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Evaluation of MIC, MBC, MFC and anticancer activities of acylated methyl β-D-galactopyranoside esters

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Abstract

A bacterial resistance gained by microorganisms to classical antimicrobials is current challenges. Searching of antimicrobial agents with new structure and mode of action is an essential strategy of research. Therefore, some acylated derivatives of methyl β-D-galactopyranoside were employed as test compounds for *in vitro* antimicrobial functionality test against five human pathogenic bacteria and two phytopathogenic fungi.For comparative studies, biological activity of standard antibiotics, Azithromycin and Nystatin were also carried out against these microorganisms. The study revealed that the tested samples exhibited moderate to good antibacterial and antifungal activities. It was also observed that the test substances were more effective against fungal phytopathogens than those of the bacterial strains. Encouragingly, a good number of test compounds exhibited better antimicrobial activity than the standard antibiotics employed. Minimum Inhibition Concentration (MIC) and MBC test of methyl 2,3,4-tri-*O*-(3-chlorobenzoyl)-6-*O*-pivaloyl-β-D-galactopyranoside 12 was conducted good result against *S. aureus* and MIC, MBC were found to be 312.5 µg/disc and 625 µg/disc, respectively. In addition, MFC was invented to be 1250 µg/disc against *Candida albicans*. *In vitro* MTT assays revealed that compound 8 was effective against Ehrlich's ascites carcinoma (EAC) cells, resulting in 12.72% and 2.11% cell growth inhibition at concentrations of 200 and 6.25 µg/ml, respectively. So the acylated derivatives of D-galactopyranoside (2-14) may be considered as a potential source for developing new and better antimicrobial agents against a number of pathogenic organisms.

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Introduction

The ubiquity of carbohydrates in cell biology and the diversity of their biological functions make them a fascinating yet complex research subject. Carbohydrate-based drug design is one of the new frontiers of pharmacology and medicinal chemistry (Chi-Huey, 2003). The development of synthetic carbohydrates and carbohydrate derivatives (or glycomimetics) with better pharmaco-kinetic properties is a technical challenge. Carbohydrates have opened a potent research filed at interface between organic chemistry and medical sciences due to their actions as antibacterial (Nogueira *et al.*, 2009), antifungal (Chi-Huey, 2003), antitumor (Shen *et al.*, 2012), antiviral, anti-diabetic, anti-inflammatory antineoplastic and antiprotozoal activities (Aboelmagd *et al.*, 2011; Helmoz *et al.*, 2013; Nguyen *et al.*, 2015). In this respect, acylated monosaccharide was demonstrated to be stronger antimicrobial agents than the corresponding non-acylated monosaccharide derivatives.

In recent years, synthesis of several carbohydrates containing monosaccharide moiety with aglycon group at specific hydroxyl position have been reported that mimic to biologically important natural products. The acylated monosaccharide derivatives showed broad spectrum biological activities (Kawsar *et al.*, 2012; Kabir *et al.*, 2005).

During the last few decades, considerable works have been done in the field of biological evaluation of various chemical compounds (Singh *et al.,* 1990). Carbohydrates, especially- acylated glycoses and glycosides, are very important due to their effective biological activity. It is known that if an active nucleus is linked to another nucleus, the resultant molecule may possess greater potential for biological activity (Gupta *et al.*, 1997). From literature survey, it was revealed (Ghorab *et al.*, 2004) that a large number of biological compounds possess aromatic, heteroaromatic and acyl substituents. Nitrogen, sulphur and halogen containing substituents are also known to enhance the biological activity of the parent compound (Ghorab *et al.*, 2004).

Over the last few years, researchers in our laboratory carried out selective acylation of monosaccharide derivatives (Kabir *et al.*, 2005a, 2001; Kawsar *et al.*, 2015a, 2014) and also biological evaluation of the synthesised compounds (Kabir *et al.*, 2004, 2003, 1998; Kawsar *et al.*, 2016, 2015b).

It was observed that the combination of two or more acyl substituents in a single molecular framework enhances the biological activity many fold than their parent nuclei. For example, some acylated derivatives of D-glucopyranose were found more active than those of the standard antibiotics (Kabir *et al.*, 2005b; Kawsar *et al.*, 2015c).

