schiff base is less crystalline than CS, which indicates that it is amorphous, confirming Schiff base formation.[28] This occurred due to strong hydrogen bond deformation in the backbone of CS with the (M1, 2, 3, and 4)-substituted aldehyde group on CS N atoms,[29] which confirms the formation of CS Schiff bases by using thermal and ultrasound methods. In order to make comparisons easier, we calculated the index of crystallinity (CI) using the equation given below

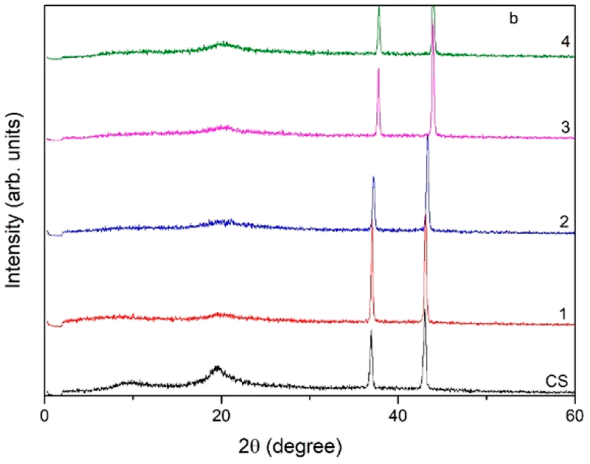
$$\text{crystallinity index (\%)} = \frac{(I_{110} - I_{am})}{I_{110}} \times 100$$

where the highest level of intensity observed at $2\theta \sim 19.5°$ represents I_{110} and the amorphous diffraction intensity at $2\theta \sim 8.49°$ represents I_{am}.[30] The crystallinity index (%) for CSM1T, CSM2T, CSM3T, and CSM4T has been calculated, and the following outcomes have been obtained, 18, 39, 35, and 45%, respectively, and for CSM1U, CSM2U, CSM3U, and CSM4U, they were 11.2, 34, 45, and 39%, respectively. The formation of Schiff base is the main factor that affects the change in crystallinity, and according to this fact, the ultrasound method is the best way to prepare the Schiff base.

Thermogravimetric Analysis. Figure 3a,b shows TGA thermograms of CS (CSM1T, CSM2T, CSM3T, and CSM4T) and (CSM1U, CSM2U, CSM3U, and CSM4U), respectively. TGA was used to exhibit the thermal stability of Schiff base of CS in comparison with pure CS. The thermogram curve of CS shows three weight loss stages, first in the range between 27 and 105 °C with a loss of weight of 11.5%, which refers to moisture loss. The second stage is from 2557 to 363 °C, with a loss of weight of 51%, which refers to polysaccharide depolymerization.[9,31] The third stage loss of weight is about 22%, and the obtained weight of residual is 8%, indicating that till 700 °C, not all CS was decomposed. Thermograms of



XRD of CS, (1) CSM1T, (2) CSM2T, (3) CSM3T& (4) CSM4T



XRD of CS, (1) CSM1U, (2) CSM2U, (3) CSM3U& (4) CSM4U

Figure 2. (a) XRD of CS, (1) CSM1T, (2) CSM2T, (3) CSM3T, and (4) CSM4T. (b) XRD of CS, (1) CSM1U, (2) CSM2U, (3) CSM3U, and (4) CSM4U.

prepared Schiff bases (CSM1T, CSM2T, CSM3T, and CSM4T) show three stages of weight loss (Figure 3a). The first stage loss of weight was found to be at 92.2, 94.4, 92.8, and 93.17 °C. The Schiff bases show lower water capacity than pure CS. The second stage of loss of weight was at 174.6, 157.91, 249, and 229.6 °C, with weight losses of 37.87, 40.5, 45.8, and 47%, respectively. This degradation occurs due to the loss of heterocyclic rings and phenyl groups attached to the backbone of chitosan during depolymerization.[9] The last stage of weight loss can be seen at 537 °C, with weight losses of 17.3−12%. The weight of residual was found to be 20.1, 32.4, 29.8, and 36.89. This decomposition occurred because Cs' unreacted amino groups were destroyed. Figure 3b shows thermogram behavior of CSM1U, CSM2U, CSM3U, and CSM4U, which shows three different stages. The first stage occurs due to moisture loss at 79.7 °C for CSM1U, CSM2U, CSM3U, and CSM4U. The second stage, as mentioned before, occurred due to heterocyclic rings and phenyl attached to the

