Journal of Research in Medical and Dental Science
2022, Volume 10, Issue 8, Page No: 10-15

Available Online at: www.jrmds.in
eISSN No. 2347-2367: pISSN No. 2347-2545

GAG-like Moiety Derived from Marine Mollusks with Antibacterial Activities

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ABSTRACT

Despite the increased bacterial resistance of various antibiotics, there is a need to develop new antibiotic drugs with improved pharmacological profiles that can also overcome drug-resistant forms of bacteria. In this research project, we have identified and characterized a marine polysaccharide with the potential to be developed as an antibacterial agent. Sulphated polysaccharides isolated from the New Zealand mussel Perna canaliculus were used against five strains of bacteria and showed an antibacterial effect on three strains of gram-positive bacteria, Staphylococcus aureus, Enterococcus faecalis and Methicillin-resistant Staphylococcus aureus. The analysis of these marine polysaccharides confirmed the presence of glycosaminoglycan-like structures that contained antibacterial activity. This antibacterial activity was shown to be highly susceptible to fucose but not to chondroitin sulphated. This enzymatic and antibacterial activity pattern has not previously been seen in either marine or mammalian glycosaminoglycans. As such, our findings suggest that we have identified a new type of marine-derived fucose chondroitin sulphated–like polysaccharide with potent antibacterial properties.

Key words: Marine molluscs, Glycosaminoglycans, Antibacterial, *Staphylococcus aureus, Enterococcus faecalis,* MRSA

HOW TO CITE THIS ARTICLE: Abdullah F Aldairi, Razan A. Al-Ahmadi, Ayman Al-Hazmi, Radi T Alsafi, Aisha Bagasi, Ghaiyda T Basfar, Hamdi M Alsaid, Ahmad A Alghamdi, Abdulrahman Mujalli, Banan Atwah, Sami S Ashgar, GAG-like Moiety Derived from Marine Mollusks with Antibacterial Activities, J Res Med Dent Sci, 2022, 10 (8):10-15.

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Received: 09-July-2022, Manuscript No. JRMDS-22-68991;

Editor assigned: 11-July-2022, PreQC No. JRMDS-22-68991 (PQ);

Reviewed: 26-July-2022, QC No. JRMDS-22-68991;

Revised: 29-July-2022, Manuscript No. JRMDS-22-68991(R);

Published: 05-August-2022

INTRODUCTION

Glycoconjugate is a carbohydrate structure chemically bound to a non-carbohydrate structure as a side-chain; for instance, carbohydrates may be attached to lipids or proteins to make glycolipids and glycoproteins, respectively [1]. There is a specific subtype of glycoproteins known as proteoglycans (PG), which contain particular amino-sugars attached to the core protein as side-chains, known as glycosaminoglycans (GAGs) [2]. Typically, GAGs consist of long, unbranched heteropolysaccharides, with repeated disaccharide building blocks of uronic acid covalently attached to amino-sugar via glycosidic linkages [3]. GAGs are classified according to the repeated disaccharide building blocks into heparin, heparan sulphate (HS), chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS) and hyaluronan [4].

Heparin is composed of disaccharide units of iduronic acid (IdoA) covalently attached to glucosamine (GlcN) residues via (α 1→4) glycosidic linkage with different patterns of sulphation [5]. HS is structurally similar to heparin; however, the IdoA residues were epimerased at carbon-5 to glucuronic acid (GlcA), which is attached to glucosamine (GlcN) residues via (α 1→4) glycosidic linkage [6]. CS is expressed in various locations of the cells, including intracellularly, membrane-bound, and in the extracellular matrix; it is found to be bound to the protein, resulting in the formation of CS-proteoglycan

