

Full Length Research Paper

Antimicrobial and antioxidant activities of substituted halogenated coumarins

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Pathogens frequently display resistance to current drugs, which frequently lack selectivity/efficacy and have detrimental side effects. Thus, there is a constant need for novel therapeutic agents. Coumarins belong to the family of lactones, having a benzopyrone system that can be isolated from plants as well as total synthesis that can be carried out in the laboratory. To date, many chemical reactions have been established that can be used to synthesize coumarins. The synthesis of coumarins and their derivatives has attracted the attention of organic and medicinal chemists, as these are widely used as fragrances, pharmaceuticals and agrochemicals. In the present study, the antimicrobial and antioxidant activities of substituted coumarin analogue compounds have been screened. 3-(2-bromoacetyl)-2H-chromen-2-one (CMRN3) and 3-(2, 2-dibromoacetyl)-2H-chromen-2-one (CMRN6) showed bacterial growth inhibition for all the tested species except *Klebsiella pneumonia* and *Bacillus stearothermophilus*. CMRN4 and CMRN5 displayed moderate bacterial inhibition against *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*. CMRN3 and CMRN6 had a minimum inhibition concentration at 0.75 mg/ml against *B. cereus*, *Bacillus coagulans*, and *Streptococcus faecalis*. They displayed a minimum inhibitory concentration (MIC) of 1.5 mg/ml against *Escherichia coli* and *S. aureus*. (CMRN5) displayed an MIC at 0.75 mg/ml against *M. luteus* and 1.5 mg/ml against *S. aureus*. Compounds 3-(2-bromoacetyl)-6-chloro-2H-chromen-2-one (CMRN4), 3-(2-aminothiazol-4-yl)-6-bromo-2H-chromen-2-one (CMRN7), 3-acetyl-6-bromo-2H-chromen-2-one (CMRN1) exhibited potent antioxidant activity at 85, 61 and 56%, respectively, as evaluated by the DPPH free radical method.

Key words: Coumarin, antibacterial, antifungal, antioxidant.

INTRODUCTION

The synthesis of coumarins and their derivatives has attracted considerable attention from organic and medicinal chemists (Gacche and Jadhav, 2012; Paramjeet et al., 2012) because of the scope of possible beneficial effects on human health (Melagraki et al., 2009). The development of concomitant polymorphism in 3-acetyl coumarin also attracted chemists due to their potential application in various single crystal X-ray studies (Munshi et al., 2004).

Coumarins consist of a large class of phenolic substances

found in plants and are made of fused benzene and α -pyrone rings (Venugopala et al., 2013), as represented in Figure 1 (Smyth et al., 2009). Coumarins were first synthesized in 1868 via the Perkin reaction, and many simple coumarins are still prepared through this method. In early 1900, the Knoevenagel reaction emerged as an important synthetic method to synthesize coumarin derivatives with carboxylic acid at the 3-position. Nowadays, different methods for synthesis of coumarins have been reported, including the Pechmann, Reformatsky and Wittig

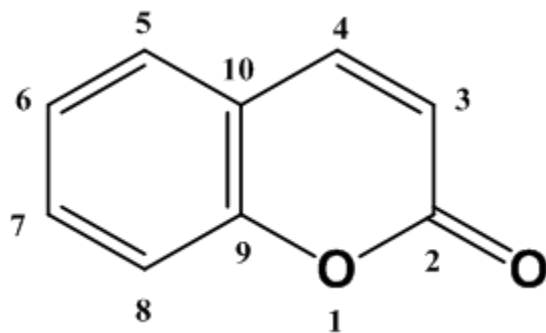


Figure 1. Chemical structure of 2H-chromen-2-one.

reactions (Nikhili et al., 2012).

As substitutions can arise at any of the six available sites of their basic molecular moiety (1, 2-benzopyrone), these compounds are exceptionally variable in structure. This structural variety leads to compounds displaying multiple pharmacological and biological properties such as antibacterial (Creaven et al., 2006), antifungal (Basanagouda et al., 2010), anti-inflammatory (Melagraki et al., 2009) and antioxidant (Abdel-Wahab et al., 2011) activities.

