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Antibacterial and Antifungal Evaluation of Chloroflavones

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Abstract

Two chloroflavones, **6** and **7** along with their corresponding chalcones, **4** and **5** have been tested for antibacterial and antifungal activities against six human pathogenic bacteria viz. *Bacillus cereus* (G+), *Staphylococcus aureus* (G+), *Escherichia coli* (G-), *Vibrio choloriae* (G-), *Pseudomonas aeruginosa* (G-), and *Salmonella typhi* (G-), and four plant as well as mold fungi viz. *Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger and Rhizopus spp.*. The antibacterial and antifungal screens of the synthesized compounds were performed *in vitro* by the filter paper disc diffusion method and the poisoned food technique, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these synthesized compounds in comparison to ampicillin were also determined by broth micro-dilution method. Some of them were found to possess significant activity, when compared to standard drugs.

Keywords: Antibacterial activity; Antifungal activity; MBC; MIC; Flavones.

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1. Introduction

The flavonoid compounds are a group of natural products widely occurring in natural plant pigments and medicinal plants [1,2]. They are found in fruits, vegetables, nuts, seeds and flowers as well as in teas and wines and are important constituent of human diet [3]. These products have been demonstrated to possess many biological and pharmacological activities such as antioxidant [4], anti-inflammatory [5], antimutagenic, antiallergic activities, and inhibitory activities on several enzymes [6,7]. Synthesis of these compounds has attracted considerable attention for their significant biocidal [8] and pharmaceutical [9,10] effects. Because of the exciting biological activities, many flavonoid compounds have been synthesized and studied their antibacterial and antifungal activities [11,12]. Our previous articles [13,14] have also reported the antibacterial and antifungal effects of some flavonoids containing various groups in their ring system. Beneficial effects of flavonoids on human health have gained increasing interest among researchers over the last few years.

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Various strategies for the synthesis of flavone derivatives have been reported. The main synthetic methods known for flavones are oxidative cyclization [15,16] of 2'-hydroxychalcones, the cyclodehydration [17] of 1-(2-hydroxyphenyl-3-phenyl-1,3-propanedione) and via intermolecular Wittig reaction [18]. However, many of these methods suffered from harsh reaction conditions, toxic reagents, strong acidic / basic conditions, prolonged reaction time, poor yield and low selectivity. Recently, microwave irradiation has gained the attention of chemists for its unique advantages, such as shorter reaction times, cleaner reaction products, higher yields and better selectivity [19-21]. A survey of the literature provides information that chloroflavones **6** and **7** were synthesized under microwave irradiation [22] but their biological activities were not evaluated. So there is still a need for the evaluation of antibacterial and antifungal activities of these chloroflavones **6** and **7** compounds.

Encouraged by these observations and in continuation of search for antimicrobial active molecules [23-27], we are interested to synthesis as well as to evaluate the antibacterial and antifungal activities of the chloroflavones **6** and **7** along with their corresponding chalcones **4** and **5** in light of green protocol.

2. Experimental

2.1. *Materials and method*

Melting points were recorded with electro thermal melting point apparatus and were uncorrected. TLC was performed on Kieselgel GF_{254} and visualization was done by iodine vapour or UV light. The IR spectra were measured by FTIR spectrophotometer (Model-8900, Shimadzu, Japan) using KBr matrix in the range 4000-200 cm^{-1}. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on JEOL GS×400, GEOL JNM-AL 400 (400 MHz) and JEOL GS×400, GEOL JNMAL 400 (100 MHz) spectrometer with TMS as internal standard by using $CDCl_3$ as solvent. All reactions were carried out in a commercially available LG microwave oven (MB-3947C) having a maximum power output of 800 W operating at 2450 MHz.

2.2. *Synthesis of 1-(2-hydroxyphenyl)-3-(chlorophenyl)-prop-2-en-1-one (2'-hydroxy-chlorochalcone) derivatives*

A mixture of *o*-hydroxyacetophenone and aromatic chloroaldehydes (1:1 mol) dissolved in minimum amount of rectified spirit and KOH (10%) were placed in an Erlenmeyer flask. The flask was covered with a funnel and then it was taken in a domestic microwave oven. The reaction mixture was irradiated under 320 watt microwave irradiation for 100-140 sec. The progress of the reactions was monitored by TLC (*n*-hexane: ethyl acetate, 6:1) every 30 sec. The reaction mixture was cooled and diluted with ice cold water, acidified with dil. HCl and extracted with ether (3×15 mL). The ether layer was washed with water and dried over anhydrous Na_2SO_4 and the solvent was evaporated under

reduced pressure. The product was obtained as solid and recrystallized from ethyl acetate and *n*-hexane solvent mixture.