Encouraged by these results and literature reports, we synthesised some acyl derivatives of methyl β-D-galactopyranoside (Fig. 1) containing various acyl groups (e.g. pivaloyl, acetyl, propionyl, butyryl, nonanoyl, myristoyl, palmitoyl, trityl, 4-*t*-butylbenzoyl, 4-methoxybenzoyl, 3-chlorobenzoyl, benzenesulphonyl and cinnamoyl) in a single molecular framework. Antimicrobial activities of these compound were carried out using a variety of bacterial and fungal strains and the results are reported here. In addition anticancer activities were also evaluated and described in this paper as first time.

Materials and methods

Tested chemicals

Methyl β-D-galactopyranoside (1) and its acylated derivatives (2-14) were used as test chemicals for the determination of antimicrobial (bacteria and fungi) activities. The test chemicals (Fig.-1, 2-14) were synthesized, isolated and purified at the Laboratory of Carbohydrate and Nucleoside Chemistry of the Department of Chemistry, University of Chittagong. The test tube cultures of bacterial and fungal pathogens were collected from the Molecular Microbiology Laboratory, Department of Microbiology, University of Chittagong. Dimethyl sulfoxide (DMSO) was used as a solvent for the test chemicals and a 10% (10 mg/ml) solution of the compound was used in the investigation. Proper

control was maintained with DMSO without chemicals.

Used bacteria and fungi

Test chemicals 2-14 were subjected to antibacterial screening studies against two Gram-positive and three Gram-negative bacterial strains viz ,. *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella abony* NCTC 6017 and *Pseudomonas aeruginosa* ATCC 9027. The following two fungal phytopathogens were used as test fungi: *Candida albicans* SC 5314 and *Aspergillus niger* ATCC 16404.

Antibacterial assay

The *in vitro* sensitivity of the bacteria to the test materials was done by disc diffusion method (Bauer *et al*., 1966). Mueller Hinton agar (MHA) media was distributed in sterilized Petri dishes. Bacterial suspension (0.1 ml) was taken in the sterile Petri dish and about 15-20 ml agar media was poured. Then it was rotated clock and anti-clockwise and waited for solidification. The paper discs (5 mm in diameter) have been soaked (20 µl/disc) with leaf extracts for antibacterial analysis. In performing the sensitivity spectrum analysis agar medium plate have been selected uniformly with the test organisms. Then the discs prepared with definite solvent extract have been placed on the medium surface. On the other hand, a disc containing each solvent was used as control (C). These plates are then kept at low temperature (4°C) for two to four hours to allow maximum diffusion of the compound. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the media. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel. The plates were then incubated at 37°C for 24 hours at the inverted position to allow maximum growth of the microorganisms. After incubation, the plates have been observed and results were noted as the "Zone of Inhibition" (clear distinct zone around the discs) in mm in diameter with transparent scale including the diameter of the discs. The diameter of the zone of inhibition was observed and measured in mm by a transparent scale and the experimentation was done in triplicates. All of the results were compared against the standard antibiotic azithromycin (Beximco Pharmaceuticals Ltd., Bangladesh).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC)

The MIC and MBC of the compounds that showed activity against the aforementioned organisms were determined by applying different concentrations of the compounds alongside the same bacterial loads in a nutrient broth. MIC and MBC were determined via the broth microdilution method (Amsterdam D, 2005).

Antifungal assay

The “poisoned food” technique (Grover and Moore, 1962) was used to screen for antifungal activity in which potato dextrose agar (PDA) was used as the culture medium. The test compounds were dissolved in dimethyl sulfoxide (DMSO) to a 1% (w/v) concentration. From this, a sterilized pipette was used to transfer 0.1 mL (containing 1 mg of the respective compound being tested) to a sterile petri dish, after which 20 mL of the medium was poured into the petri dish and allowed to solidify. Inoculation was performed at the center of each petri dish with a 5-mm mycelium block of each fungus. The mycelium block was prepared by applying a corkscrew to the growing area of a 5-day-old culture of the test fungi on PDA. The blocks were placed at the center of each petri dish in an inverted position to maximize contact between the mycelium and the culture medium.