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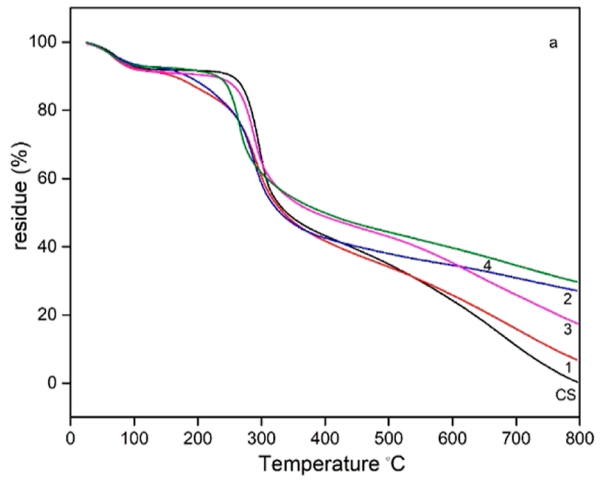
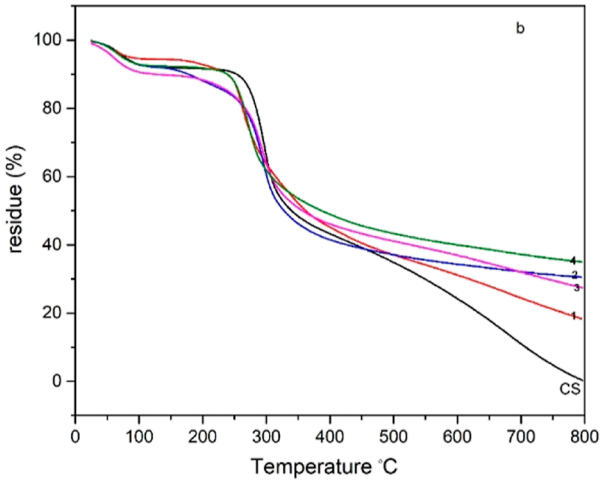


TGA of CS, (1) CSM1T, (2) CSM2T, (3) CSM3T& (4) CSM4T



TGA of CS, (1) CSM1U, (2) CSM2U, (3) CSM3U& (4) CSM4U

Figure 3. (a) TGA of CS, (1) CSM1T, (2) CSM2T, (3) CSM3T, and (4) CSM4T. (b) TGA of CS, (1) CSM1U, (2) CSM2U, (3) CSM3U, and (4) CSM4U.

backbone of CS, which have been lost with depolymerization of CS starting from 239, 168, 234, and 239 °C to nearly 408 °C, with losses of weight of 44, 41, 45, and 48%, respectively. The third stage starts from 408 °C to complete degradation with weight loss of 15−9%. The weight of residual was found to be 24.56, 32.1, 32, and 37.3. According to the previous data, the prepared Schiff base in the presence of CS has higher thermal stability than pure CS, which proves the formation the Schiff base prepared with different methods.

