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Synthesis, characterization, antimicrobial and biofilm inhibitory studies of new esterquats

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ABSTRACT

Novel esterquats (monoesterquats and diesterquats) were synthesized from 11-bromo undecanoic acid (11-BUA) and different alkyl amines. The prepared compounds were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis. 11-BUA was converted into methyl 11-bromo undecanoate which was further converted into amine ester (amine monoester and diester) by reacting with different aliphatic amines (hexyl, dodecyl, octadecyl, dioctyl and dicyclohexyl amine). Finally, the obtained amine esters were converted into esterquats (monoesterquat and diesterquat) by reacting with methyl iodide followed by ion exchange to afford chloride counter ion esterquats (**5a**–**h**). The synthesized esterquat products were studied for their antimicrobial and biofilm inhibitory activities. Among all the compounds, amine ester **3a** and esterquat **5d** showed potent antimicrobial activity towards pathogenic Gram-positive bacterial strains with minimum inhibitory concentration (MIC) values in the range of 3.9–15.6 μg mL^{-1} and 1.9–7.8 μg mL^{-1}, respectively. The esterquat **5d** also showed promising antifungal activity against *Candida albicans* MTCC 3017, *Candida albicans* MTCC 4748 and *Candida aaseri* MTCC 1962 strains with MIC value of 7.8 μg mL^{-1} which was identical to standard Miconazole. The compounds which exhibited antimicrobial activity were also effective in anti-biofilm activity and it was found that compound **5d** exhibited excellent biofilm inhibitory activity with IC_{50} value of 0.9 μg mL^{-1} against *Staphylococcus aureus* MLS16 MTCC 2940.

The amine moiety has played a central role in chemotherapeutics of numerous diseases due to its unique biological properties.[1,2] Nitrogen containing fatty acid derivatives like long chain aza, aziridine and heterocyclic based fatty esters, and quaternary ammonium compounds are important chemical entities synthesized and broadly used as antimicrobial agents.[3–5] Quaternary ammonium compounds (QACs) are ubiquitous in nature and find application in surfactants, antimicrobials, disinfectants and dyes.[6] QACs are widely used as bioactive agents and biocides that possess antimicrobial effect against a broad range of microorganisms and function over a wide range of pH and find use in domestic, industrial, agricultural, and medical applications such as wood preservatives, pesticides, hard-surface cleansers,[7–11] sanitizers/disinfectants,[12] bacterial biofilm eradicating agents[13] and fungicides.[14] The antimicrobial activity of QACs is primarily related to their cationic surfactant properties. The main mode of antimicrobial action of QACs is due to interaction with cell membranes, predominantly at the level of the cytoplasmic membrane causing disruption and leakage of the cellular content.[15,16] Another related class of soft antimicrobial amphiphilic compounds are esterquats (EQs) which have been shown to exhibit good antibacterial activity. The studies suggest that modification of head groups had a positive effect on the antimicrobial activity.[17,18] EQs were obtained via reactions between alkylation agents and tertiary amines synthesized from fatty acid and methyldiethanolamine (MDEA) or triethanolamine.[19] EQs and their derivatives have been reported since olden days and in the recent years these compounds gained attention due to their biodegradable properties and also to develop newer surfactants with higher bioactivity.[20–22] These molecules find extensive use as surfactants,[23–26] antimicrobial agents,[17,27–29] antistatic agents, corrosion inhibitors, fabric softeners.[30–32] Several reports showed the synthesis of nitrogen containing fatty esters like long chain aza, aziridine, azetidine and heterocyclic (piperidine and pyridine) based fatty esters, but reports on their biological activity are limited.[3,33] It is important to explore the possibilities of developing newer bio-based antimicrobials which can overcome the increased resistance of pathogenic microbes and biofilms. Therefore, the present study is aimed to synthesize fatty acid based novel amine esters and esterquats from

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11-BUA, and to evaluate their antimicrobial and anti-biofilm activities.