The inoculation plates were incubated at 25°C ± 2°C, and the experiment was conducted in triplicate. A control sample (i.e., PDA without any test chemicals applied) was also maintained under the same conditions. After 5 days of incubation, the diameter of the fungal radial mycelia growth was measured. The average of three measurements was taken as the radial mycelia growth of the fungus in mm. The percentage inhibition of mycelia growth of the test fungus was calculated as follows:

$$I = \left\{\frac{C-T}{C}\right\} \times 100$$

Where, I is the percentage of inhibition, C represents the diameter of the fungal colony in the control (DMSO), and T is the diameter of the fungal colony during treatment. The results obtained were compared with those of the standard antifungal agent nystatin.

Minimum fungal concentration (MFC)

MFC were also assessed by testing various concentrations of the derivatives against fungal cultures.

Anticancer activity

MTT colorimetric assay

In this study, adult Swiss albino mice were first obtained from the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDRB). Cells were harvested from the mice, and their viability was checked using the trypan blue exclusion assay. *In vivo* proliferation of Ehrlich's ascites carcinoma (EAC) cells was performed according to the method reported by (Ahmed *et al.*, 2017). MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay was used to detect *in vitro* proliferation of EAC cells. Viable EAC cells (5×10^5 in 200 µl RPMI-1640 media) were placed in a 96-well culture plate in the presence and absence of different concentrations of the respective derivative under investigation (12.5–200 µg/ml) and incubated at 37°C in a CO_2 incubator for 24 h. After removal of an aliquot from each well, 10 mM of PBS (180 µl) and MTT (20 µl, 5 mg/ml MTT in PBS) was added, and the plate was incubated at 37°C for 4 h. Then, an aliquot was removed again, and 200 µl of acidic isopropanol was added to each well. The plate was agitated for 5 min and incubated at 37°C for 1 h before absorbance values were measured at 570 nm using a titer plate reader. The cell proliferation inhibition ratio was calculated as follows:

Proliferation inhibition ratio (%) = {(A – B) × 100}/A,
Where, A is the OD_{570} nm of the cellular homogenate (control) without the derivative and B is the OD_{570} nm of the cellular homogenate with the derivative added.

Results and discussion

The results of antimicrobial and anticancer activity studies of the test compounds (Fig. 1) are presented in Table 1-7 and Fig. 2-4.

Effects of test chemicals against bacteria

The test chemicals exhibited a promising inhibitory activity against a number of both Gram-positive and Gram-negative bacterial strains (Table 1 and 2; Fig. 2).

Table 1. Zone of inhibition observed for Gram-positive bacteria.

Compound no.	Diameter of the zone of inhibition in mm	
	Bacillus subtilis	*Staphylococcus aureus*
2	8.0 ± 0.19	NI
3	NI	NI
4	NI	3.0 ± 0.21
5	7.0 ± 0.13	NI
6	5.0 ± 0.22	7.0 ± 0.16
7	*14.0± 0.30	*17 ± 0.12
8	8.0 ± 0.21	8.0 ± 0.11
9	NI	NI
10	6.0 ± 0.23	NI
11	NI	NI
12	*21.0 ± 0.15	*12.0 ± 0.18
13	NI	NI
14	NI	NI
Azithromycin	**18.0 ± 0.16	**20.0 ± 0.19

N.B.: NI = No inhibition.

The inhibition data indicated that the chemical 12 was more active (21 mm) than the standard antibiotic, Azithromycin (18 mm). However, chemical 12 was also more active on *S. abony* (15 mm) and *P. aeruginosa* (15 mm). On the other hand, chemical 7 showed good activity against both *B. subtilis* (14 mm), *S. aureus* (17 mm) and chemical 8 also displayed moderate activity against *S. abony* (12 mm) and *P. aeruginosa* (11 mm). However, chemicals 2, 5, 6, 9 showed either little inhibition or did not show any inhibition against the bacterial strains examined. From the study it was also observed that the chemicals 12, 7 and 8 were very effective as compared to Azithromycin that led us to carry out the MIC and MBC tests for this chemical against these bacterial strains and the results are presented in Table 3 and 4.