MATERIALS AND METHODS

All the chemicals were obtained from Aldrich and Merck chemical company and were used without further purification. Reactions were monitored by thin layer chromatography (TLC). TLC was performed on Merck 60 F-254 silica gel plates with ethyl acetate and *n*-hexane (7:3) as solvent system and visualization with ultra violet (UV)-light or iodine chamber. Melting points were determined on a Büchi melting point B-545 apparatus. Liquid chromatography–mass spectrometry (LC-MS) was performed on an Agilent Technologies 1200 series instrument. Yields refer to isolated products. Synthesis of title compounds CMRN1 – CMRN7 is illustrated in Figure 2

3-Acetyl-6-substituted-2H-chromen-2-one (CMRN1 and CMRN2)

A mixture of 5-substituted salicylaldehyde (0.1 mol) and ethylacetoacetate (0.11 mol) were taken in a conical flask, stirred and cooled. To this mixture, 1 g of piperidine was added with shaking. The mixture was then maintained at freezing temperature for 2 to 3 h, and then a yellow coloured solid mass separated out. The lumps were broken in cold ethanol and filtered. The solid was washed with cold ethanol and dried which gave satisfactory yields of CMRN1 and CMRN2. The products were recrystallized from hot glacial acetic acid and formation of the products was confirmed by the difference in melting point and R_f values on thin layer chromatography (Chopra et al., 2006).

3-(2-Bromoacetyl)-2H-chromen-2-one (CMRN3), (CMRN4), (CMRN5)

To a solution of 3-acetyl-6-substituted-2H-chromen-2-one (0.1 mol) in 20 ml of alcohol free chloroform, bromine (0.1 mol) was added to

5 ml of chloroform, with intermittent shaking. The mixture was heated for 15 min on a water bath to expel most of the hydrogen bromide, cooled and filtered. The solid on washing with ether gave satisfactory result of almost pure product. The formation of compounds was confirmed by the difference in melting point and R_f values on thin layer chromatography (Chopra et al., 2007).

3-(2,2-Dibromoacetyl)-2H-chromen-2-one (CMRN6)

To a solution of 3-(2-monobromoacetyl)-2H-chromen-2-one (0.1 mol) in 20 ml of alcohol free chloroform, bromine (0.1 mol) was added to 5 ml of chloroform, with intermittent shaking. The mixture was heated for 15 min on a water bath to expel most of the hydrogen bromide, cooled and filtered. The solid on washing with ether gave satisfactory results of almost pure product. The formation of compound was confirmed by the difference in melting point and R_f values on thin layer chromatography (Chopra et al., 2007).

3-(2-Aminothiazol-4-yl)-6-bromo-2H-chromen-2-one (CMRN7)

Suspension of 6-bromo-3-(2-bromoacetyl)-2H-chromen-2-one (0.1 mol) in 17.5 ml of hot ethanol was treated with thiourea (0.11 mol) a mild exothermic reaction took place, leading to a clear solution that soon deposited as crystals. The deposit was removed, washed with ethanol and then boiled with water containing sodium acetate which yielded (80%) of 3-(2-aminothiazol-4-yl)-6-bromo-2H-chromen-2-one and the product obtained was recrystallized from absolute ethanol. The formation of compound was confirmed by the difference in melting point and R_f values on thin layer chromatography (Chopra et al., 2009).

Biological screening

The compounds were screened for their anti-bacterial, anti-fungal and anti-oxidant activity according to standard protocols.

Antimicrobial activity

The development of antimicrobial resistance by many organism demands novel compounds from the pharmaceutical industry that are inexpensive and possess a broad spectrum activity. The antimicrobial activity and the minimum inhibitory concentration (MIC) of the compounds were carried out using the method of Cos et al. (2006) using the agar disc diffusion method. The bacterial strains used were based on the standard recommendations and were obtained from the stock collection in the Department of Biotechnology and Food Technology, Durban University of Technology. *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Streptococcus faecalis*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus stearothermophilus*, *Citrobacter freundii*, *Staphylococcus aureus*, and *Micrococcus luteus* were used in this study.