The novel esterquats (**5a**–**h**) were prepared via a new chemical synthetic approach starting from 11-BUA using different aliphatic amines and methyl iodide as shown in Figure 1. The synthesized intermediate amine esters (**3a**–**h**) and final esterquats (**5a**–**h**) were characterized by spectroscopic studies. The synthesized compounds were studied for their antimicrobial and biofilm inhibitory activities in the following sections. Three classes of esterquats (**5a**–**h**) were prepared and the synthetic route for each class involved four steps as shown in Scheme 1. In the first step, 11-BUA (**1**) was converted into methyl 11-bromoundecanoate (11-BUME) (**2**), followed by the synthesis of methyl-11-(alkylamino) undecanoates (**3a**–**c**, secondary amine monoester and **3g**–**h**, tertiary amine monoester) and dimethyl-11,11′-(alkylazanediyl)diundecanoates (**3d**–**f**, tertiary amine diester) by the reaction of 11-BUME with different aliphatic amines. In the third step, the synthesized secondary amine monoesters (**3a**–**c**), tertiary amine mono (**3g**–**h**) and diesters (**3d**–**f**) were converted into monoesterquats (**4a**–**c** and **4g**–**h**) and diesterquats (**4d**–**f**) by reacting with methyl iodide. The resultant esterquats (**4a**–**h**) were converted to the final esterquats (**5a**–**h**) with chloride counter ion by passing through Amberlyst A-26 Cl^- ion exchange resin.[34] Amberlyst A-26 is a macroporous, styrene divinylbenzene Cl^- anion exchanger, which enables the halide switch. During the process of passing the esterquats through the resin, the iodide counter ion is swapped and exchanged with the chloride counter ion. Further, all the synthesized compounds were obtained in good yields in the range of 85–96%.

Antimicrobial activity: The synthesized amine esters and esterquats were screened for their antimicrobial activity against both Gram-positive as well as Gram-negative bacterial strains along with *Candida albicans* fungal strain.[35] The results to this regard are shown in Table 1 which indicate that some of the compounds exhibited variable inhibitory effects with minimum inhibitory concentration (MIC) values ranging between 1.9 and 31.2 μg mL^{-1}. While, the compounds prepared from dodecyl/C12 carbon chain (**3b**, **3e** and **5e**) and octadecyl/C_{18} carbon chain (**3c**, **3f**, **5c** and **5f**) did not show any antimicrobial activity on the tested strains up to the maximum tested concentration of >125 μg mL^{-1} except for the compound **5b**. Earlier reports also suggest that the quaternized compounds with alkyl chain length above C_{12} did not show any antimicrobial property indicating that the antimicrobial activity is dependent on the alkyl chain lengths.[28] For Gram-positive bacteria, promising activity was observed with chain lengths of n = 12–14, while for Gram-negative bacteria the chain lengths of n = 14–16 showed better activity.[15]

The results from the present study showed that the synthesized esterquats with hexyl amine **5a** and **5d** showed good antimicrobial activity on Gram-positive bacteria with MIC values ranging between 1.9 and 31.2 μg mL^{-1}. Intermediate amine esters, i.e., secondary amine ester (**3a**), tertiary amine diester (**3d**) prepared using hexyl amine showed good antimicrobial activity on Gram-positive bacteria with MIC values ranging between 3.9 and 62.5 μg mL^{-1}, while the tertiary amine monoesters **3g**–**h** (synthesized from dioctyl and dicyclohexyl amine, respectively) showed MIC values ranging between 15.6 and 62.5 μg mL^{-1}. However, in comparison between amine esters and esterquats, the antimicrobial activity of esterquats was improved by quaternization of amine ester. We observed that after the quaternization of the amine esters **3a**–**h**, the antimicrobial activity of esterquats **5a**–**h** increased significantly. Among all the synthesized esterquats, **5a**, **5b**, **5d**, **5g** and **5h** displayed broad spectrum antimicrobial activity exhibiting excellent inhibitory effects on Gram-positive bacterial strains and less activity against Gram-negative bacterial strains.

