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An *in vitro* assessment of antibacterial, antifungal and cytotoxic effects of D-glucopyranoside derivatives

Sarkar MA Kawsar*[1], Md M Rahman[1], Mariam Islam[1], Mohammad A Manchur[2], Imtiaj Hasan[3], Sultana Rajia[4]

[1]*Laboratory of Carbohydrate and Nucleoside Chemistry, Department of Chemistry, Faculty of Science, University of Chittagong, Chittagong, Bangladesh*

[2]*Department of Microbiology, Faculty of Biological Science, University of Chittagong, Chittagong, Bangladesh*

[3]*Department of Biochemistry and Molecular Biology, Faculty of Biological Science, University of Rajshahi, Rajshahi, Bangladesh*

[4]*Department of Pharmacy, Faculty of Science and Engineering, Varendra University, Rajshahi, Bangladesh*

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Abstract

Methyl α-D-glucopyranoside and its twelve acylated derivatives were employed as test chemicals for *in vitro* antimicrobial functionality test against four human pathogenic bacteria and two phytopathogenic fungi. The evaluation study revealed that the tested chemicals exhibited moderate to good antibacterial and antifungal activities. It was observed that the test chemicals were more effective against fungal phytopathogens than those of the bacterial strains. Encouragingly, a good number of test chemicals exhibited better antimicrobial activity than the standard antibiotics employed. It was also found that selectively acylated derivatives 12 and 13 showed promising inhibition against both Gram-positive and Gram-negative bacteria. That is with the introduction of various acyl groups such as 4-chlorobenzoyl, 2-bromobenzoyl, benzenesulphonyl the antibacterial functionality of the compound 2 increases. The mortality of brine shrimp was found to increase with the increase of concentrations of compounds. It is evident from the results of brine shrimp lethality testing that the test chemicals methyl 2,3,4-tri-*O*-decanoyl-6-*O*-octanoyl-α-D-glucopyranoside (5) and methyl 2,3,4-tri-*O*-lauroyl-6-*O*-octanoyl-α-D-glucopyranoside (6) showed highest levels of toxicity (i.e., 50% death) indicating its higher mortality and compound 8 displayed the good mortality as 40%.

* **Corresponding Author:** Sarkar M. A. Kawsar ✉ akawsarabe@yahoo.com

Introduction

Carbohydrates have opened a dynamic research filed at interface between organic chemistry and medical sciences due to their actions as antibacterial, antifungal, antitumor, antiviral, anti-diabetic, anti-inflammatory antineoplastic and antiprotozoal activities (Nogueira *et al.*, 2009; Chi-Huey Wong 2003; Shen *et al.*, 2012; Aboelmagd *et al.*, 2011; 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 (e.g. D-glucose, D-mannose etc.) 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). Microbial food contamination problems have been the cause of much public concern over the last few decades because of an increase in the number of infections and diseases originating from the consumption of spoiled food (Wilson 2007). Antibacterial and antifungal agents are necessary for food preservation, especially for food processors, because bacterial and fungal growths are important causes of food spoilage. For this reason, many investigators have focused their research efforts on finding new efficient, low toxicity and environmentally friendly antibacterial and antifungal agents.

Carbohydrates, especially acylated glycosides, are very important due to their effective biological activity (Andary *et al.*, 1982; Kabir *et al.*, 2009). Literature survey revealed that a wide variety of biologically active substances contain aromatic, heteroaromatic and acyl substituents (Gupta *et al.*, 1997). The benzene and substituted benzene nuclei play important role as common denominator for various biological activities. In the context of our studies, we observed that some acylated derivatives of monosaccharides (Kawsar *et al.*, 2016, 2015a, 2014; Kabir *et al.*, 2004) and nucleosides (Kawsar *et al.*, 2015b; Kabir *et al.*, 1998) also exhibited effective antimicrobial activities.

Over the last few years, researchers in our laboratory carbohydrate and nucleoside chemistry carried out selective acylation of monosaccharide derivatives (Kabir *et al.* 2002-2004) and also biological evaluation of the synthesized compounds (Kabir *et al.* 2004-2005). 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 ((Kawsar *et al.*, 2015a; Kabir *et al.* 2001).

It was also observed that substituted monosaccharide derivatives containing aromatic, heteroaromatic and acyl substituents along with chlorine, bromine, sulphur enhances the biological activity many fold than the precursor compounds (Kabir *et al.* 2003-2005). In this background, the present attempt has been undertaken to explore the antibacterial, antifungal and cytotoxic potential of some derivatives of methyl α-D-glucopyranoside.