Cultures were plated out and verified. Stock cultures were stored in micro bank vials (Davies diagnostics, South Africa) using 50% glycerol. When needed they were plated out on Nutrient Agar (Biolab) plates and grown in nutrient broth (Biolab) for 24 h at 37°C. The concentrations of bacterial cells were adjusted to MacFarland standard of 0.5 absorbance which corresponded to 10^8 cfu/ml. A suspension (100 μ l of 10^8 cfu/ml) of the test bacteria was spread on Mueller Hinton Agar plates (Fluka, Biochemika). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper and they were dried in open sterile petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were inoculated with 10 μ l of sample at a concentration of 3 mg/ml and placed onto inoculated agar plates and incubated at 37°C for 24 h.

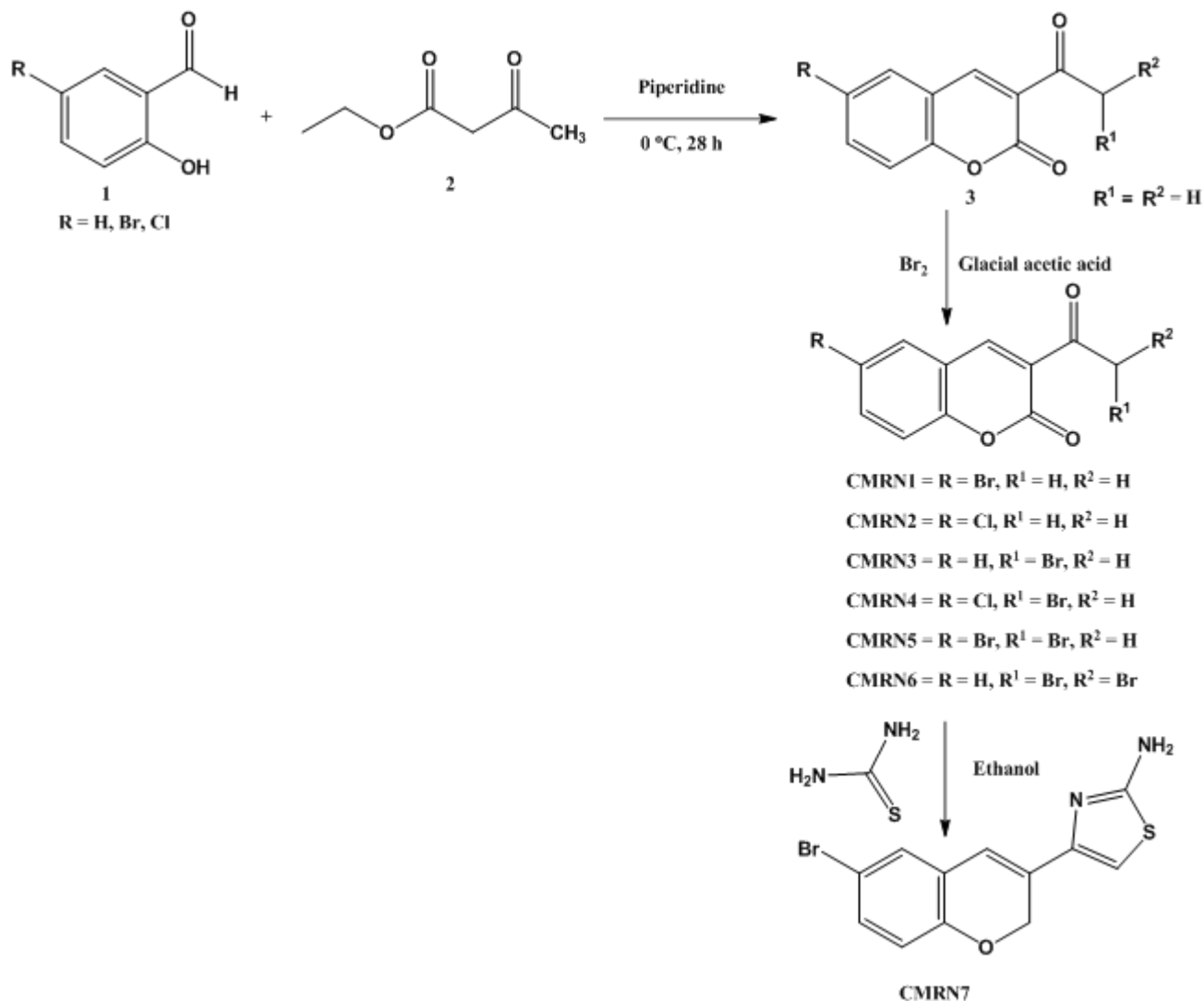


Figure 2. Schematic illustrating the pathway for the synthesis of substituted halogenated coumarins.

Each concentration was tested in triplicate. 100% dimethyl sulphoxide (DMSO) (10 μ l) was used as the negative control and ciprofloxacin 3 mg/ml (Fluka, Biochemika) was used as a positive control.

Preliminary disk diffusion assays were carried out in order to determine the level in inhibition of all compounds (CMRN1 to CMRN7) on the bacterial species. Where zones of inhibition equalled or exceeded 8 mm, these results were recorded and further analysis by MIC assay was conducted. The minimum inhibitory concentration (MIC) determination of the tested compounds was investigated with ciprofloxacin as a positive control. Dilutions of the test compounds (CMRN3, CMRN5 and CMRN6) were prepared in DMSO at the following concentrations 3 mg/ml, 1.5; 0.75; 0.37; 0.18 and 0.09 