Among these five compounds, **5d** was found to exhibit excellent antimicrobial activity with MIC values ranging between 1.9 and 7.8 μg mL^{-1}. The compound **5d** showed excellent antimicrobial activity against *Micrococcus luteus* MTCC 2470 with the MIC value of 1.9 μg mL^{-1}. The compound **5d** showed good inhibitory activity on *Staphylococcus aureus* MTCC 96 and *Staphylococcus aureus* MLS16 MTCC 2940 strains with the value of 3.9 μg mL^{-1} and against *Bacillus subtilis* MTCC 121 the MIC value was 7.8 μg mL^{-1}. Moreover, it also showed excellent antifungal activity on *Candida albicans* MTCC 3017. The amine ester and esterquat prepared from dicyclohexyl amine, **3h** and **5h** showed promising antimicrobial activity with MIC values ranging between 3.9 and 62.5 μg mL^{-1}. The compounds prepared from dioctyl amine, **3g** and **5g** also showed good antimicrobial activity with MIC values ranging between 3.9 and 62.5 μg mL^{-1}. The antimicrobial screening results indicate that the esterquats are more potent than intermediate amine esters. Further, the minimum bactericidal concentration (MBC) was also evaluated for the synthesized amine esters and esterquats and the MBC values ranged between 3.9 and

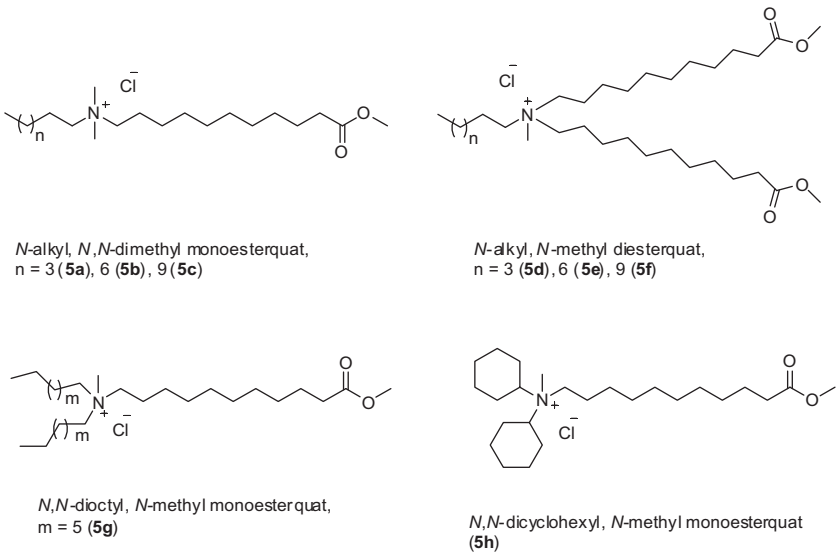
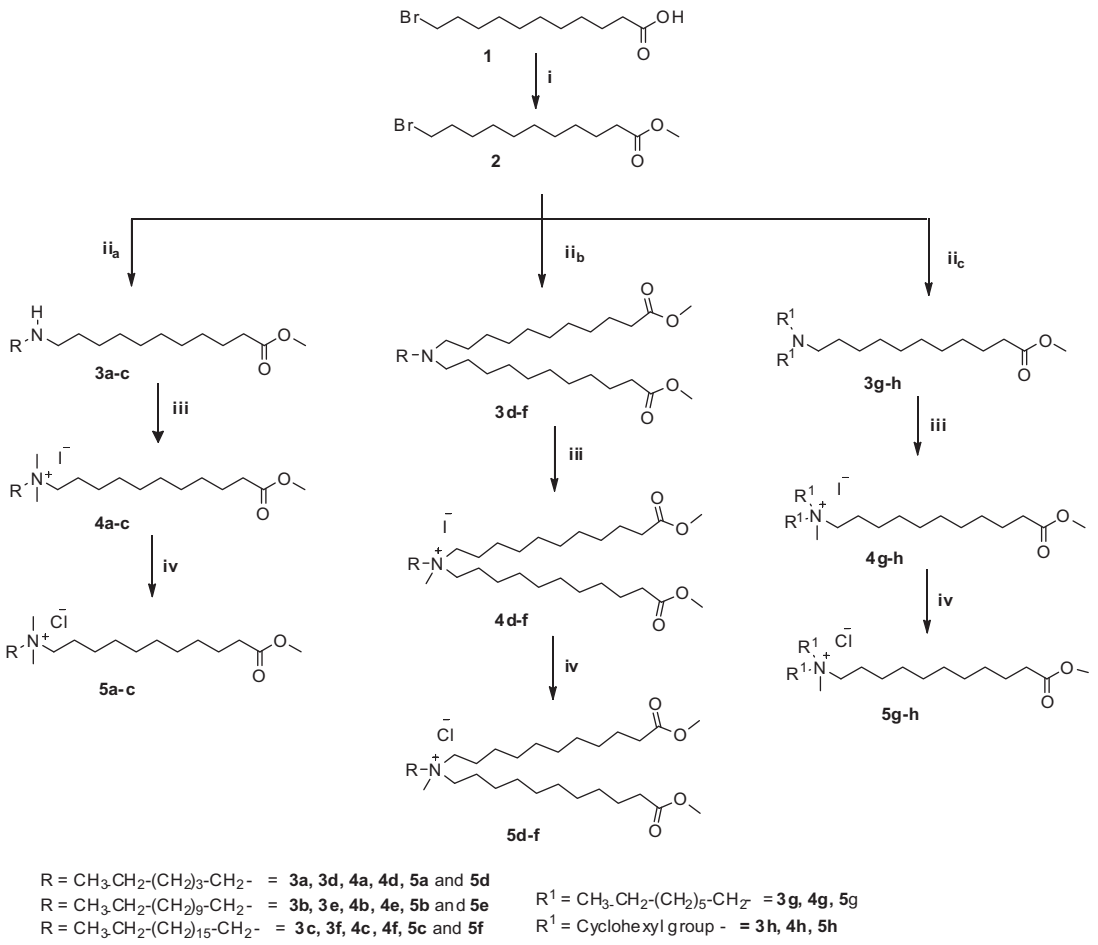