Bioactivity of Schiff Bases against the Pathogenic Bacteria and Fungus *Candida* spp. The bioactivities of prepared Schiff bases against the selected pathogenic microbes were investigated in the current study in comparison with CS. According to the latest studies, CS possesses antimicrobial and antifungal properties. There are various explanations for how chitosan can fight against bacteria. One possible mechanism is that CS molecules, which carry a positive charge, are capable of interacting with the membranes of microbial cells, which carry a negative charge. Electrostatic forces are thought to be responsible for the development of this interaction, which can be traced back to the NH_3^+ groups that are present in CS as well as the charges that are present on the surface of the cells.[32] Another hypothesized mechanism suggests that CS exhibits an interaction with microbial DNA, resulting in the inhibition of both protein synthesis and mRNA. This is believed to occur, as chitosan penetrates into the microorganisms' nuclei.[33] The last hypothesis, which considered a more acceptable mechanism, involves inhibiting spore components, binding essential nutrients, and chelating metals to prevent rapid growth of microorganisms.[32] Each Schiff base in the well was used at a concentration of 2.5 mg per well. Only four Schiff bases have antipathogenic microbial activity without the others. Results in Table 1 and Figure 4 show that the Schiff bases CSM3T(1), CSM4T(14), CSM3U(15), and CSM4U(16) had bioactivity against *S. aureus*, *E. faecalis*, *E. coli*, *S. typhimurium*, *Candida albicans*, and *Candida tropicalis*, with inhibition zones ranging from 7 to 35 mm. CSM3T and CSM3U had low antifungal activity against *C. albicans* (7 mm), but all had high antimicrobial activity against the other pathogenic microbes tested. CSM4U had the highest activity against all microbes compared to Gentamicin, an antibacterial agent, which caused inhibition in the range of 19 to 27 mm, and Terbinafine, an antifungal agent, which caused inhibition zone at 21 mm for both *C. albicans* and *C. tropicalis*. Based on the resulting data, it can be observed that the Schiff base, which is produced through the process of ultrasound, exhibits a greater degree of antimicrobial activity when compared to the Schiff base produced through high temperature. This comparison indicates that the ultrasound method can be considered to be more effective in producing Schiff bases with superior antimicrobial properties.

Table 1. Primary Antimicrobial Activity for Schiff Bases at 2.5 mg/Well

pathogens		inhibition zone (mm)					
		Schiff bases					
		CSM3T	CSM4T	CSM3U	CSM4U	Gentamicin (50 μg)	Terbinafine (50 μg)
bacterial pathogens	<>S. aureus<>	20	35	15	21	27	
	<>E. faecalis<>	21	27	10	20	21	
	<>E. coli<>	17	32	6	11	25	
	<>S. typhimurium<>	16	27	8	15	19	
fungal pathogens	<>C. albicans<>	7	32	7	15		21
	<>C. tropicalis<>	18	32	17	16		21

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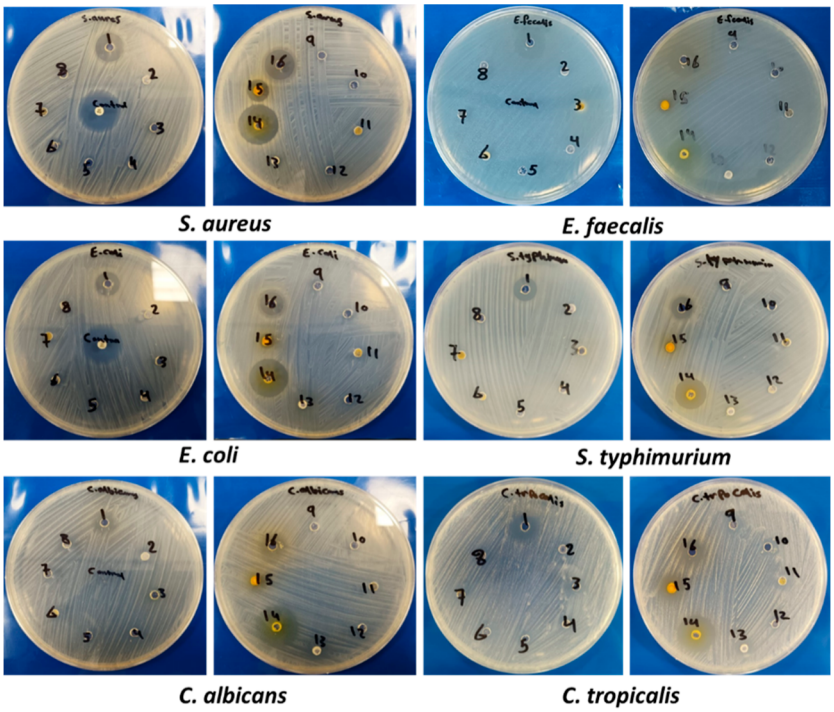

Figure 4. Photographs of inhibition zones on plates as a result of Schiff bases effect on the targeted pathogenic microbes. (1 means CSM3T, 14 means CSM4T, 15 means CSM3U, and 16 means CSM4U).