mg/ml. Ciprofloxacin was used as the positive control, tested at a concentration of 3 mg/ml against all bacterial species. All concentrations were tested against the bacterial species *B. cereus*, *B. coagulans*, *S. faecalis*, *E. coli* and *M. luteus*. MIC values for each compound against the bacteria tested are shown in Table 4.

The antifungal activity was evaluated on three yeast cultures, *Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae* and

two fungal species, *Aspergillus flavus* and *Aspergillus niger*. The yeast cultures were grown in Sabouraud dextrose broth for 24 h at 37°C. The fungi were incubated at 28°C for 4 to 7 days in Sabouraud Dextrose Agar until sporulation. The spores were collected in 10 ml sterile distilled water, counted in a Neubauer counting chamber and the concentration adjusted to 10⁶ spores/ml. Sterile distilled water containing the fungal spores (10⁶ spores/ml) was poured over the Sabouraud dextrose agar base plates (Biolab, Merck, South Africa). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper and they were dried in open sterile petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were inoculated with 10 μ l of sample at a concentration of 3 mg/ml and placed onto inoculated agar plates and incubated at 25°C for 24 h. Each concentration was tested in triplicate. 100% DMSO (10 μ l) was used as the negative control and whilst amphotericin B, 3 mg/ml (Fluka, Biochemika) was used as a positive control.

The effect of the compound was determined by measuring the diameter of clearing around the disks in mm. This zone of inhibition indicates the level of antimicrobial activity of the tested compound and the MIC value was the lowest concentration at which a zone of