Figure 1. Molecular structures of the synthesized novel esterquats.



Scheme 1. Reagents and conditions: (i) methanol, PTSA, reflux, 6 h, 99.5%; (ii$_a$) R-NH$_2$ (1.1 equiv), K_2CO_3, 90–110 °C, 12 h, 85–90%; (ii$_b$) R-NH$_2$ (0.55 equiv), K_2CO_3, 90–110 °C, 12 h, 88–91%; (ii$_c$) R^1-NH-R^1 (1.1 equiv), K_2CO_3, 90–110 °C, 12 h, 95–96% (iii) CH_3I, K_2CO_3, rt, 97–99%; (iv) Amberlyst A 26 chloride ion resin, 96–98%.

Table 1
Antimicrobial activity of synthesized amine esters and esterquats

Test compounds	Minimum inhibitory concentration (μg mL^{-1})							
	[a]S. a.	[a]B. s.	[a]S. m.	[a]M. l.	[b]K. p.	[b]E. c.	[b]P. a.	[c]C. a.
3a	3.9	7.8	3.9	15.6	>125	>125	>125	15.6
3b	>125	>125	>125	>125	>125	>125	>125	>125
3c	>125	>125	>125	>125	>125	>125	>125	>125
3d	31.2	62.5	62.5	31.2	>125	>125	>125	>125
3e	>125	>125	>125	>125	>125	>125	>125	>125
3f	>125	>125	>125	>125	>125	>125	>125	>125
3g	15.6	>125	62.5	31.2	>125	>125	>125	>125
3h	31.2	62.5	31.2	15.6	>125	>125	>125	>125
5a	3.9	7.8	7.8	3.9	15.6	31.2	>125	31.2
5b	15.6	15.6	7.8	7.8	7.8	15.6	>125	31.2
5c	>125	>125	>125	>125	>125	>125	>125	>125
5d	3.9	7.8	3.9	1.9	>125	>125	>125	7.8
5e	>125	>125	>125	>125	>125	>125	>125	>125
5f	>125	>125	>125	>125	>125	>125	>125	>125
5g	3.9	7.8	7.8	3.9	31.2	>125	>125	15.6
5h	3.9	7.8	3.9	7.8	>125	>125	>125	15.6
Ciprofloxacin	0.9	0.9	0.9	0.9	0.9	0.9	0.9	—
Miconazole	—	—	—	—	—	—	—	7.8

MIC-minimum inhibition concentration and the values are mean of three determinations. [a] Gram-positive bacteria; [b] Gram-negative bacteria; [c] fungus; S. a. (*Staphylococcus aureus* MTCC 96); B. s. (*Bacillus subtilis* MTCC 121); S. m. (*Staphylococcus aureus* MLS16 MTCC 2940); M. l. (*Micrococcus luteus* MTCC 2470); K. p. (*Klebsiella planticola* MTCC 530); E. c. (*Escherichia coli* MTCC 739); P. a. (*Pseudomonas aeruginosa* MTCC 2453); C. a. (*Candida albicans* MTCC 3017).