Table 2. Zone of inhibition observed for Gram-negative bacteria.

Compound no.	Diameter of the zone of inhibition in mm		
	Escherichia coli	*Salmonella abony*	*Pseudomonas aeruginosa*
2	9.0 ± 0.28	NI	NI
3	NI	NI	NI
4	NI	NI	NI
5	*13.0 ± 0.25	8.0 ± 0.13	8.0 ± 0.11
6	NI	NI	NI
7	NI	NI	NI
8	8.0 ± 0.29	*12 ± 0.30	*11 ± 0.19
9	11.0 ± 0.18	9.0 ± 0.18	9.0 ± 0.21
10	NI	9.0 ± 0.31	NI
11	10 ± 0.30	NI	NI
12	NI	*15.0± 0.15	*15.0 ± 0.17
13	NI	NI	NI
14	NI	NI	NI
Azithromycin	**18 ± 0.39	**19 ± 0.38	**19 ± 0.39

N.B.: NI = No inhibition.

In general, it has been observed that antibacterial results of the selectively acylated D-galactopyranoside derivatives obtained by using various acylating agents followed the order of 12 > 7 > 8 > 5 > 10 > 6 > 2 > 4 for Gram-positive organisms and for Gram-negative bacteria the order was 12 > 5 > 8 > 7 > 10 > 2 = 11.

Table 3. MIC of test chemicals against five bacteria.

Bacteria	8 (µg/ml)	7 (µg/ml)	12 (µg/ml)
B. subtilis	625.0	625.0	625.0
S. aureus	1250.0	625.0	312.5
E. coli	625.0	ND	ND
S. abony	625.0	ND	625.0
P. aeruginosa	1250.0	ND	1250.0

N.B.: ND = Not done.

The MIC values of the chemicals 8, 7 and 12 were found to be 312.5 µg /disc and 625.0µg /disc. The MIC is indicative of the usefulness of these compounds as antimicrobial drugs but some other experiments must be carried out before these can be used as effective drugs.

Table 4. MBC of test chemicals against five bacteria.

Bacteria	8 (µg/ml)	7 (µg/ml)	12 (µg/ml)
B. subtilis	1250.0	1250.0	625.0
S. aureus	1250.0	625.0	625.0
E. coli	625.0	ND	ND
S. abony	1250.0	ND	1250.0
P. aeruginosa	1250.0	ND	1250.0

N.B.: ND = Not done.

Table 5. Antifungal activity by the test compounds.

Compound no.	% Inhibition of fungal mycelial growth	
	Aspergillus niger	*Candida albicans*
2	NI	45.0 ± 3.2
3	15.07 ± 1.8	NI
4	NI	40.0 ± 1.3
5	NI	NI
6	*95.10 ± 1.7	20.0 ± 1.8
7	NI	45.0 ± 1.4
8	20.05 ± 0.9	40.0 ± 0.9
9	60.0 ± 1.3	60.0 ± 1.2
10	65.27 ± 1.2	*90.0 ± 3.6
11	60.33 ± 1.6	*80.0 ± 2.4
12	*90.21 ± 1.1	45.0 ± 1.8
13	55.34 ± 1.5	60.0 ± 1.3
14	60.0 ± 1.3	NI
Nystatin	**66.4 ±1.8	**63.1 ± 1.9

N.B.: NI = No inhibition.

In addition the minimum bactericidal concentrations (MBC) values of the chemicals 8, 7 and 12 were found to be 625.0µg /disc in each case. As the tested chemicals have shown remarkable inhibitory activity against three potential pathogenic bacteria i.e. *B. subtilis, S. aureus, E. coli* and *S. abony*, the compounds should be subjected to further experiments to evaluate their efficacy.

Table 6. MIC of test chemicals against two fungi.

Bacteria	8 (µg/ml)	12 (µg/ml)
Candida albicans	625.0	1250.0
Aspergillus niger	ND	ND

N.B.: ND = Not done

Table 7. MFC of test chemicals against two fungi.

Fungi	8 (µg/ml)	12 (µg/ml)
Candida albicans	1250.0	1250.0
Aspergillus niger	ND	ND

N.B.: ND = Not done.