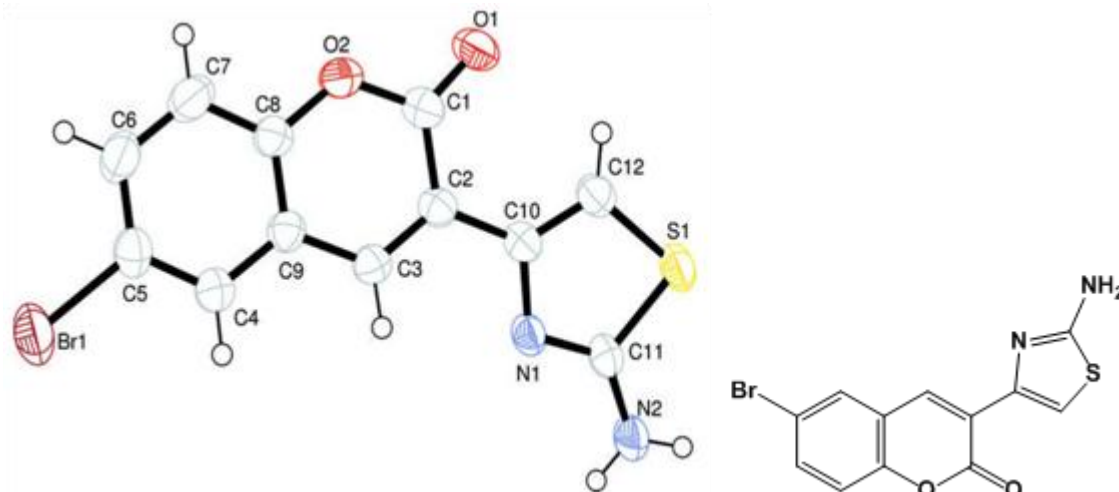


Figure 3. The structure of the title compound drawn with 50% probability displacement ellipsoids.

inhibition was noticed.

Antioxidant activity

The radical scavenging activity of the compounds were measured using the stable free radical scavenger, DPPH (2, 2 diphenyl-2-picryl hydrate), decolouration assay described by (Choi et al., 2002). Stock solutions of the substituted coumarins analogues were made in methanol and were diluted to final concentrations of 1000, 500, 250, 100, 80, 60, 40, 20 and 1 µg/ml in methanol. The results were compared with Quercetin-3-rutinoside which is a potent antioxidant. The radical scavenging was measured as the decolourization percentage of the test sample using the following formula:

$$\text{Scavenging capacity (\%)} = \frac{100 - (\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of negative control}} \times 100$$

RESULTS AND DISCUSSION

Characterization of CMRN7

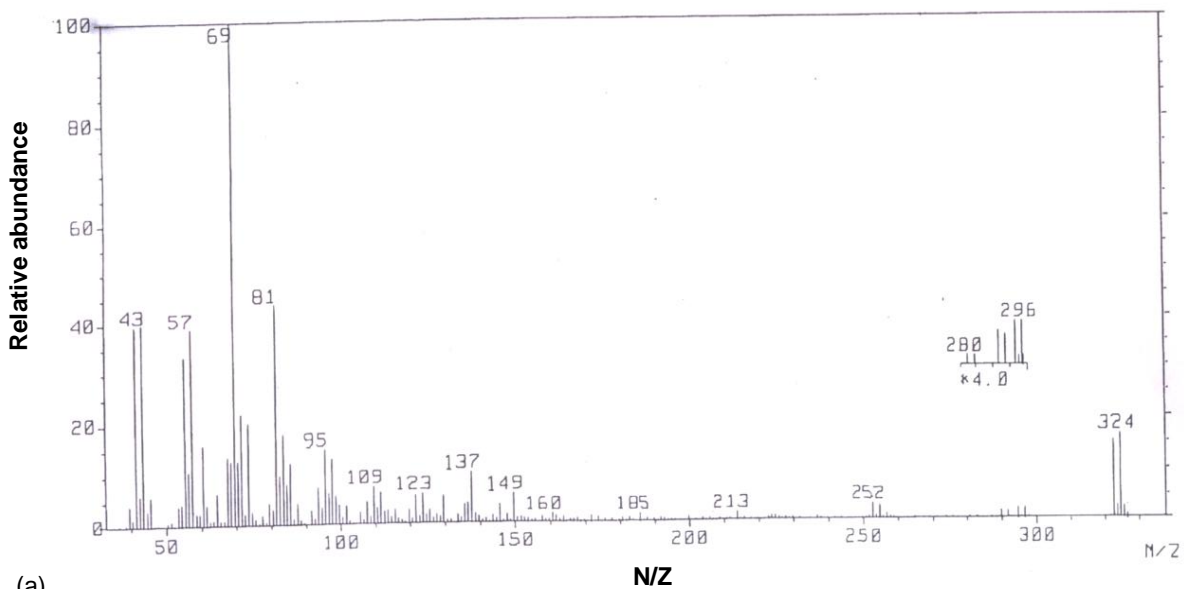
The crystal data of CMRN 7 and physicochemical characteristics of CMRN 1-7 are shown in Tables 1 and 2, respectively. The 50% probability displacement ellipsoids structure, mass spectrum and probable fragmentation pattern of CMRN 7 is shown in Figures 3, 4a and b, respectively. In the present study, the antimicrobial activities and the MIC values of a series of seven coumarins derivatives were screened for their antimicrobial activity using the broth micro dilution method against ten strains of bacteria. The results obtained depicted in Table 1 revealed that compounds 3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN3), 3-(2,2-dibromoacetyl)-2*H*-chromen-2-one (CMRN6), 6-bromo-3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN5) and 3-(2-bromoacetyl)-6-chloro-2*H*-chromen-2-one (CMRN4) exhi-