Materials and methods

Tested chemicals

Some partially protected derivatives of D-glucopyranoside (2-13) (Fig. 1) were used as test chemicals. The chemicals were synthesized, isolated, purified and characterized in the Laboratory of Carbohydrate and Nucleoside Chemistry, Department of Chemistry, University of Chittagong. The antimicrobial assay of the chemicals was done in the Microbiology Research Laboratory, Department of Microbiology, Chittagong University and the tested micro-organisms (bacteria and fungi) were collected from this Laboratory. In all the cases, 2% solution (w/v) of the chemicals in chloroform ($CHCl_3$) was used.

Used bacteria and fungi

The following Gram-positive and Gram-negative bacteria were used as test organisms for antibacterial evaluation of the test chemicals (2-13): *Bacillus cereus* BTCC 19, *Bacillus subtilis* BTCC 17, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* CRL (ICDDRB).

The following fungal phytopathogens were used as test fungi: *Aspergillus niger* ATCC 16430 and *Rhizopus nigricans* ATCC 6227b.

Antibacterial activity studies

In vitro antibacterial activities of the synthesized D-glucopyranoside (2-13) were carried out by disc diffusion method (Bauer *et al.* 1966) with little modification (Miah *et al.*, 1990). Standard Nutrient Agar (NA) was used as basal medium for antibacterial tests throughout the study. Sterilized paper discs of 4mm in diameter and Petri dishes of 150mm in diameter were used throughout the experiment. The autoclaved Mueller-Hinton agar medium, cooled to 45˚C, was poured into sterilized Petri dishes to a depth of 3 to 4mm and after solidification of the agar medium; the plates were transferred to an incubator at 37˚C for 15 to 20 minutes to dry off the moisture that developed on the agar surface.

The plates were inoculated with the standard bacterial suspensions (as McFarland 0.5 standard) followed by spread plate method and allowed to dry for three to five minutes. Dried and sterilized filter paper discs were treated separately with 50µg dry weight/disc from 2% solution (in $CHCl_3$) of each test chemical using a micropipette, dried in air under aseptic condition and were placed at equidistance in a circle on the seeded plate. A control plate was also maintained in each case without any test chemical. These plates were kept for 4-6 hours at low temperature (4-6˚C) and the test chemicals diffused from disc to the surrounding medium by this time. The plates were then incubated at 35±2˚C for 24 hours to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the mean diameter of zone of inhibitions in millimeter. Each experiment was repeated thrice. All the results were compared with the standard antibacterial antibiotic ampicillin (20µg/disc, BEXIMCO Pharm Bangladesh Ltd).

Antifungal activity studies

The *in vitro* antifungal activities of the synthesized D-glucopyranoside (2-13) were investigated against two plant pathogenic fungi.

The investigation was based on poisoned food technique (Grover and Moore 1962) and the technique in some modification (Miah *et al.* 1990). Potato Dextrose Agar (PDA) was used as basal medium for test fungi throughout the study. Chloroform was used as a solvent to prepare the desired solution of the compound initially.

Control was maintained with chloroform. Two percent solution of the test chemical (in $CHCl_3$) was mixed with sterilized melted Saburaud agar medium to obtain the desired concentration (2%) and this was poured in sterilized Petri dishes. At the center of each plate, 5 days old fungal mycelial block (4mm in diameter) was inoculated and incubated at 27˚C. A control set was also maintained in each experiment. Linear mycelial growth of fungus was measured after 3-5 days of incubation. The percentage inhibition of radial mycelial growth of the test fungus was calculated as follows:

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

Where, I = Percentage of inhibition, C = Diameter of the fungal colony in control ($CHCl_3$), T = Diameter of the fungal colony in treatment. All the results were compared with the standard antifungal antibiotic nystatin (100µg/ml medium, BEXIMCO Pharm Bangladesh Ltd.).