Table 2. Minimal Inhibitory and Minimal Bactericidal/Fungicidal Concentrations of Bioactive Schiff Bases

pathogens			Schiff bases			
			CSM3T (mg/mL)	CSM4T (mg/mL)	CSM3U (mg/mL)	CSM4U (mg/mL)
bacterial pathogens	<>S. aureus<>	MIC	0.58	0.195	0.58	0.195
		MBC	**0.78**	**0.39**	**0.78**	**0.39**
	<>E. faecalis<>	MIC	1.17	0.78	0.78	0.78
		MBC	**1.56**	**1.56**	**1.56**	**1.56**
	<>E. coli<>	MIC	0.58	0.098	3.12	0.58
		MBC	**0.78**	**0.195**	**6.25**	**0.78**
	<>S. typhimurium<>	MIC	0.58	0.145	9.37	0.58
		MBC	**0.78**	**0.195**	**12.5**	**0.78**
fungal pathogens	<>C. albicans<>	MIC	3.12	0.049	2.34	1.56
		MFC	**6.25**	**0.098**	**3.12**	**3.12**
	<>C. tropicalis<>	MIC	2.34	0.049	1.56	1.17
		MFC	**3.12**	**0.098**	**3.125**	**1.56**

The present study on chitosan Schiff bases' antimicrobial activity is consistent with recent research that has provided valuable insights into their potential as antimicrobial agents against Gram-negative bacteria such as *E. coli* and *K. pneumoniae*, Gram-positive bacteria including *S. aureus* and *Salmonella mutans*, as well as fungi like *A. fumigatus* and *C. albicans*.[9] In another study, Sabaa et al.[34] investigated the antibacterial activities of chitosan-graft-poly(acrylonitrile) modified with Schiff bases. The researchers assessed the effects of these modified chitosan compounds against Gram-positive bacteria such as *Salmonella pneumoniae* and *S. aureus*, Gram-negative bacteria like *E. coli*, and fungi including *A. fumigatus*, *C. albicans*, and *G. candidum*. Furthermore, Anush et al.[35] conducted a comparative analysis between chitosan and Schiff bases in terms of their antimicrobial activity against a range of microorganisms (*S. aureus*, *B. subtilis*, *K. pneumoniae*, *E. coli*, and *C. albicans*). The results revealed that the Schiff bases exerted a stronger inhibitory effect on these microorganisms compared to chitosan alone. Additionally, the extent of inhibition varied depending on the specific substitution within the Schiff bases.[9,12] This is consistent with the findings of an earlier study by Hamed et al.,[9,12] who also demonstrated the superior antimicrobial efficacy of chitosan Schiff bases compared to chitosan.

The efficacy of antimicrobial activity could be affected by various factors, for instance, the functional group interactions are located on both the CS surface and bacterial cells, modification degree of CS, the hydrophobic/hydrophilic characteristics of the surface, and the dispersion of modified CS inside the medium. Furthermore, the charge of cell surfaces is essential to determine the maintaining microbial homeostasis and resistance to antimicrobial agents.[36,37] Schiff bases are known to contain π-electrons in their imine group. When combined with heterocyclic moiety-conjugated systems such as pyridine, thiophene, furan, and pyridine, as well as rings containing phenyl and pyrazole, imine gp density of electrons