31.2 μg mL^{-1} which is twice the MIC value for the compounds **3a**, **5a**, **5b**, **5d**, **5g** and **5h**. The results to this regard are shown in Supplementary data Table S1.

Antifungal activity: The synthesized compounds, i.e., amine ester (**3a**) and esterquats (**5a**, **5b**, **5d**, **5g** and **5h**) showed good antifungal activity against *Candida albicans* MTCC 3017 (Table 1), which

Table 2
Antifungal activity of synthesized amine esters and esterquats

Fungal strain	Minimum inhibitory concentration (MIC, μg mL^{-1})						
	3a	**5a**	**5b**	**5d**	**5h**	**5g**	Miconazole
C. a. 3017	15.6	15.6	31.2	7.8	15.6	15.6	7.8
C. a. 183	31.2	15.6	31.2	15.6	15.6	31.2	7.8
C. a. 227	31.2	31.2	31.2	15.6	15.6	15.6	7.8
C. a. 854	15.6	15.6	31.2	31.2	31.2	15.6	7.8
C. a. 1637	15.6	31.2	62.5	15.6	31.2	31.2	7.8
C. a. 3018	31.2	15.6	62.5	15.6	15.6	31.2	7.8
C. a. 3958	31.2	31.2	62.5	15.6	15.6	31.2	7.8
C. a. 4748	62.5	15.6	15.6	7.8	15.6	15.6	7.8
C. a. 7315	62.5	15.6	31.2	15.6	15.6	15.6	7.8
C. p. 1744	31.2	15.6	62.5	15.6	15.6	62.5	7.8
C. as. 1962	15.6	31.2	31.2	7.8	15.6	15.6	7.8
C. g. 3019	31.2	15.6	31.2	15.6	31.2	31.2	7.8
C. k. 3020	15.6	15.6	62.5	31.2	31.2	31.2	7.8
I.s. 4755	31.2	15.6	31.2	15.6	15.6	31.2	7.8

MIC values are the mean of three estimations. C.a. 3017 (*C. albicans* MTCC 3017); C. a. 183 (*Candida albicans* MTCC 183); C. a. 227 (*C. albicans* MTCC 227); C. a. 854 (*C. albicans* MTCC 854); C. a. 1637 (*C. albicans* MTCC 1637); C. a. 3018 (*C. albicans* MTCC 3018); C. a. 3958 (*C. albicans* MTCC 3958); C. a. 4748 (*C. albicans* MTCC 4748); C. a. 7315 (*C. albicans* MTCC 7315); C. p. 1744 (*Candida parapsilosis* MTCC 1744); C. as. 1962 (*Candida aaseri* MTCC 1962); C. g. 3019 (*Candida glabrata* MTCC 3019); C. k. 3020 (*Candida krusei* MTCC 3020) and I. s. 4755 (*Issatchenkia hanoiensis* MTCC 4755).

prompted us to further test these compounds against thirteen more fungal strains and the results to this regard are tabulated in Table 2. As evident from the results, all the six compounds showed excellent to good antifungal activity with MIC values ranging between 7.8 and 62.5 μg mL^{-1}. The compound **5d** was found to be most effective among the all tested compounds which showed excellent activity identical to the standard antifungal drug (Miconazole) with a MIC value of 7.8 μg mL^{-1} against *C. albicans* MTCC 3017, *C. albicans* MTCC 4748 and *Candida aaseri* MTCC 1962.