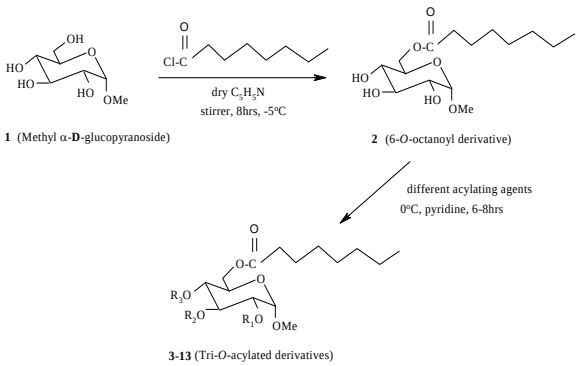
Cytotoxic activity studies

The cytotoxic assessment of the uridine derivatives were performed by brine shrimp lethality assay according to the McLaughlin *et al.* method (1991). Stock solutions of the acylated derivatives of D-glucopyranoside (2-13) (dissolved in DMSO) were prepared and 20, 40, 80 and 160µl were placed in different vials containing artificial sea water and 10 brine shrimp nauplii were added to each vial and finally the volume of each vial was adjusted to 4ml by the addition of artificial sea water and vials were designated as type-A, B, C and D, respectively. The experiment was performed in triplicate and a negative control (artemia nauplii in artificial sea water in absence of uridine derivatives) was used. After 24 and 48 hrs at room temperature, the number of dead nauplii was counted for each concentration using a magnifying glass.

From the data, the average percentage of mortality of nauplii was calculated for each concentration. No deaths were found in the controls. From the data, the average percentage of mortality of nauplii was calculated for each concentration.

Results and discussion

In the present investigation we selected the ten acylated derivatives (2 and 3-13, Fig. 1) of methyl α-D-glucopyranoside (1) as the test chemicals. The test chemicals contained different type of acyl groups in the molecular framework. All the twelve test chemicals and two standard antibiotics were screened *in vitro* against four human pathogenic bacteria and two plant pathogenic fungi. Antimicrobial or antibiotic resistant pathogen bacteria and fungi are causing major health problem worldwide. For these reasons the search for new antimicrobial agents with novel modes of action represents a major target in chemotherapy. The results of antibacterial and antifungal activities of the test chemicals (2-13) were measured in terms of zone of inhibition in mm and are presented in Table 1 and Table 2.



1 (Methyl α-**D**-glucopyranoside)

2 (6-*O*-octanoyl derivative)

3-13 (Tri-*O*-acylated derivatives)

Compound no.	$R_1=R_2=R_3$	Compound no.	$R_1=R_2=R_3$
3.	$CH_3(CH_2)_3CO$-	9.	$(CH_3)_3CCO$-
4.	$CH_3(CH_2)_4CO$-	10.	CH_3SO_2-
5.	$CH_3(CH_2)_8CO$-	11.	$C_6H_5SO_2$-
6.	$CH_3(CH_2)_{10}CO$-	12.	4-Cl.C_6H_4CO-
7.	$CH_3(CH_2)_{12}CO$-	13.	2-Br.C_6H_4CO-
8.	$CH_3(CH_2)_{14}CO$-		

Fig. 1. The structure of synthesized D-glucopyranoside derivatives (2-13).

A) Effect of test chemicals on Gram-positive bacteria

The results of the inhibition zone (diameter in mm) against the selected bacteria due to the effect of chemicals are mentioned in Table 1.

Bacillus cereus BTCC 19

The inhibition zone for this Gram-positive organism by different monosaccharide derivatives treatments is mentioned in Table 1 and Fig. 2. It was observed that only 2-bromobenzoyl derivative 13 (16mm) and 4-chlorobenzoyl derivative 12 (14mm) were move prone towards inhibition against this bacterium than that of the other chemicals, although much lower than that of the standard antibiotic, Ampicillin (21mm). Most of the chemicals indicated lower potentiality against this human pathogens,

Bacillus subtilis BTCC 17

The in *vitro* growth inhibitions of this Gram-positive bacterium due to the treatment of different test chemicals are shown in Table 1. It was found that, the inhibition zone of the 4-chlorobenzoyl derivatives 12 (15mm) (Fig. 2) more effective than that of other chemicals such as 11, 13, 6, 7, 8, 5, 4, and 2 which were somewhat less effective. The rest of the chemicals such as 3, 9, and 10 did not show any inhibition. All of these test chemicals were, however, less active against this bacterial strain than standard antibiotic, Ampicillin (25mm) in case of this bacterial strain.

B) Effect of test chemicals on Gram-negative bacteria

Escherichia coli ATCC 25922

The diameter zone of inhibition of *Escherichia coli* after different chemicals treatments is presented in Table 2 and Fig. 3. The inhibition zone of the 4-chlorobenzoyl (12) (14mm) were more effective than that of other chemicals such as 3, 5, 6, 7, 8 and 11 which were somewhat less effective. All of these test chemicals were, however, less active against this bacterial strain than standard antibiotic, Ampicillin (25mm) in case of this bacterial strain.