(E)-1-(2-hydroxyphenyl)-3-(2-chlorophenyl)-prop-2-en-1-one (2'-hydroxy-2-chlorochalcone), 4: Yellow crystals (yield: 99.63%); m.p. 53-54°C; IR (KBr), υ_{max} (cm^{-1}): 3468 (-OH), 3059, 1639 (>C=O), 1562 (C=C), 1485 (C=C, Ph), 1257, 1161, 1026, 829, 756. ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H (ppm): 6.96 (t, 1H, *J*=8 Hz, $C_{5'}$-H), 7.07 (d, 1H, *J*=8 Hz, $C_{3'}$-H), 7.40 (d, 2H, *J*=8 Hz, C_4-H and C_5-H), 7.52 (m, 2H, C_3-H and $C_{4'}$-H), 7.67 (d, 1H, *J*=15.6 Hz, C_α-H), 7.80 (d, 1H, *J*=8 Hz, C_6-H), 7.93 (d, 1H, *J*=8 Hz, $C_{6'}$-H), 8.33 (d, 1H, *J*=15.6 Hz, C_β-H), 12.73 (s, 1H, $C_{2'}$-OH). ^{13}C-NMR (100 MHz, $CDCl_3$), δ_C (ppm): 117.2 (C-3'), 121.2 (C-5'), 124.1 (C-α), 124.6 (C-1'), 126.2 (C-5) 127.2 (C-6), 129.1 (C-3), 130.3 (C-4), 131.2 (C-6'), 132.2 (C-2), 135.6 (C-1), 137.3 (C-4'), 143.4 (C-β), 158.2 (C-2'), 186.1 (>C=O).

(E)-1-(2-hydroxyphenyl)-3-(4-chlorophenyl)-prop-2-en-1-one (*2'-hydroxy-4-chlorochalcone),* 5: Bright yellow crystals (yield: 98.64%); m.p. 148-149°C; IR (KBr), υ_{max} (cm^{-1}): 3464 (-OH), 3066, 1639 (>C=O), 1581 (C=C), 1485 (C=C, Ph), 1203, 1153, 1018, 825, 752. ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H (ppm): 6.98 (t, 1H, *J*=7.2 Hz, $C_{5'}$-H), 7.05 (d, 1H, *J*=8 Hz, $C_{3'}$-H), 7.44 (d, 2H, *J*=8 Hz, C_3-H and C_5-H), 7.54 (m, 1H, $C_{4'}$-H), 7.63 (d, 2H, *J*=8 Hz, C_2-H and C_6-H), 7.65 (d, 1H, *J*=16 Hz, C_α-H), 7.93 (d, 1H, *J*=16 Hz, C_β-H), 8.04 (d, 1H, *J*=8 Hz, $C_{6'}$-H), 12.76 (s, 1H, $C_{2'}$-OH). ^{13}C-NMR (100 MHz, $CDCl_3$), δ_C (ppm): 117.2 (C-3'), 122.1 (C-5'), 123.6 (C-α), 124.1 (C-1'), 127.1 (C-2 & C-6), 128.6 (C-3 & C-5), 130.1 (C-6'), 133.2 (C-1), 133.5 (C-4), 137.1 (C-4'), 142.7 (C-β), 158.3 (C-2'), 186.3 (>C=O).

2.3. *Synthesis of 2-chlorophenyl-chromen-4-one (chloroflavone) derivatives*

1-(2-hydroxyphenyl)-3-(chlorophenyl)-prop-2-en-1-one derivatives (1 mmol) were suspended in (DMSO, 2 mL) and placed in an Erlenmeyer flask and iodine (0.02 mmol) was added. Then the covered flask was taken in the microwave oven. The mixture was irradiated under 320 watt microwave irradiation for 300-325 sec. The progress of the reactions was monitored by TLC (*n*-hexane: ethyl acetate, 4:1). The reaction mixture was diluted with water (excess), and extracted with diethyl ether (2 × 15 mL). The ether layer was washed with aqueous 20% sodium thiosulphate, water and dried over anhydrous Na_2SO_4 and the solvent was evaporated. The product was obtained as solid crystals. The solid products were recrystallized using ethyl alcohol and it gave blue fluorescence in UV light.