Moreover, this compound also displayed good antifungal activity against *Candida albicans* MTCC 183, *C. albicans* MTCC 227, *C. albicans* MTCC 1637, *C. albicans* MTCC 3018, *C. albicans* MTCC 3958, *C. albicans* MTCC 7315, *Candida parapsilosis* MTCC 1744, *Candida glabrata* MTCC 3019 and *Issatchenkia hanoiensis* MTCC 4755 with the MIC value of 15.6 μg mL^{-1} and showed moderate activity against *C. albicans* MTCC 854 and *Candida krusei* MTCC 3020 with MIC value of 31.2 μg mL^{-1}. The synthesized compounds from amines like dicyclohexyl amine (**5h**) and dioctyl amine (**5g**) showed good to moderate antifungal activity with MIC values ranging between 15.6 and 31.2 μg mL^{-1}, while the other compounds like **3a**, **5a** (C_6 carbon chain) and **5b** (C_{12} carbon chain) also showed good to moderate antifungal activity. Based on the antifungal screening results, it can be concluded that the compounds with above C_{12} carbon chain length did not show any antifungal activity.

The selected compounds from the above study were further tested for their minimum fungicidal concentration (MFC). The compound **5d** displayed a MFC value of 7.8 μg mL^{-1} identical to the standard Miconazole against *Candida albicans* MTCC 4748. Moreover, the MFC results correlated well with the antifungal activity data and the MFC values ranged between 7.8 and 62.5 μg mL^{-1} which is twice the MIC value for all the tested compounds. The results to this regard are shown in Supplementary data Table S2.

Anti-biofilm assay: Biofilms are complex communities of bacteria that exist in a self-produced matrix of polysaccharides, proteins, and extracellular DNA responsible for the growing resistance in bacteria towards antibiotics.[36] Several clinically significant pathogens producing biofilms are implicated in more than 80% of the bacterial infections.[37] Hence, the synthesized amine esters and esterquats were studied for their biofilm inhibitory activity against four bacterial strains, namely *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MLS16 MTCC 2940 and *Micrococcus luteus* MTCC 2470. The results on the biofilm inhibitory activity for the synthesized amine esters and esterquats shown in Table 3 indicated that these compounds exhibited good to moderate anti-biofilm activity against all the four tested strains. In particular, the compound **5d** demonstrated promising anti-biofilm activity with IC_{50} values of 0.9, 1.9, 1.4 and 4.1 μg mL^{-1} against *Staphylococcus aureus* MLS16 MTCC 2940, *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 2470 and *Bacillus subtilis* MTCC 121, respectively. Other compounds such as **3a** (IC_{50} values ranged between 2.2 and 4.2 μg mL^{-1}), **5a** (2.1–4.8 μg mL^{-1}), **5h** (2.5–4.4 μg mL^{-1}) and **5g** (2.0–4.8 μg mL^{-1}) showed good biofilm inhibitory activity on all the tested bacterial strains. It is important to note that the ability to inhibit biofilm formation of these compounds to a maximum extent is in coherence with the antimicrobial activity against the respective strain. Previous literature reports also suggest that the QACs exhibited promising biofilm inhibitory activity.[38] Further, it is reported earlier that reactive oxygen species (ROS) is an important inducer of apoptosis in bacteria which may cause oxidative damage to cellular compounds and lead to cellular dysfunction or cell death.[39] Therefore, to elucidate whether oxidative stress is involved in the apoptotic cell death, the intracellular ROS accumulation within the cells of mature biofilms was measured using the fluorescent probe 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), a general ROS fluorescent probe. The DCFH-DA dye conversion mainly depends on the metabolically active cells in the biofilm. DCFH-DA is deacetylated in the cells where it can react quantitatively with intracellular

Table 3
Biofilm inhibition assay of synthesized compounds

Test compounds	IC_{50} values (μg mL^{-1})			
	S.a	B.s	S.m	M.l
3a	2.6 ± 0.12	4.2 ± 0.18	3.9 ± 0.16	2.2 ± 0.24
3d	20.1 ± 0.52	—	—	18.6 ± 0.32
3g	14.5 ± 0.19	—	—	17.2 ± 0.12
3h	21.3 ± 0.27	—	20.8 ± 0.35	19.9 ± 0.62
5a	2.5 ± 0.44	4.8 ± 0.32	3.2 ± 0.25	2.1 ± 0.22
5b	9.1 ± 0.33	8.5 ± 0.26	22.2 ± 0.56	8.8 ± 0.44
5d	1.9 ± 0.08	4.1 ± 0.18	0.9 ± 0.11	1.5 ± 0.16
5g	3.2 ± 0.26	4.6 ± 0.22	4.8 ± 0.24	2.0 ± 0.16
5h	2.5 ± 0.14	4.4 ± 0.38	3.6 ± 0.34	2.5 ± 0.18
Ciprofloxacin	0.6 ± 0.07	0.5 ± 0.06	0.4 ± 0.06	0.6 ± 0.08