Pseudomonas aeruginosa CRL (ICDDR,B)

The screening data presented in Table 2 suggests that the inhibition zone of the 2-bromobenzoyl derivatives (13) (14mm) (Fig. 3) were maximum inhibition against this bacterium. It was found that the chemicals 3, 5, 6, 7, 11 and 12 showed some inhibition against this pathogen. The rest of the chemicals were unable to show any inhibition.

Table 1. Zone inhibition observed against Gram-(+) test organisms by the test chemicals

Compound no.	Determination of inhibition zone in mm 200 µg dw/disc					
	Bacillus cereus			*Bacillus subtilis*		
	Treatment	Control	Net inhibition	Treatment	Control	Net inhibition
2	8	--	8	7	--	7
3	--	--	--	--	--	--
4	10	--	10	10	--	10
5	11	--	11	10	--	10
6	10	--	10	*13	--	*13
7	*12	--	*12	11	--	11
8	10	--	10	10	--	10
9	--	--	--	--	--	--
10	--	--	--	--	--	--
11	*12	--	*12	*14	--	*14
12	*14	--	*14	*15	--	*15
13	*16	--	*16	*14	--	*14
**Ampicillin	*21	--	*21	*25	--	*25

N.B: '*' = marked inhibition, '**' = standard antibiotic, '--' = not found, 'dw' = dry weight.

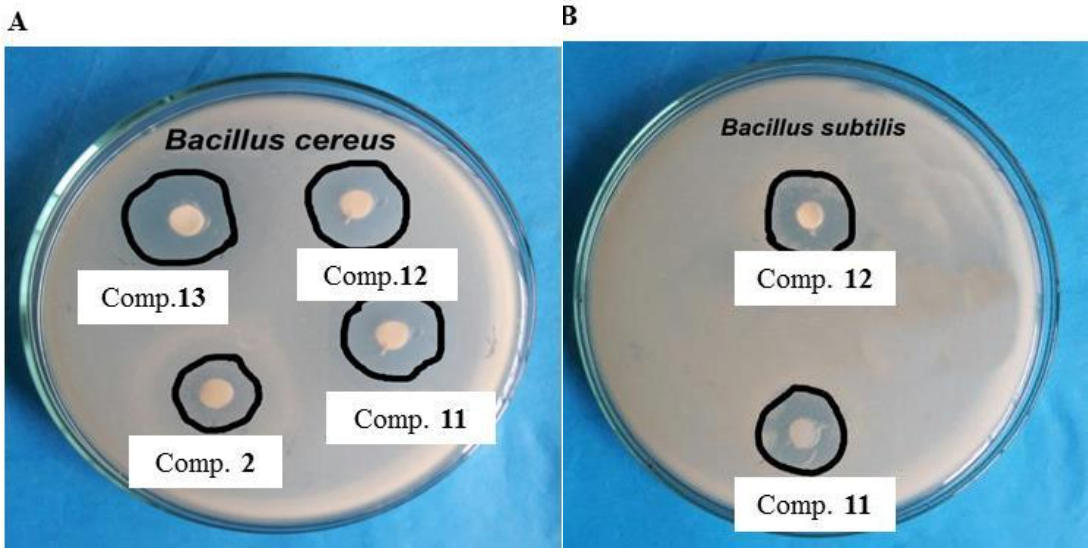


Fig. 2. A: Zone of inhibition observed against *Bacillus cereus* by four test chemicals 2, 11, 12 and 13. **B:** Zone of inhibition observed against *Bacillus subtilis* by two test chemicals 11 and 12.

Table 2. Zone inhibition observed against Gram-(-) test organisms by the test chemicals

Compound no.	Determination of inhibition zone in mm 200 µg dw/disc					
	Escherichia coli			*Pseudomonas aeruginosa*		
	Treatment	Control	Net inhibition	Treatment	Control	Net inhibition
2	--	--	--	--	--	--
3	10	--	10	8	--	8
4	--	--	--	--	--	--
5	8	--	8	7	--	7
6	*12	--	*12	11	--	11
7	11	--	11	10	--	10
8	10	--	10	8	--	8
9	--	--	--	--	--	--
10	--	--	--	--	--	--
11	8	--	8	10	--	10
12	*14	--	*14	*13	--	*13
13	10	--	10	*14	--	*14
**Ampicillin	*25	--	*25	*17	--	*17

N.B: '*' = marked inhibition, '**' = standard antibiotic, '--' = not found, 'dw' = dry weight.