(CSPG). Structurally, GlcA is attached to galactosamine (GalN) makes up the CS typical repeating disaccharide building blocks with the chemical formula [→4 β-D-GlcA (1→3) β-D-N-GalNAc (1→] [7]. DS can be distinguished from CS in cell type and disaccharide building blocks, as DS is composed of epimerised GlcA carbon-5 forming IdoA attached to GalNH2, with the chemical formula as [→4 α-L-IdoA (1→3) β-D-GalNAc (1→] [7]. KS is found in nature in the extracellular matrix of certain tissues, such as cartilage, bone, and cornea. KS repeated building blocks are composed of galactose (Gal) and GlcNH2, which is chemically formulated as [→3) β-D-galactose (1→4) β-D-GlcNAc (1] [3]. KS is the only GAG type that does not acquire acidic residue, a common requirement seen in GAGs and can be found in cornea and participates in intracellular signalling and developmental [8]. Finally, hyaluronan was considered a unique GAG polysaccharide, as it is not attached to proteoglycan [9]. Various structural modifications such as branching and chain decoration with sialic acid would result in tremendous biological functions of polysaccharides, such as antiproliferative activities [10], antiviral activity [11], prevent Plasmodium falciparum Cytoadhesion [12].

Protein glycosylation is defined as the addition of sugar molecules to a protein structure [1]. These modifications change protein structure, function, and localisation using polysaccharides. In eukaryotic cells, the endoplasmic reticulum and Golgi apparatus, secretory and surface proteins would be post-translationally modified by adding specific carbohydrates either via N-linked or O-linked glycosylation. N-linked glycan chain is formed by adding a particular carbohydrate sequence to the polypeptide residues of a core protein resulting in N-glycosidic linkages. Thus, it occurs via the dolichol-phosphate pathway starting within the rough endoplasmic reticulum surface, followed by the sugar sequence added to the protein sequence. Dolichol phosphate is an essential pathway for glycoprotein glycosylation in the process of synthesizing N-linked glycans [13]. On the other hand, the O-linked glycan chain occurs in the Golgi apparatus. The addition of the sugars molecules to the O-linked sequence is cell type-specific, which requires sugar transferase enzymes to transfer sugars to amino acid residues [14].

The marine life body is composed of proteins, glycoproteins, carbohydrates, amino acids, polyphenols, and mineral salts [15]. Marine life's carbohydrate structure would vary from its human counterpart, making it a potential source of biologically active carbohydrates [10], making it a subject of research interest [16]. Biologically active carbohydrates have been studied in pharmaceutical applications as therapeutic agents [17]. There are different compounds extracted from marine organisms, such as alginate, which is extracted from brown algae, chitosan that was used as a therapeutic agent to treat hypertension [18] and as an antifungal [19], fucoidan, which was extracted from several species of brown algae that used as an anti-cancer therapy [20], an anti-inflammatory [21] and antithrombotic [22]. In addition, carrageenan, found in red algae, is a sulphated galactan with antiviral activity against different viruses, such as human papillomavirus (HPV) and influenza A virus [20, 23].

The demand for new natural components that possess antimicrobials activity based on polysaccharides derived from marine organisms is increased [3,10,22,24,25]. A severe rise in antibiotic resistance occurred in bacterial species worldwide [26]. The response to antibiotics in infections with resistant anti-bacteria has been associated with higher morbidity and mortality rates, expensive treatment, and more extended hospital stays, which place a more significant burden on healthcare systems [27]. These facts make the increase in bacterial resistance one of the biggest healthcare challenges of the past hundred years. The ability of microorganisms to withstand antibiotics' effects is known as antibiotic resistance, which can be classified into two categories: natural (intrinsic) or acquired resistance [28]. Natural resistance naturally occurs in all bacterial organisms [29]. Inherent resistance coexisted with the resistance mediated by the bacterial outer membrane and active efflux [30]; however, acquired resistance could occur due to chromosomal mutations or as a result of external genetic determinants of resistance acquired through a plasmid or transposon containing resistance determinants [26]. In addition, multidrug-resistant (MDR) bacteria are resistant to more than one antimicrobial group [31] that currently is considered a severely high risk to public health [32], which are commonly related to nosocomial infections in hospitals [33]. However, they have grown in prevalence as a source of community-acquired infection. The spread of MDR bacteria into the population is critical because it is linked to increased morbidity and mortality, leading to higher healthcare costs [34].

In this study, GAG-like polysaccharides were extracted from marine life known as *P. canaliculus* to evaluate its antimicrobial effects on three bacterial strains.