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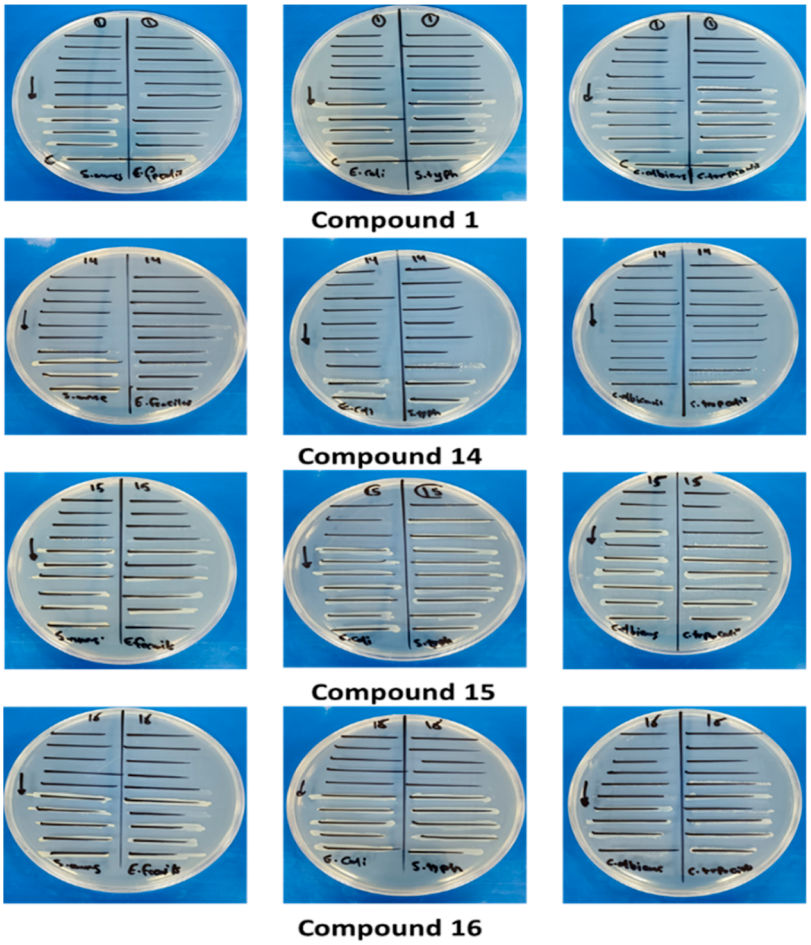

Figure 5. Growth of targeted pathogenic microbes on plates after treatment with Schiff bases at different concentrations to determine MIC and MBC/MFC (1 means CSM3T, 14 means CSM4T, 15 means CSM3U, and 16 means CSM4U).

increases. This increase in electron density can have a significant impact on the respiration process of microbial cells, resulting in the disruption of bacterial growth. The disruption occurs due to the blocking of protein synthesis, which is essential for the growth and survival of bacteria.[38,39]

Inhibitory of Minimum and Bacteriostatic/Fungicidal Concentration of Bioactive CS Schiff Bases toward Target Pathogenic Microbes. Table 2 and Figure 5 show that Schiff bases CSM3T, CSM4T, CSM3U, and CSM4U had MICs and MBCs against *S. aureus* of 0.58 and 0.19, 0.58 and 0.195, 0.78 and 0.39, and 0.78 and 0.39 mg/mL, respectively. MICs and MBCs of Schiff bases CSM3T, CSM4T, CSM3U, and CSM4U against *E. faecalis* were 1.17 and 0.78, 0.78 and 0.78, 1.56 and 1.56, and 1.56 and 1.56 mg/mL, respectively. Schiff bases CSM3T, CSM4T, CSM3U, and CSM4U had MICs and MBCs against *E. coli* of 0.58 and 0.098, 3.12 and 0.58, 0.78 and 0.195, and 6.25 and 0.78 mg/mL, respectively. Schiff bases CSM3T, CSM4T, CSM3U, and CSM4U had MICs and MBCs of 0.58 and 0.145, 9.37 and 0.58, 0.78 and 0.195, and 12.5 and 0.78 mg/mL against *S. typhimurium*, respectively. Schiff bases CSM3T, CSM4T, CSM3U, and CSM4U had MICs and MFCs of 3.12 and 0.049, 2.34 and 1.56, 6.25 and 0.098, 3.12 and 3.12 mg/mL, respectively, against *C. albicans*. Schiff bases CSM3T, CSM4T, CSM3U, and CSM4U had MICs and MFCs of 2.34 and 0.049, 1.56 and 1.17, 3.12 and 0.098, and 3.125 and 1.56 mg/mL, respectively, against *C. tropicalis*.