2-(2-chlorophenyl)-chromen-4-one (*2'-chloroflavone),* 6: Pale yellow crystals (yield: 98.12%), m.p. 118-119°C; IR (KBr), υ_{max} (cm^{-1}): 3066, 2981, 2939, 1639 (>C=O), 1604, 1573 (C=C), 1465 (C=C, Ph), 1373, 1222 (C-O), 1091, 1037, 825, 752. ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H (ppm): 6.86 (s, 1H, C_3-H), 7.47 (d, 1H, *J*=7.2 Hz, C_8-H), 7.73 (d, 2H, *J*=8 Hz, $C_{4'}$-H & $C_{5'}$-H), 7.60 (d, 1H, *J*=8.4 Hz, C_6-H), 7.74 (t, 1H, *J*=8 Hz, C_7-H), 7.90 (d,

2H, *J*=8.4 Hz, $C_{3'}$-H & $C_{6'}$-H), 8.26 (d, 1H, *J*=7.2 Hz, C_5-H). ^{13}C-NMR (100 MHz, $CDCl_3$), δ_C (ppm): 97.3 (C-3), 118.2 (C-8), 123.1 (C-6), 124.9 (C-4a), 126.9 (C-5'), 127.1 (C-6'), 128.7 (C-3'), 129.3 (C-4'), 130.2 (C-5), 131.2 (C-2'), 135.8 (C-7), 136.2 (C-1'), 158.3 (C-8a), 163.5 (C-2), 178.9 (>C=O).

2-(4-chlorophenyl)-chromen-4-one (*4'-chloroflavone),*7: Pale yellow crystals (yield: 97.41%), m.p. 185-186°C; IR (KBr), υ_{max} (cm^{-1}): 3066, 2924, 2854, 1654 (>C=O), 1624, 1573 (C=C), 1469 (C=C, Ph), 1369, 1219 (C-O), 1126, 1033, 848, 752. ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H (ppm): 6.68 (s, 1H, C_3-H), 7.44 (m, 3H, $C_{3'}$-H, $C_{5'}$-H & C_8-H), 7.54 (t, 2H, J=8.8 Hz, $C_{2'}$-H & $C_{6'}$-H), 7.68 (dd, 1H, J=7.2 Hz and 1.6 Hz, C_6-H), 7.73 (t, 1H, J=8.8 Hz, C_7-H), 8.28 (dd, 1H, J=9.6 Hz & J=1.6 Hz, C_5-H). ^{13}C-NMR (100 MHz, $CDCl_3$), δ_C (ppm): 97.4 (C-3), 118.1 (C-8), 123.6 (C-6), 126.1 (C-4a), 127.1 (C-2' & C-6'),127.9 (C-3' & C-5'), 129.9 (C-5), 133.2 (C-1'), 133.3 (C-4'), 136.2 (C-7), 157.6 (C-8a), 167.7 (C-2), 178.3 (>C=O).

2.4. *Antimicrobial activities*

2.4.1. *Antibacterial screening*

The antibacterial activity of the synthesized compounds **4-7** were studied against six human pathogenic bacteria viz. *Bacillus cereus* (G+), *Staphylococcus aureus* (G+), *Escherichia coli* (G-), *Vibrio choloriae* (G-), *Pseudomonas aeruginosa* (G-), and *Salmonella typhi* (G-). For determination of this activity the filter paper disc diffusion method [28-29] was employed. Ampicillin was used as standard antibiotic for the antibacterial test. Nutrient agar (NA) was used as the basal medium for test bacteria. These agar media were inoculated with 0.5 mL of the 24 hr liquid cultures containing 10^7 micro-organisms/mL. The diffusion time was 24 hr at 5°C and the incubation time was 12 hr at 37°C for bacteria. Discs with only DMSO were used as control. The diameter (in mm) of the observed inhibition zones were taken as a measure of inhibitory activity.