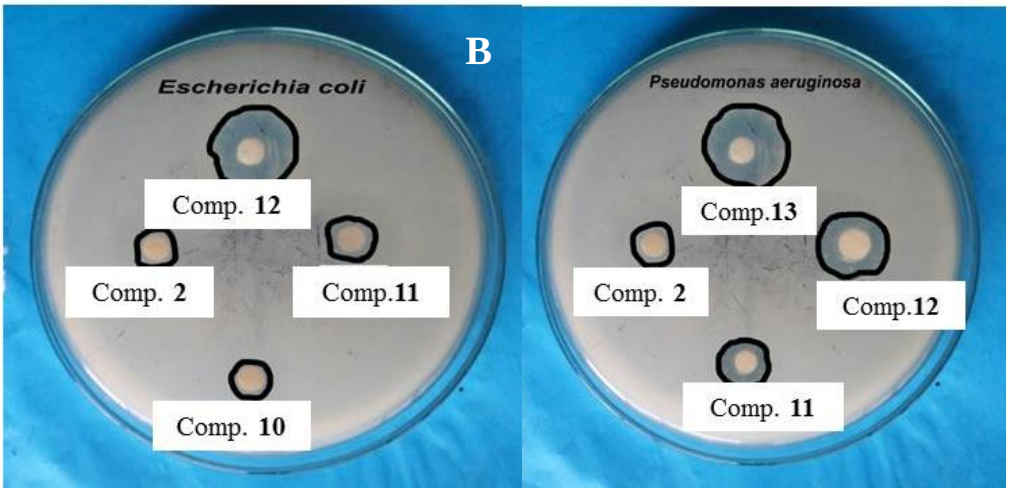


Fig. 3A: Zone of inhibition observed against *Escherichia coli* by four test chemicals 2, 10, 11 and 12. **B:** Zone of inhibition observed against *Pseudomonas aeruginosa* by four test chemicals 2, 11, 12 and 13.

From the above experimental results obtained by using a number of selected human pathogenic bacteria (as shown in Table-6 and Table-7), we found that selectively acylated derivatives 13 and 12 showed highest inhibition against Gram-positive bacteria while compounds 6, 7, and 11 were also very active against Gram-negative bacteria. We also observed that the compound 13 and 11 are highly active against both the Gram-positive and Gram-negative organisms. So these compounds may be targeted for future studies for their usage as broad spectrum antibiotics.

Table 3. Antifungal activities of the test chemicals & nystatin

Compound no	% Inhibition of fungal mycelial growth[a] (100 µg (dw)/ml medium)	
	Aspergillus niger	*Rhizopus nigricans*
2	40	45.25
3	47	46
4	45	44.4
5	42.45	46
6	*52.25	*50.25
7	42.5	45
8	*50	*51.2
9	10.5	15
10	16	19
11	44	40.3
12	*58.5	*54.5
13	*52.5	*56.5
**Nystatin	*66.41	*63.10

N.B: '*' = marked inhibition, '**' = standard antibiotic, 'NF' = not found, 'dw' = dry weight,
[a]growth measured-radial growth in cm.

In general, it has been observed that antibacterial results of the selectively acylated monosaccharide derivatives obtained by using various acylating agents follow the order for Gram-positive organisms: 13 > 12 > 11> 7 > 6> 4=8 and Gram-negative bacteria follow the order: 12 > 13 > 7 > 6 > 3=11. Thus, a comparative study on antibacterial activities of a number of selectively acylated derivatives of methyl α-D-glucopyranoside (1) has been carried out successfully.

As seen in our previous investigations (Kawsar *et al.*, 2015c) the presence of some acyl groups in the test chemicals increased the antimicrobial capacity, here in this investigation we found that the presence of chlorobenzoyl-, bromobenzoyl-, sulphonyl- etc. acyl groups improved the antibacterial power of the test chemicals.

B) Effect of test chemicals on fungi

The results of the percentage inhibition of fungi (*Aspergillus niger* and *Rhizophers nigricans*) due to treatment of compounds (2-13) are presented in Table 3.