bited inhibitory effect on the growth of the tested strains *in vitro*. Thus, these compounds showed more or less pronounced antibacterial potencies, affecting both Gram-positive and Gram negative microorganisms.

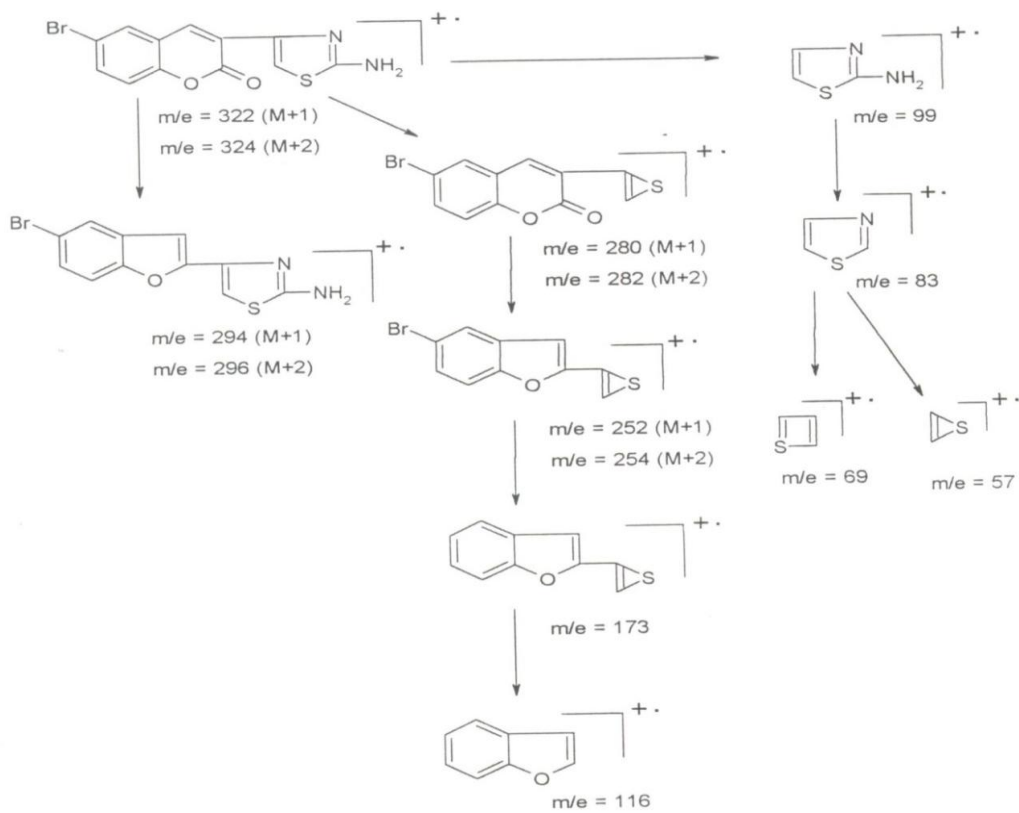
Among the active coumarin (Table 3), compounds 3-(2-Bromoacetyl)-2*H*-chromen-2-one (CMRN3) and 3-(2, 2-Dibromoacetyl)-2*H*-chromen-2-one (CMRN6) displayed bacterial inhibition growth to most of the tested microorganisms with the exception of *B. stearotherophilus* and *K. pneumonia*. The MIC was the lowest concentration of the tested compound that inhibited bacterial growth. It was observed that compound (CMRN3) and (CMRN6) had a minimum inhibition concentration (Table 4) of 0.75 mg/ml against *B. cereus*, *B. coagulans*, and *S. faecalis*. Compound CMRN5 showed an MIC of 0.75 mg/ml against *M. luteus*. Compounds (CMRN3) and (CMRN6) displayed an MIC of 1.5 mg/ml against *E. coli*, *S. aureus*, and CMRN5 displayed an MIC of 1.5 mg/ml against *S. aureus*.