2.4.2. *Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation and minimum bactericidal concentrations (MBCs) as the lowest concentration of an antimicrobial that will prevent the growth of an organism after subculture onto antibiotic-free media. The MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials. The present study was performed to determine the MIC and MBC values of the synthesized compounds against six human pathogenic bacteria viz. *Bacillus cereus* (G+), *Staphylococcus aureus* (G+), *Escherichia coli* (G-), *Vibrio choloriae* (G-), *Pseudomonas aeruginosa* (G-), and *Salmonella typhi* (G-). The MIC and MBC of these

tested compounds in comparison to ampicillin were determined against those selected bacteria by broth micro-dilution method [30]. The main advantage of the "micro dilution method" for the MIC determination lies in the fact that it can readily be converted to determine the MBC as well. The media used in this respect were nutrient broth. Dilution series were setup with 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/mL of nutrient broth medium. To each well 100 µL of standardized suspension of the testing bacteria (10^7 cell/mL) were added and incubated at 30°C for 24 hr.

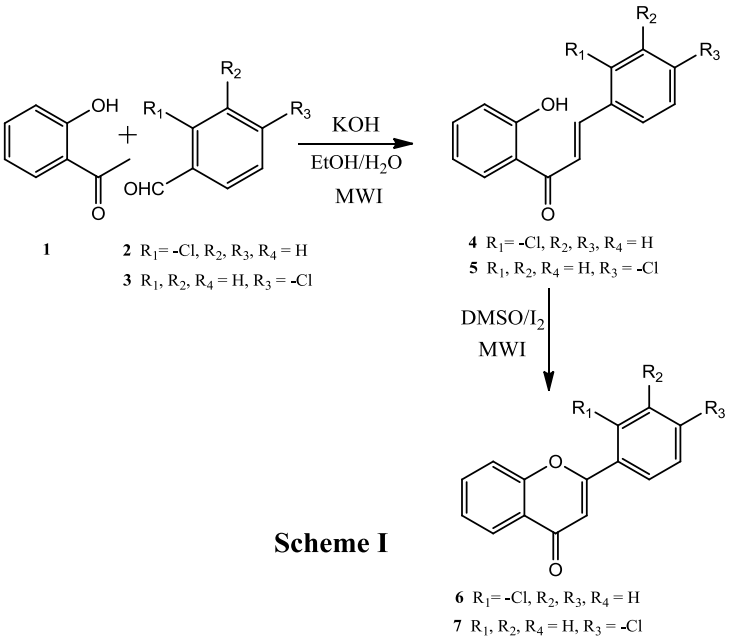
2.4.3. Antifungal screening

The antifungal activity of compounds **4-7** were evaluated towards four plant pathogenic and mold fungi, viz. *Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger and Rhizopus spp.*. The antifungal activities of the synthesized compounds were assessed by poisoned-food technique [31]. Nystatin (50 µg/disc) was used as standard fungicide for the antifungal test. Potato Dextrose Agar (PDA) was used as basal medium for test fungi. The test chemicals (100 µg) were mixed with sterilized potato dextrose agar (PDA) medium (42°C) of the rate 100 mg/mL PDA. The medium was poured in sterilized petri-plates. Inoculation was done by just touching the sample with the needle and then touching the needle at the middle of the plate containing solidified PDA media and then incubated at 27°C for 5 days. Radial growth of fungal colony was measured in mm after 3-5 days of incubation at (25±2) °C. A control set was maintained in each experimental using only PDA with DMSO as growth medium. The percentage inhibition of mycelial growth of the test fungi was calculated.