Effects of test chemicals against fungi

The tested compounds displayed marked toxicities towards a number of fungal phytopathogens. The antifungal screening data (Table 5-7 and Fig. 3) suggests that the test chemicals 6 (95.10 ± 1.7%) and 12 (90.21 ± 1.1%) showed marked toxicities towards *Aspergillus niger* even higher than the standard antibiotic, Nystatin (66.4 ±1.8%); compounds 10 (90.0 ± 3.6%) and 11 (80.0 ± 2.4%) showed excellent inhibition against *Candida albicans,* being higher than or comparable to Nystatin (63.1 ± 1.9%). However, the inhibition of mycelial growth of the chemicals 9 (60.0 ± 1.3%), 10 (65.27 ± 1.2%), 11 (60.33 ± 1.6%) and 14 (60.0 ± 1.3%) against *Aspergillus niger* and chemicals 9 (60.0 ± 1.2%) and 13 (60.0 ± 1.3%) against *Candida albicans* were reasonably high, though not as high as the standard antibiotic, Nystatin.

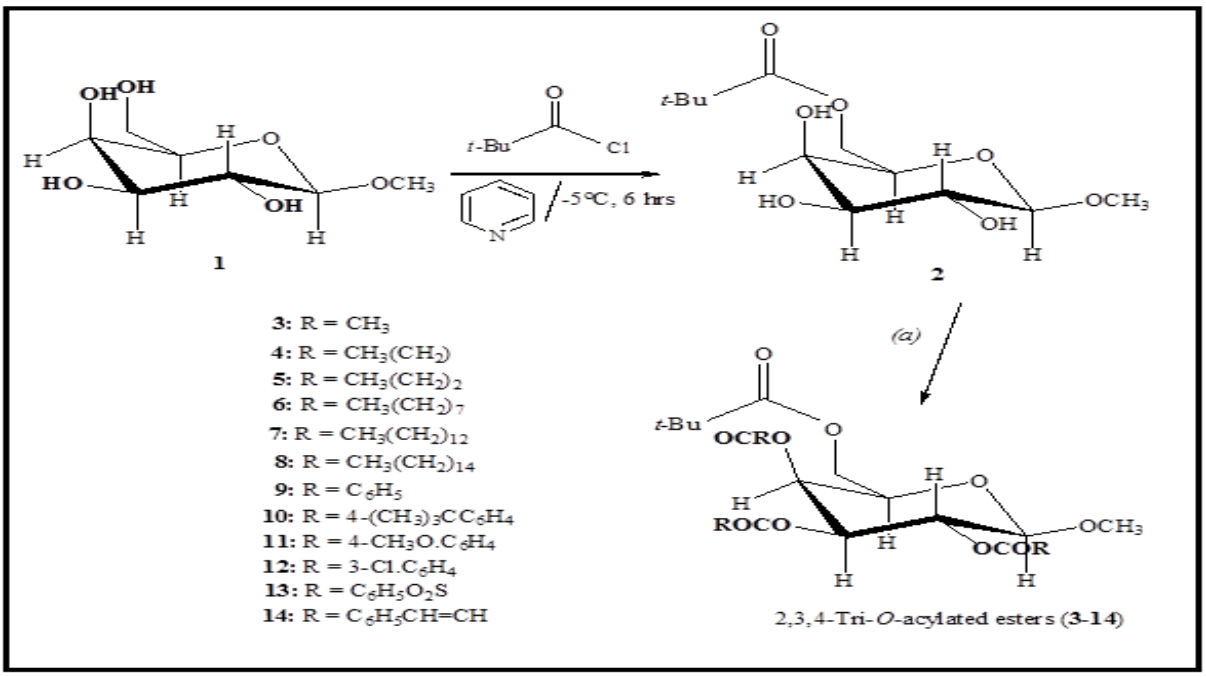

Fig. 1. Synthesis of acylated D-galactopyranoside esters. *Reagents and conditions*: (*a*) dry Pyridine, various acyl chlorides (2-14), 0°C, stirrer 6-8 hrs.