This antibacterial activity was due to the incorporation of 3-(2-bromoacetyl) and 3-(2, 2-dibromoacetyl) on carbon three of the coumarin nucleus. Unpredictably the presence of bromide and chloride on carbon six of coumarin nucleus (Table 6) in compound CMRN4 and CMRN5 made the compounds exhibit less antibacterial activity against bacterial species tested. The substitution of the bromine on compound (CMRN6) and (CMRN3) at position 3 to the carbon atom at position 6 on the coumarin nucleus and the addition of 3-(2-aminothiazol-4-yl) on carbon 3 of the compound (CMRN7) contributed to a notable loss of antimicrobial efficiency. According to previous studies conducted by (Zavrsnik et al., 2011), a substitution in the para position of the compound's ring structure improves antibacterial activity. Our study indicates that when bromine is present in the ortho position of the coumarin compound's ring structure (Table

MASS SPECTRUM
 Sample: COMPOUND D
 RT: 0.18" EI (Pos.) GC 264.0c BP: m/z 69.0000 Int. 50.7142 LV 0.00
 Scan# (10)



(a)



(b)

Figure 4. (a) LC-MS and (b) probable fragmentation pattern of 3-(2-aminothiazol-4-yl)-6-bromo-2H-chromen-2-one (CMRN 7).

Table 1. Crystal data and measurement details for CMRN 7.

Crystal data	CMRN 7
Radiation	Mo K α
Crystal system	Monoclinic
Space group	<i>P</i> 2 ₁ / <i>n</i>
<i>a</i> (Å)	7.031 (4)
<i>b</i> (Å)	13.804 (8)
<i>c</i> (Å)	12.453 (7)
α (°)	90
β (°)	90.047 (9)
γ (°)	90
Volume (Å ³)	1208.6(12)

Table 2. Physicochemical characteristics of 3-mono/dibromoacetyl-6-halogenated coumarin analogues CMRN 1-7.

Code	MF (M. Wt.)	Yield (%)	mp. (°C)
CMRN 1	C ₁₁ H ₇ BrO ₃ (265)	96	120-122
CMRN 2	C ₁₁ H ₅ Br ₃ O ₃ (421)	95	146-148
CMRN 3	C ₁₁ H ₇ BrO ₃ (265)	98	220-222
CMRN 4	C ₁₁ H ₆ Br ₂ O ₃ (343)	95	204-206
CMRN 5	C ₁₁ H ₆ BrClO ₃ (299)	94	180-182
CMRN 6	C ₁₁ H ₇ ClO ₃ (222)	95	218-220
CMRN 7	C ₁₂ H ₇ BrN ₂ O ₂ S(321)	87	210-212

Table 3. Antimicrobial activity of substituted halogenated coumarin derivatives.