MATERIALS AND METHODS

Materials

Bacterial strains

Genetically characterized American Type Culture Collection (ATCC) isolates of *E. faecalis* ATCC (29212), *S. aureus* ATCC (25923), MRSA ATCC (43300), *P. aeruginosa* ATCC (15442), and *E. coli* ATCC (35218), were used in this study.

Culture media

Mueller-Hinton agar (Oxoid, CM0337) and Mueller-Hinton broth media (Oxoid, CM0405) were used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), which prevented any growth of organisms.

Marine life

In this study, polysaccharides were extracted from New Zealand mussels, *Perna canaliculus* (*P. canaliculus*), which were exported frozen to Saudi Arabia via Sea land company.

METHODS

Extraction of sulphated polysaccharides

GAG-like polysaccharides were extracted from *P. canaliculus* using the method according to Aldairi, et al. [10] and Kim et al. [35].

Glycosyl monosaccharide composition analysis

Glycosyl composition analysis was performed using Gas-chromatography coupled with mass spectrometry (GC-MS) of Per-O-trimethylsilyl (TMS) derivatives of methyl glycosides. According to Santander et al. (2013), acidic methanolysis was used to produce TMS derivatives from the sample. The GAG-like polysaccharides extracted from *P. canaliculus* (400 g) were freeze-dried with inositol. The dried sample was heated for 18 hours at 80 °C in 3M methanolic-HCl. The sample was treated with methanol and dried several times after cooling and drying under nitrogen. After that, the samples were combined with methanol, pyridine, and acetic anhydride before being left to sit for 30 minutes. These solvents were thoroughly dried out, and these solvents were dried down fully; Tri-Sil (Pierce) was used to derivatise the sample at 80 °C for 30 minutes. The sample was then added to hexane, centrifuged, and the supernatant was removed and dried for examination. TMS methyl glycosides were analysed using a Supelco Equity-1 fused silica capillary column on an Agilent 7890A GC interfaced to a 5975C MSD (30 m x 0.25 mm).

Preparation of standard inoculum

The target species were grown on Muller-Hinton agar medium for 24 hours at 37°C. The organism was then standardized to 0.5 McFarland using calibrated VITEK 2 DENSICHEK and a single colony was collected using a sterile loop and inoculated in Muller Hinton broth to form a homogeneous suspension.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of compounds (crude)

On micro titration plates, 100 µL of the dissolved pure crude compound at the highest concentration (10 mg/mL) was mixed with 100 µL Mueller Hinton broth in the first well of the first column. Dissolved pure compounds were serially diluted by transferring 100 µL to the subsequent wells that contained 100 µL Mueller Hinton broth to produce the final concentration of (5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 mg/mL). Then, in the dilution series, 10 µL of 0.5 modified McFarland bacterial suspensions was introduced to each well containing 100 µl of dissolved compounds, as well as a positive control well, and mixed. As a negative control, Sterilized Mueller Hinton broth was used. Micro-dilution plates were then incubated at 37°C overnight. The MIC and MBC of each tested compound were recorded against five tested bacterial strains after being sub-cultured on a Muller Hinton agar plate according to the clinical and laboratory standards institute (CLSI M26-A, 1998).

Contact time assay

Using a micro titration plate, 100µl of the tested compound was added to the first column in a microtiter plate and 100µl Mueller Hinton broth to other wells. 10µl tested bacterial strain that was suspended in Mueller Hinton broth and adjusted at 0.5 McFarland was mixed with tested compound. At the end of 30 seconds, then 10µl of the suspension transferred from the 1st well to the next well in the second column. This step was repeated after 60, and 90 seconds from zero time to the third, and fourth columns. A Mueller-Hinton broth without additions was used as a negative control and broth with tested bacterial strain for positive control. The plate was incubated at 37ºC for 24 hours. After the incubation period, sub-cultured on Muller Hinton agar plate according to clinical and laboratory standards institute (CLSI M26-A, 1998) by taken transferring 10µl from each well to Muller Hinton agar plates. All pates were incubated for 24 h at 37°C and to determine the killing time for tested bacterial strains. The presence of bacterial growth showed no effect of the tested compound against the bacterial strains during the exposure time of 90 seconds.