3. Results and Discussion

3.1. *Synthesis of 2-(chlorophenyl)-chromen-4-one (chloroflavone) derivatives*

The main aim of the present work involves the synthesis as well as antimicrobial activity of two derivatives of chloroflavones viz., 2'-chloroflavone **6**, 4'-chloroflavone **7**. The synthesis of the above derivatives has been accomplished under microwave irradiation, as shown in Scheme 1. An important feature of this method is the survival of variety of functional group i.e. chloro group under the reaction conditions. The precursor chalcones, **4-5**, were prepared from 2-hydroxyacetophenone and aromatic chloroaldehydes using literature procedure [32]. Alkaline condensation of 2-hydroxyacetophenone **1** with 2-chlorobenzaldehyde **2** under microwave irradiation gave the corresponding 1-(2-hydroxyphenyl)-3-(2-chlorophenyl)-1-prop-2-en-one **4** with very high yield (99.63%). It was purified by recrystallization from *n*-hexane- ethyl acetate (7:1) mixture to obtain yellow crystals with m.p. 53-54°C. The IR absorption band at 3468 cm^{-1} indicated the presence of hydroxyl group. A positive ferric chloride test also indicated that compound **4** has a free hydroxyl group and a band at 1639 cm^{-1} showed the presence of a conjugated carbonyl group (>C=O).



1

2 R_1= -Cl, R_2, R_3, R_4 = H

3 R_1, R_2, R_4 = H, R_3 = -Cl

4 R_1= -Cl, R_2, R_3, R_4 = H

5 R_1, R_2, R_4 = H, R_3 = -Cl

6 R_1= -Cl, R_2, R_3, R_4 = H

7 R_1, R_2, R_4 = H, R_3 = -Cl

Scheme I

The ^{1}H-NMR spectrum of **4** displayed a triplet signal resonated at δ_H 6.96 (*J*=8 Hz) corresponding to one aromatic proton, C_5'-H *ortho* to the carbonyl group. The presence of olefinic protons of an α,β unsaturated ketone which were clearly observed at δ 7.67 (*J*=15.6 Hz) and δ 8.33 (*J*=15.6 Hz) corresponding to C_α-H and C_β-H, respectively. The higher coupling values show that the olefinic protons are in a *trans*- relationship. One doublet resonated at δ_H 7.07 (*J*=8 Hz) corresponding to one aromatic proton, C_3'-H. The presence of two-proton doublet signal and two-proton multiplet resonated at δ_H 7.40 and δ_H 7.52 were designated to C_4-H, C_5-H, and C_3-H, C_4'-H, respectively. The two doublet signals at a lower field, δ_H 7.80 (*J*=8 Hz) and δ_H 7.93 (*J*=8 Hz) were attributed to two aromatic protons, C_6-H and $C_{6'}$-H, respectively. A characteristic singlet at δ 12.73 indicated the presence of a chelated phenolic proton at $C_{2'}$-OH integrating for one proton. The ^{13}C-NMR spectrum of compound **4** showed the presence of fifteen signals attributed to fifteen carbons corresponding molecular formula $C_{15}H_{11}O_2Cl$**.** The ^{13}C-NMR spectrum showed the existence of a carbonyl group (>C=O) at δ 186.3. At the same time the olefinic carbon of C_α and C_β resonated at δ 124.1 and δ 143.4, respectively. Remaining signals were assigned to the rest of the aromatic carbons in the molecule.

Oxidative cyclization of 1-(2-hydroxyphenyl)-3-(2-chlorophenyl)-prop-2-en-1-one **4** into the corresponding 2-(2-chlorophenyl)-chromen-4-one **6** was carried out using $DMSO/I_2$ reagent under microwave irradiation. It was purified by recrystallization from ethyl alcohol and obtained as light brown needles with a very good yield (98.12%) and m.p.118-119°C. The formation of **4** has been supported by spectral data. The IR absorption at 1639 cm^{-1} showed the presence of a carbonyl group (>C=O) and the absence of a hydroxyl group band confirmed the oxidation of chalcone **4** into flavone **6**. Signal of

OH was also not observed in ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum of **6** also displayed a singlet resonated at δ_H 6.86 for one proton corresponding to C_3-H and showed that the C_β-H of the corresponding chalcone **4** involved in cyclization of chalcone to form corresponding flavone. The rest of the ^{1}H- and entire ^{13}C-NMR spectral data were in accordance with the structure of 2-(2-chlorophenyl)-chromen-4-one **6** (see experimental section).

Similarly, the structures of the compounds **5** and **7** have been elucidated by IR, ^{1}H- and ^{13}C-NMR spectral data (see experimental).