Bacteria	Zone of inhibition (mm)							Control ciprofloxacin
	CMRN1	CMRN2	CMRN3	CMRN4	CMRN5	CMRN6	CMRN7	
<i>B.cereus</i>	NA	NA	10	6	7	10	NA	29
<i>E.coli</i>	NA	NA	8	NA	NA	8	NA	35
<i>M.luteus</i>	NA	NA	6	7	8	6	NA	29
<i>S.aureus</i>	NA	NA	10	7	10	11	NA	25
<i>S.marcescens</i>	NA	NA	6	NA	NA	6	NA	35
<i>C.freundii</i>	NA	NA	6	7	NA	6	NA	30
<i>B.coagulans</i>	NA	NA	10	NA	7	10	NA	28
<i>B.stearothermophilus</i>	NA	NA	NA	NA	NA	NA	NA	30
<i>S.faecalis</i>	NA	NA	10	NA	NA	11	NA	22
<i>K.pneumoniae</i>	NA	NA	NA	NA	NA	NA	NA	36
Yeast								
<i>C.albicans</i>	NA	NA	NA	6	6	NA	NA	30
<i>C.utilis</i>	NA	NA	NA	6	6	NA	NA	28
<i>S.cerevisiae</i>	NA	NA	NA	NA	6	6	NA	28
Fungi								
<i>A.flavus</i>	NA	NA	NA	NA	NA	NA	NA	19
<i>A.niger</i>	NA	NA	NA	NA	NA	NA	NA	19

Data are mean \pm SD (n = 3); NA: no activity

Table 4. Minimum inhibition concentration of substituted halogenated coumarin derivatives (MIC).

Bacteria	MIC mg/ml		
	CMRN3	CMRN5	CMRN6
<i>B. cereus</i>	0.75	-	0.75
<i>E. coli</i>	1.5	-	1.5
<i>M. luteus</i>	-	0.75	-
<i>S. aureus</i>	0.75	1.5	1.5
<i>B. coagulans</i>	0.75	-	0.75
<i>S. faecalis</i>	0.75	-	0.75

Table 5. Antioxidant activity of the substituted halogenated coumarins.

Compound code	Concentration ($\mu\text{g/ml}$)								
	1	20	40	60	80	100	250	500	1000
CMRN1	7	8	10	13	16	18	30	45	56
CMRN2	6	7	8	9	15	16	17	18	23
CMRN3	4	6	7	8	9	10	12	14	28
CMRN4	6	17	23	29	30	34	56	72	86
CMRN5	12	14	16	17	18	20	22	23	25
CMRN6	24	26	28	30	31	32	33	34	36
CMRN7	14	21	25	26	27	30	40	46	61
- control	-	-	-	-	-	-	-	-	0
+ control (Rutin)	-	-	-	-	-	-	-	-	95

6), the antibacterial activity is still pronounced, but not to the same level obtained by (Zavrsnik et al., 2011), who obtained MIC's ranging from 0.03 to 0.001 mg/ml for all para substituted coumarin compounds tested against bacterial species.

In vitro antifungal effects of the investigated compounds were tested against three yeast cultures, *Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae* and two fungi, *A. flavus* and *A. niger*. It was observed that compound (CMRN5) was found to have slight activity against the three yeast species tested, compound (CMRN4) showed a slight activity against *C. albicans* and *C. utilis* and compound (CMRN6) displayed a slight activity against *S. cerevisiae*.

The results of the DPPH radical scavenging activities of the coumarin analogue are summarized in Table 5. The results obtained clearly indicate that some of the compounds showed considerable free radical-scavenging activities. The order of the reactivity was CMRN4 (86%) > CMRN7 (61%) > CMRN1 (56%). However, the remaining substituted coumarins analogue showed activity in the range of 23 to 36% at 1 mg/ml as compare to the Rutin which was 95%.

Conclusion

The investigation of antimicrobial screening data reveals

that the synthesized compounds, particularly CRMN3 and CRMN6, showed antibacterial activity against both Gram-positive and Gram-negative bacteria. Based on results, functional substitutions on the benzene ring selectively enhance or decrease inhibition of coumarin activity and the bromine on the acetyl group at carbon three on the coumarin nucleus seems to be a highly significant factor in influencing the biological activity of the compound. Compounds CMRN4, CMRN7 and CMRN1 displayed considerable antioxidant activity, 86, 61 and 56%, respectively.

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