RESULTS

Monosaccharide composition analysis using TMS–glycoside

Porcine bovine kidney HS-salts (Sigma, UK) was used as a standard to evaluate the crude GAG structure from *P. canaliculus*. The glycosyl residues from the bovine HS are shown to be composed of xylose (Xyl) (11.3 mol%), GlcA (25.1 mol%), Gal (17.6 mol%), GlcNAc (46 mol%) with a total amount of carbohydrate (9.4 µg). Regarding crude polysaccharide structure that derived from *P. canaliculus*, the monosaccharide composition analysis showed to be composed of GAG-like structure, anamely, Fuc, Xyl, GlcA, Mannose (Man), Gal, Glucose (Glc), GalNAc and GlcNAc (Table 1).

Antibacterial activity of the crude GAG-like structure against different bacterial strains

The antibacterial activity of the crude GAG-like structure against *E. faecalis* showed the effect of the extract as a MIC value of 5 mg/mL, and the bactericidal activity MBC value of 10 mg/mL. The antibacterial activity against *S. aureus* showed that the extract's potency was demonstrated at a MIC value of 0.625 mg/mL and the MBC value of 1.25 mg/mL. The antibacterial activity against MRSA extract's potency was demonstrated at a MIC value of 1.25 mg/mL and the MBC value of 2.5 mg/mL; however, no effect was determined against *P. aeruginosa* or *E. coli* (Table 2).

Contact time assay

This method showed no effect of crude GAG-like structure

Table 1: Total amount and mole percentage of monosaccharide composition of HS standard and crude GAG sample.

Sample	Glycosyl residue	Mass (µg)	Mol %1
HS standard	Xyl	0.8	11.3
	GlcA	2.3	25.1
	Gal	1.5	17.6
	GlcNAc	4.8	46
	SUM	9.4	100
	Total carbohydrate % by weight		2.30%
Crude	Ribose (Rib)	0.95	3.8
	Fuc	1.9	6.9
	Xylose (Xyl)	0.66	2.6
	Glucuronic Acid (GlcA)	2.5	7.6
	Mannose (Man)	0.45	1.5
	Galactose (Gal)	6.4	21.2
	Glucose (Glc)	12.6	41.7
	N-Acetyl Galactosamine (GalNAc)	3.5	9.6
	N-Acetyl Glucosamine (GlcNAc)	1.9	5.1
	SUM	30.9	100
	Total carbohydrate % by weight		7.70%

[1]Values are expressed as mole percent of total carbohydrate. The total Mol% may not add up to exactly 100%.

Table 2: Assessment of the antibacterial activity of the crude GAG-like against five ATCC bacterial strains using MIC and MBC.

Organisms	MIC	MBC
E. faecalis	5 mg/mL	10 mg/mL
S. aureus	0.625 mg/mL	1.25 mg/mL
MRSA	1.25 mg/mL	2.5 mg/mL
P. aeruginosa	Resistant	Resistant
E. coli	Resistant	Resistant

Table 3: Assessment of the antibacterial activity of the crude GAG-like against three bacterial strains, *E. faecalis, S. aureus* and MRSA.

Tested Strains	Contact Time		
	30 Sec	60 Sec	90 Sec
E. faecalis	+	+	+
S. aureus	+	+	+
MRSA	+	+	+
Notes: (-) effective, (+) non effective			
Abbreviation: Sec, seconds.			

against three bacterial strains during the exposure time of 90 seconds, this indicates that it may need more time than 90 seconds (Table 3).

DISCUSSION

GAGs are long unbranched polysaccharides that play an essential role in several biological activities, such as anticancer [3], antiviral [36] and antithrombotic activities [37-39]. This study aimed to purify GAGs from marine life *P. canaliculus* and evaluate its effectiveness as an antibacterial agent against five bacterial strains. Regarding structural characterization, the GC/MS data suggested the presence of GlcA, GlcNAc, and GalNAc in the crude sample, which support the presence of GAG-like moiety within the polysaccharide chain. Thus, it would be different from the mammalian GAGs composed of either GlcNAc or GalNAc; however, common monosaccharide's were determined, such as xylose, Glc, and Gal [1]. More interestingly, the results showed fucose residues within the chain, which would be linked to the GAG chain. This phenomenon has been shown in various GAG structures found in marine life known as fucosylated-GAGs [36,37,40]

The literature suggested the presence of fucosylated-CS with potent biological Activity [37,41-44], in addition to HS, which also suggested having several biological functions [10,25,45,46]. However, the GAG-like structure from *P. canaliculus* was suggested to have both CS and HS monosaccharides residues within the chain.