CONCLUSIONS

In summary, by using thermal or ultrasound methods, the CS Schiff base was prepared and achieved successfully. Amino groups in CS was reacted with the carbonyl group in the four chosen heterocyclic compounds to produce Schiff base. The final Schiff bases were characterized by FT-IR, XRD, and TGA. XRD patterns show that Schiff base has an amorphous structure, and by comparing the amorphous degree for each of the Schiff bases, the data show that the ultrasound method was optimal. Thermal behavior of the prepared Schiff bases was investigated and showed that CS Schiff bases are more thermally stable than CS. CS Schiff bases were tested against +ve Gram bacteria, namely, *S. aureus* and *E. faecalis*, and −ve Gram bacteria, namely, *E. coli* and *S. typhimurium*. According to our resulting data, the Schiff base, which is produced through the process of ultrasound, exhibits a greater degree of antimicrobial activity compared to the Schiff base produced through the thermal approach.

EXPERIMENTAL SECTION

Chemicals. Ethyl acetoacetate, salicylaldehyde, 3-methyl-1-phenyl-1,5-dihydro-4*H*-pyrazol-4-one, piperidine, and Vilsmeier reagent [phosphorus oxychloride ($POCL_3$) and dimethylformamide (DMF)] were used; medium molecular weight chitosan (less than 100 kDa) was soluble only in dilute acid with a 90% degree of deacetylation; they were all purchased from Sigma-Aldrich, USA.

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Instruments. Fourier transform infrared spectroscopy (FTIR) (Shimadzu FTIR-8101 A) was used to characterize the composition of the chitosan Schiff base in the range of 4000−400 cm^{-1}. A TGA-50H thermogravimetric analyzer was used in TGA. Sample characterizations were carried out in Shimadzu, Japan. In a platinum pan prepared Schiff bases were heated up to 800 °C with a heating rate of 10 °C/min, in N_2 atm of the flow rate 25 mL/min. Using a Thermo Nicolet AVATAR 330 spectrophotometer, FT-IR spectra were recorded in the range 400−4000 cm^{-1} and using the KBr pellet method. An X-ray diffractometer (Philips PW 1710) equipped with Cu Kα radiation (λ = 1.54060 Å) was used to determine the crystallinity, and phases of the resulting Schiff base were examined with a voltage of 40 kV.

Preparation of Heterocyclic Compounds (M1, M2, M3, and M4). Synthesis and characterization of 1,3-dimethyl-2,4,6-trioxohexahydropyrimidine-5-carbaldehyde (M1), 3-acetyl-2*H*-chromen-2-one (M2), 5-chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde (M3), and 4-oxo-4*H*-chromene-3-carbaldehyde (M4) were done as mentioned in refs 40−43, respectively.

Chitosan Schiff Bases Preparation by Using Two Different Methods. First Method: High Temperature Method. Chitosan Schiff bases (CSM1T, CSM2T, CSM3T, and CSM4T) were prepared as follows. Into a 100 mL round flask, 0.2 g of CS (medium mol. Weigh) was added to a mixture solution of (1%) acetic acid and ethanol and then stirred for 6 h at room temperature until full dissolution. Heteroaldehyde (M1, M2, M3, and M4) equivalent mole was dissolved in methanol or ethanol and gradually added to the mixture. At 70 °C for 12 h, the reaction mixture was reacted with continuous stirring; after 12 h, the reaction was left for cooling. After cooling, Schiff bases were precipitated. The unreacted heteroaldehyde was removed by washing using anhydrous ethanol many times. To confirm purification of Schiff bases, unreacted aldehydes traces were extracted in a Soxhlet apparatus for 3 days using ethanol. CS Schiff bases were left in vacuum for 24 h at 60 °C till they were completely dry. The Schiff base yield was obtained as follows: 92.3, 96.4, 94.87, and 95.1% for CSM1T, CSM2T, CSM3T, and CSM4T, respectively.