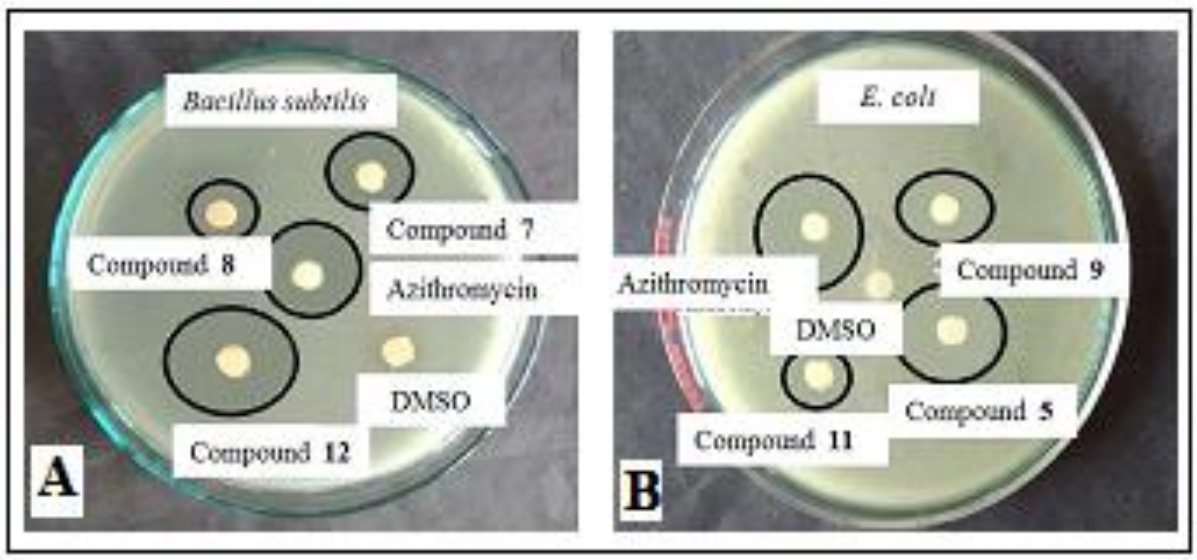

Fig. 2. Percentage of inhibition observed for A) *B. subtilis* by compounds 7, 8, and 12; B) *E. coli* by compounds 5, 9, and 11. DMSO was the negative control, whereas azithromycin represented the positive control.

Since compound 12 showed a maximum zone of inhibition against both bacteria and fungus, we choose this chemical to determine the MIC (Table 6) and MFC (Table 7) against the fungus *Candida albicans*. Moreover, we considered 8 for further work as it showed potential anticancer activity.