Aspergillus niger ATCC 16430

The percent inhibitions of mycelial growth of plant pathogenic *Aspergillus niger* for different chemical treatment are shown in Table 3. It is evident that chemicals 12 (58.50%), 13 (52.50%), 6 (52.25%) and 8 (50.00%) were found to be very active against the test fungus. Whereas, the remaining test chemicals such as 2, 3, 4, 5, 7, 9, 10 and 11 were found less effective against these plant pathogenic fungi. Chemicals 2-bromobenzoyl derivatives 12 (58.50%) showed the highest inhibition, which is less inhibition than the standard antibiotic, Nystatin (66.41%).

Rhizopus nigricans ATCC 6227b

The mycelial growth inhibition of *Rhizopus nigricans* due to the treatment by different test chemicals are mentioned in Table 3. It was found that, test chemical 13 (56.50%), 12 (54.50%), 8 (51.20%) and 6 (50.25%) showed marked inhibition against this fungus. The chemicals 2, 3, 4, 5, 6, 9, 10 and 11 were less effective against this fungal species. Among these compounds 4-chlorobenzoyl derivatives 13 (56.50%) showed the highest inhibition against this fungus. It was observed that the 4-chlorobenzoyl derivative (12) showed maximum inhibition (58.50%) against *Aspergillus niger* and 2-bromobenzoyl derivative (13) showed the highest inhibition (56.50%) against *Rahizopus nigricans*.

From the results we observed that the introduction of some specific functionalities in the test chemicals improved their antimicrobial activities. In this series the presence of benzenesulphonyl, 4-chlorobenzoyl and 2-bromobenzoyl acyl groups might be responsible for the improvement of the antifungal capacity of the test chemicals. We found that the presence of 2-bromobenzoyl, 4-chlorobenzoyl etc. acyl groups improved the antifungal activities of the test chemicals which was in accordance with our previous work (Kawsar *et al.*, 2014).

C) Effect of test chemicals on cytotoxicity

The cytotoxic activity of the acylated of D-glucopyranoside derivatives in the brine shrimp lethality bioassay was performed and showed the percentage of mortality of shrimps at 24 hrs and 48 hrs. Mortality of the nauplii was noticed in the experimental groups at the same time the control group remained unchanged. The number of survived nauplii in each vial was counted and the results were noted. From these data the percent of mortality of the shrimp was calculated for every concentration of each chemical. In the bioassay, the test chemicals showed a significant cytotoxicity activity in the brine shrimp lethality bioassay indicating that these compounds are biologically active. The compounds showed different rate mortality with different concentrations, this results also accordance with our previous result (Kawsar *et al.*, 2015a).

The mortality of brine shrimp was found to increase with the increase of concentrations of compounds. It is evident from the results of brine shrimp lethality testing that the test chemicals methyl 6-*O*-octanoyl-2,3,4-tri-*O*-pentanoyl-α-D-glucopyranoside (3), methyl 2,3,4-tri-*O*-hexanoyl-6-*O*-octanoyl-α-D-glucopyranoside (4) and methyl 2,3,4-tri-*O*-methanesulfonyl-6-*O*-octanoyl-α-D-glucopyranoside (10) showed the average lowest levels of toxicity (i.e., 0.0% death) indicating its lower mortality and compounds 11 and 12 also exhibited the lower toxicity (only 5%). On the other hand, methyl 2,3,4-tri-*O*-decanoyl-6-*O*-octanoyl-α-D-glucopyranoside (5) and methyl 2,3,4-tri-*O*-lauroyl-6-*O*-octanoyl-α-D-glucopyranoside (6) showed highest levels of toxicity (i.e., 50% death) indicating its higher mortality and compound 8 displayed the good mortality as 40%. To our knowledge, this is the first report on cytotoxic study of the newly synthesized D-glucopyranoside derivatives by brine shrimp lethality.

Conclusions

Our synthesized and reported chemicals (2-13) have not been tested before against the selected bacterial and fungal pathogens. This is the first report regarding the effectiveness of the selected chemicals against the selected pathogens. The results of the present investigation showed that some of the newly synthesized acylaled derivatives of methyl α-D-

glucopyranoside (1) may be tested against a wide range of bacteria and phytopathogenic fungi, before sending them to pesticide producing companies for further tests. It is also expected that this piece work employing carbohydrate derivatives as test chemicals will help further work to the development of pesticides and medicine for human and plant disease control. So it is hoped that the tested D-glucopyranoside derivatives (2-13) might show potential antiviral, antituberculatic and anti-inflammatory activities. Cytotoxic study showed that the most of the D-glucopyranoside derivatives are non-toxic.

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