Referring to the biological function derived from GAG-like from marine life, this study was aimed to evaluate their antibacterial effects.

The results showed that GAG-like structure deribed from *P. canaliculus* would act as antimicrobial agent as it shows high sensitivity to gram-positive bacteria, particularly MRSA with MIC 1.2 mg/mL, which is reported to be highly resistant to several antibiotics [47]. In addition to *E. faecalis* with MIC 5 mg/mL and *S. aureus* with MIC 0.6 mg/mL.

The antibacterial effect of the crude GAG-like chain on MRSA shown to have greater substantial inhibitory effects than the ethyl-acetate extracts of Acacia aroma with MIC 2.5 mg/mL [48]; in addition, β-asarone extracts from Acorus calamus rhizome showed MIC 2.5 mg/mL [49]. However, the Bauhinia kockiana tree from Malaysia and the ethanolic extracts of the Canarium patentinervium leaves both showed more potent antibacterial activities against MRSA with MIC 0.25 mg/mL [50].

The antibacterial effects of the crude GAG-like chain on *S. aureus* with MIC 0.6 mg/mL is more potent than that of olive oil polyphenol extracts with MIC 1.25 mg/mL [51]. However, the D. amoenum acetone extracts from Orchids showed potent antibacterial activity with MIC 0.39 mg/mL and MBC 0.39 mg/mL. Moreover, methylglyoxal (MGO), a 1,2-dicarbonyl compound present in Manuka honey, has an effect on *S. aureus* with MIC 0.150 mg/mL [52] and the Brocazine G extracts that derived from the mangrove penicillium showed potent antibacterial activity against *S. aureus* with MIC 0.25 µg/mL [53].

The antibacterial effects of the crude GAG-like polysaccharides extract against *E. faecalis* with MIC 5 mg/mL showed a weaker effect than that of the extract of the Lamiaceae leaf, which shows a potent antimicrobial effect that is inhibited the growth of *E. faecalis* with MIC 0.26 mg/mL [38]. Polyphenolic flavonoids demonstrated antimicrobial activity against *E. faecalis* with MIC 0.512 mg/mL [54]. The leaf extract of Woodfordia floribunda showed potent antimicrobial activity against *E. faecalis* with MIC 0.256 mg/mL [38].

The gram-positive bacteria were more susceptible to the GAG-like polysaccharides than the gram-negative bacteria. This could be due to the nature of the bacterial structure, for instance, the cell wall with a high percentage of peptidoglycan (90–95%), as well as lipopolysaccharides and phospholipids, within the Gram-

positive cell, which destroys the cell membrane or protein biosynthesis units (DNA and RNA). This susceptibility could also be due to a two-layer membrane, including the outer and the inner membrane. The thicker outer murein membrane is made up of lipoprotein, phospholipids, and mucopolysaccharides, whereas the inner membrane is made up of peptidoglycan (glycopeptide) (5–10%); thus, it would suggest the high percentage of lipids (90–95%) in the cell membrane characteristic of Gram-negative bacteria could explain this phenomenon. This outer membrane prevents certain medications and antibiotics from entering the cell. On the other side, Gram-positive bacteria may be more vulnerable to the extracts because of peptidoglycan. The outside layer of the Gram-negative bacteria is not an active permeability barrier to bioactive complexes. As a result, Gram-negative bacteria have a more complex cell wall than Gram-positive bacteria, which helps explain why Gram-negative bacteria are more resistant to antibiotics in general [55].

In this regard, highly sulphated GAG-like moieties have proven antibacterial effects; however, Gram-negative bacteria showed resistance to these effects. This could be due to the difference in bacterial structure, as Gram-negative bacteria usually have a double wall [48], which may provide this resistance.

ACKNOWLEDGMENTS

This work was supported by Taif University Researchers Supporting Program Project number (TURSP-2020/350), Taif University, Saudi Arabia.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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