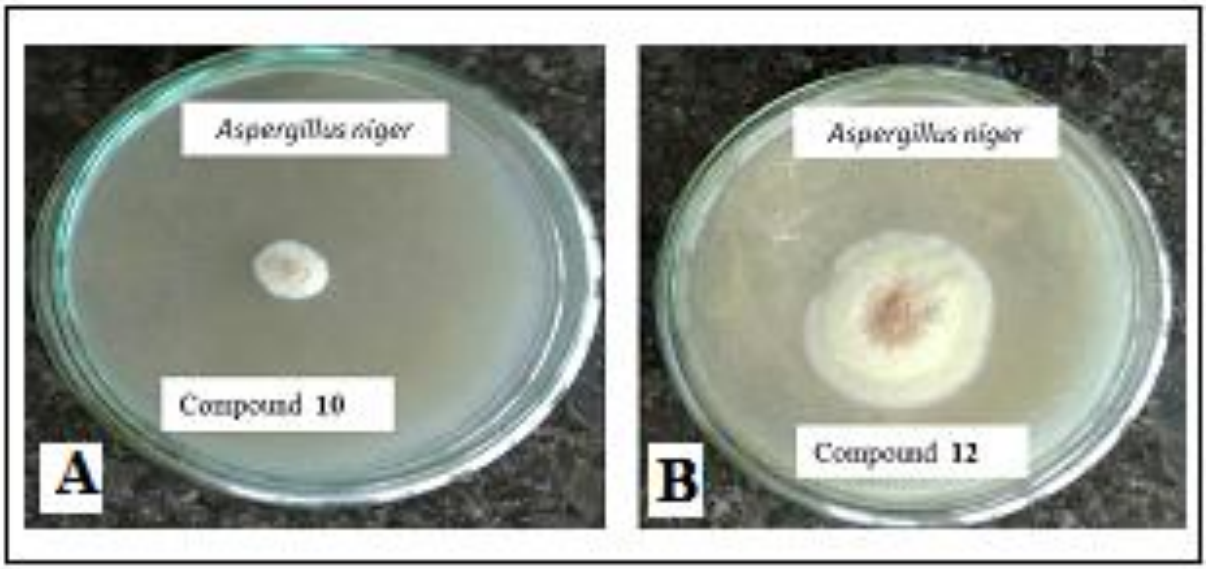

Fig. 3. Percentage zone of mycelial growth inhibition for compounds 10 and 12 against *A. niger* (C and D, respectively). DMSO represents the negative control, whereas nystatin is the positive control.

From the results we observed that the introduction of some specific functionality in the test chemicals improved their antimicrobial activities. We found that the presence of 3-bromobenzoyl, palmitoyl etc. acyl groups improved the antifungal activities of the test chemicals which was in accordance with our previous work (Kawsar *et al.*, 2014, 2015b, 2016).

It also observed that the benzene and substituted benzene nuclei play important role as common denominator for various antimicrobial activities (Kawsar *et al.*, 2018; Mirajul *et al.*, 2019).

Effect of test chemicals on EAC cells

MTT assay was used to investigate the effect of compound 8 (palmitoyl derivative) *in vitro* on EAC cells (Fig 4). At 200 µg/ml protein concentration, the inhibitory effect by compound (8) were 12.72%, at 100 µg/ml were 8.37%, at 50 µg/ml were 7.53%, at 25 µg/ml were 6.48%, at 12.5 µg/ml were 3.47% and at 6.25 µg/ml were 2.11% respectively. When the concentration decreased gradually, the inhibitory effect also reduced and finally reached 2.11% at 6.25 µg/ml of compound (8).

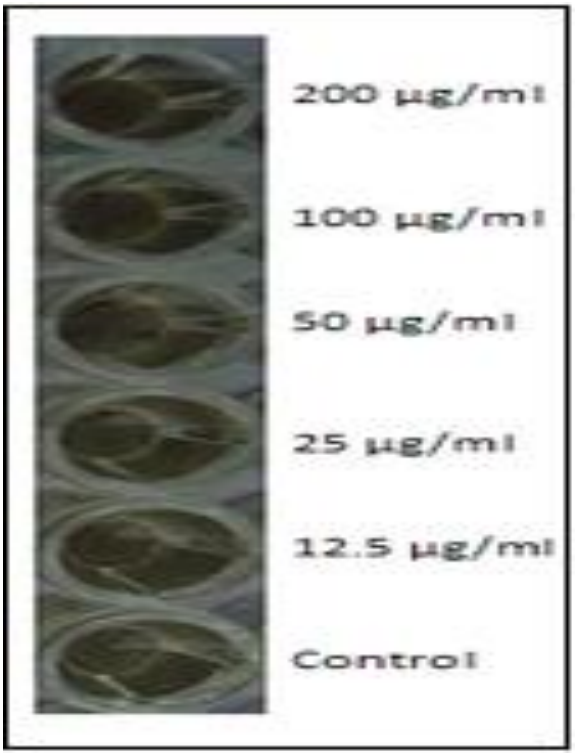

Fig. 4. Anti-cancer screening of compound 8.

Conclusion

This is the first report regarding the effectiveness of the selected chemicals against the selected pathogens. We also observed that some compounds such as 8

and 12 are active against both the Gram-positive and Gram-negative organisms and also mycelial growth efficacy. By considering of the above results, so the acylated derivatives (Fig.-1) may be considered as a potential source for developing new and better antimicrobial agents against a number of pathogenic organisms.

So it is hoped that the acylated derivatives of methyl β-D-galactopyranoside (2-14) might show potential antiviral, antituberculatic and anti-inflammatory activities.

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