3.2. *Antibacterial activity*

The antibacterial activity of compounds **4-7** has been assayed at concentrations of 100 µg disc^{-1} against strains of both, gram-positive and gram-negative pathogenic bacteria. Initially, susceptibility testing was carried out by measuring the inhibitory zone diameters on nutrient agar (NA), with conventional paper disc method and the inhibitory zone diameters were read and rounded off to the nearest whole numbers (mm) for analysis. The inhibitory effects of compounds **4-7** against these organisms are given in Table 1. Some of the compounds showed low antibacterial activities and some were unable to show inhibition.

3.3. *Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of compounds **4-7** in comparison to ampicillin against antibiotic susceptible strains of both gram-positive and gram-negative bacteria viz. *Bacillus cereus* (G+), *Staphylococcus aureus* (G+), *Escherichia coli* (G-), *Vibrio choloriae* (G-), *Pseudomonas aeruginosa* (G-) and *Salmonella typhi* (G-) were determined. Amongst all the compounds tested, **6** and **7** showed lower MIC values against both the gram-positive and gram-negative bacteria strains. The MBC of compounds **6** and **7** showed lower values and compounds **4** and **5** showed higher values for all bacteria strains. The MIC and MBC level of compounds **4-7** against these organisms are given in Tables 2 and 3, respectively.

3.4. *Antifungal activity*

The antifungal activity of compounds **4-7** has been assayed *in vitro* at a concentration of 100 µg disc^{-1} against *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus* and *Rhizopus spp.*. The result of the percentage inhibitions of mycelial growth of the selected chemicals are presented in Table 4**.** The overall results of the present investigation showed that chloroflavones are somewhat effective than their corresponding chalcones towards the selected organisms (fungi). The effective inhibition or fungicidal effects of all the tested compounds are compared to the standard antibiotic Nystatin-50.

Table 1. Inhibition against the two Gram-Positive and four Gram-Negative organisms by the compounds, **4-7.**

Compd No.	Diameter of zone of inhibition in mm (100 µg (dw)/ disc)					
	B. Cereus	*S. Aureus*	*E. Coli*	*Vibrio Choloriae*	*P. Aerugnosa*	*Salmonella Typhi*
4	…	…	*10	….	*12	*12
5	*10	*10	…	08	09	*10
6	*10	*10	*11	08	*13	*14
7	*13	*13	*12	*10	*11	*10
AMP	18	20	17	20	18	14

N.B: '(…)' Means no inhibition, '*' Means good inhibition, dw.= dry weight.

Table 2. MIC level of tested compounds against the organisms by the test chemicals, **4-7.**

Test organism	Minimum inhibitory concentration(µg mL^{-1}) of compounds				
	4	**5**	**6**	**7**	AMP
B. cereus	128	128	32	64	4
S. aureus	128	64	64	32	4
E. coli	128	128	128	64	4
Vibrio cholerae	256	128	128	64	4
P. aeruginosa	128	128	32	128	8
Salmonella typhi	128	128	64	32	4

Table 3. MBC level of tested compounds against the organisms by the test chemicals, **4-7.**

	Minimum bactericidal concentration (µg mL^{-1}) of compounds				
Test Organism	**4**	**5**	**6**	**7**	AMP
B. cereus	>256	128	64	64	8
S. aureus	256	128	128	64	8
E. coli	256	128	128	64	8
Vibrio cholerae	>256	256	128	128	8
P. aeruginosa	256	128	64	128	8
Salmonella typhi	256	256	64	64	8

Table 4. Percent inhibitions of mycelial growth of fungi treated with different chemicals, **4-7.**

Compounds No.	% Inhibition of mycelial growth (100 µg(dw)/ml PDA)			
	A. flavus	*A. niger*	*A. ochraceus*	*Rhizopus spp.*
4	47	58	60	63
5	47	61	71	66
6	53	64	71	58
7	53	67	69	71
Nystatin-50	53	61	66	68

Conclusion

In this work, we have demonstrated the synthesis of chloroflavones using microwave irradiation. The advantages of this method are high yields, relatively short reaction times, low cost, simple experimental and isolation procedures, and finally, it is in agreement with the green chemistry protocols. From the result of antibacterial and antifungal activities, it can be concluded that the chloroflavones ring system and presence of chloro groups are responsible for the antimicrobial effects. The antimicrobial activity data obtained during the study will be certainly useful to go for further research for drug designing and synthesizing new flavone derivatives.

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