IC_{50} (Half maximal inhibitory concentration) and the values are mean of three determinations. S. a. (*Staphylococcus aureus* MTCC 96); B. s. (*Bacillus subtilis* MTCC 121); S. m. (*Staphylococcus aureus* MLS16 MTCC 2940); M. l. (*Micrococcus luteus* MTCC 2470).

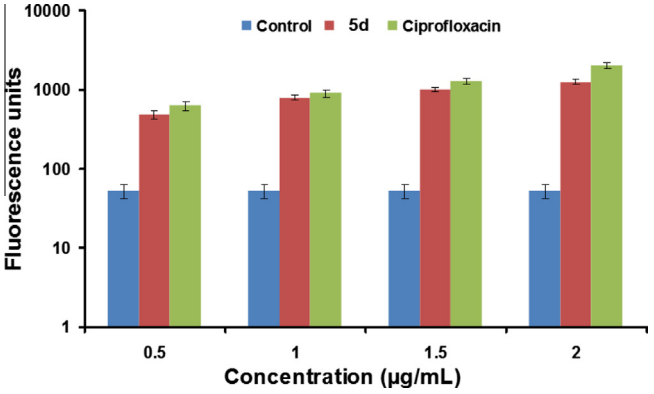


Figure 2. Intracellular ROS accumulation in *Staphylococcus aureus* MLS16 MTCC 2940 of compound **5d**.

radicals (mainly H_2O_2) to get converted to its fluorescent product (DCF), which is retained within the cells and thus provides an index of oxidation in the cell cytosol. The intracellular ROS accumulation in the biofilms of *Staphylococcus aureus* MLS16 MTCC 2940 was measured using DCFHDA dye, in which the dye conversion depends on the metabolically active cells in the biofilm.[40] After treatment with esterquat (**5d**) and Ciprofloxacin, a significant increase in the accumulation of ROS levels was observed in *Staphylococcus aureus* MLS16 MTCC 2940 as shown in Figure 2.

When compared with untreated biofilms, the esterquat (**5d**) treated biofilms showed increased levels of ROS accumulation. The ROS measurements were also carried out separately in both sessile cells and in the supernatant. The ROS induced increase in fluorescence was observed only for the sessile cells, suggesting that the ROS accumulation is of intracellular origin. This accumulated ROS may be responsible for the bactericidal activity. Recent studies also indicate that under oxidative stress, the free radicals contribute to arrest the cell growth or cause bacterial mediated cell death by damaging the specific essential metabolic enzymes (cellular respiratory chain), disrupting cellular membrane, and DNA damage ultimately leading to cell lysis and death.[41]

In conclusion, we have synthesized new amine esters and esterquats by employing a simple and convenient synthetic approach with good yields using different aliphatic amines and 11-bromoundecanoic acid. Synthesized compounds were evaluated for antibacterial, antifungal and biofilm inhibitory activities. Some of the synthesized amine esters and esterquats showed promising to moderate antimicrobial activity. Further, some of the compounds also displayed potent antifungal activity against fourteen different *Candida* strains. Especially, the esterquat **5d** was found to exhibit excellent activity against *Candida albicans* MTCC 3017, *Candida albicans* MTCC 4748 and *Candida aaseri* MTCC 1962 strains. We observed that among the synthesized compounds, the antibacterial and antifungal activities decreased with increasing chain length of amine esters and esterquats. The compounds prepared using hexylamine, **3a**, **5a** and **5d** showed antibacterial and antifungal activities, while the compounds synthesized using dodecylamine and octadecylamine did not show any antimicrobial activity except the esterquat **5b**. Furthermore, the compound **5d** was identified as a promising lead molecule for further investigation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.03.002.

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