Second Method: Ultrasound. For compounds CSM1U, CSM2U, CSM2U, and CSM4U, CS was dispersed in [MEOH/ACOH (1%)] solution left under medium intensity of ultrasound for 60 min; then a solution of heterocompound was added dropwise under high ultrasound intensity and lifted for 7 h at 25−40 °C. After completion of the reaction, the product was filtered and washed using methanol many times to get rid of unreacted heterocompounds. To confirm purification of Schiff bases, unreacted aldehyde traces were extracted in a Soxhlet apparatus for 3 days using ethanol. CS Schiff bases were left in vacuum for 24 h at 60 °C till they were completely dry. The Schiff base yield was obtained as follows: 97.41, 98, 96.6, and 98.8% for CSM1U, CSM2U, CSM2U, and CSM4U, respectively.

Test Microorganism. The antibacterial activity of the prepared Schiff base was assessed against two bacteria groups: +ve Gram bacteria, namely, *S. aureus* ATTC 29213 and *E. faecalis* ATTC 29212, and −ve Gram bacteria, namely, *E. coli* ATTC 25922 and *S. typhimurium* ATTC 14028 maintained in BHI at 20 °C; 300 mL of each stock-culture was added to 3 mL of BHI broth. Overnight cultures were kept for 24 h at 37 ± 1 °C, and after 24 h of incubation, the purity of cultures was checked. After 24 h of incubation, bacterial suspension (inoculum) was diluted with sterile physiological solution, for the diffusion and indirect bioautographic tests, to 108 cfu/mL (turbidity = McFarland barium sulfate standard 0.5). In the case of fungus *C. albicans* ATTC 60193 and *C. tropicalis* ATTC 66029, PDA is the medium that is utilized in antagonistic activity.

Primary Screening of Synthetic Schiff Bases for Bioactivity by the Agar Well Diffusion Method. The Schiff bases were prepared at a concentration of 50 mg/mL dissolved in dimethyl sulfoxide (DMSO). Sterilized Mueller Hinton agar plates seeded with pathogenic microbes were prepared; each well in the plates seeded with the tested pathogenic microbes was filled with 2.5 mg of the synthetic compound. For 24 h at 37 °C, plates were placed in an incubator. The inhibition zone, measured in (mm), was used to evaluate the antimicrobial activity.

Minimum Inhibitory and Minimum Bacteriostatic/Fungicidal Concentration Determination. The antimicrobial activity of the prepared Schiff bases was investigated by employing a microdilution method using nutrient broth. The inoculum was prepared as described previously. The prepared Schiff bases were dissolved in DMSO to a concentration of 12.5 mg/mL. Further 1:2 serial dilutions were performed by addition of culture broth to reach concentrations ranging from 12.5 to 1.3 0.048 mg/mL; 100 μL of each dilution was distributed in 96-well plates, and sterility control and a growth control (containing culture broth plus DMSO, without antimicrobial substance) were carried out. Each test and growth control well were inoculated with 10 μL of a bacterial suspension (10^8 cfu/mL). Microdilution trays were incubated at 37 °C for 24 h. Microbial growth was detected by subculture of all treatments at 37 °C for 24 h using the streaking method to determine MICs and MBCs/MFCs of the tested compounds. The results were expressed in mg/mL.

AUTHOR INFORMATION

Corresponding Author

M. Ali − *Department of Chemistry, College of Science, King Saud University, Riyadh 11451, Saudi Arabia;* orcid.org/0000-0003-1812-8953; Email: maly.c@ksu.edu.sa

Authors

Essam Nageh Sholkamy − *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Ahmed S. Alobaidi − *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Muhanna K. Al-Muhanna − *The Material Science Research Institute, King Abdulaziz City for Science and Technology (KACST), Riyadh 11442, Saudi Arabia*

Assem Barakat − *Department of Chemistry, College of Science, King Saud University, Riyadh 11451, Saudi Arabia;* orcid.org/0000-0002-7885-3201

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c08446

Notes

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