The antibacterial potential of structurally modified vitamin C on some conjunctivitis pathogens

Dr. Sinaa A. Kadhim. Lecturer, Department of Pharmacology and Therapeutics, Collage of Medicine, Al Qadisiya University.
May J. Abd. Assistant lecturer, Department of Chemistry, Collage of Medicine, Al Qadisiya University.
Shaimaa A. Behjet. Lecturer, Department of Chemistry, Collage of education, Al Qadisiya University

الخلاصة :-
الخلفية:- التهاب الملتحمة من الإمـــراض الشائعة والتي تستخدم في علاجـــــها مختلف المضادات الحيوية حيث أن البكتيريا المسببة لهذا الالتهاب أظهرت في هذه الأيام مقاومه للمضادات الحيوية المستخدمة وبهذا أصبح من الضروري إيجاد بدائل أو مضادات جديدة للسيطرة على هذا الالتهاب.
الهدف:- لمعرفة الجهد المضاد للبكتيريا لفيتامين سي المحور التركيب على مسببات التهاب الملتحمة
المـــواد وطريقـــة العمـــل:- ٣٦ مســحة بكتيريـــة اختيـــرت لبكتريـــا Haemophilus influenza، Pseudomonas aeruginosa و Klebsilla pneumoniae وبما إن مركبات سكر الازا (aza sugar) لهـا تأثير بايولوجي ضـــد بعض الالتهابات فقد استخدمنـا مركـــــب سكر الازا (A1-A2) المحضر من فيتامين سي بعد سلسلة من التفاعلات التي تمثلت بإضافة مجموعة الاستيل الجانبية للمركب وكذلك استخدام (B1-B2) والذي هو عبارة عن سكر ازا يحتوي على مجموعة الكاربونيل (من مشتقات الالديهايد)
النتائج والاستنتاجات :- وجد إن هذه المركبات لها تـأثير فعـال ($p<0.05$) في قتل البكتيريا وبمقاومة غير ملحوظة ومن المحتمل ان يعزى هذا إلى وجود مجموعة الاستيـــــل في(A1-A2) ومجموعة الكاربونيل في مشتقات الالديهايد من الاسكوربيك أسيد (B1-B2) والتي تسببت في قتل البكتيريا.

Abstract

Background: Conjunctivitis, commonly known as an inflammation of the conjunctiva. Consequently broad-spectrum antibiotics that are routinely used in the treatment of bacterial conjunctivitis showed high resistant by most of these bacterial strains, therefore most of peoples tend to use alternative therapy.

Objective: The present study was undertaken to clarify the pharmacological effect, the antibacterial potential of structurally modified vitamin C (aldehyde derivatives containing carbonyl side group) on some bacteria isolated from patient with conjunctivitis.

Materials and methods: A total of 36 isolates from patients with conjunctivitis who attended to Diwaniyah teaching hospital from October 2010 to April 2011 were studied. *Haemophilus influenza*, Klebsiella pneumonia and Pseudomonas auerogenosa were identified; bacteria were inoculated for further assessment. The structurally modified vitamin C by substitution of oxygen atom by nitrogen atom and formation of vitamin C aza sugar, after a series of chemical reactions, and by changing the side group of the newly formed structure and formation of a carbonyl side group (now this compound is aldehyde derivatives), were applied to the diagnostic bacteria and compared them with tetracycline.

Results and conclusions: The structurally modified vitamin C caused significant (p value less than 0.05) bactericidal effect with zero percent of resistance against all tested bacteria, compared to tetracycline which showed 100% of resistance against *Haemophilus influenza* and Pseudomonas auerogenosa, These actions may be due to presence of acetyl group in vitamin C aza sugar, and carbonyl group in newely modified vitamin C.

Key words: Conjunctivitis, vitamin C, antimicrobials, aldehyde.

Introduction:

Conjunctivitis, commonly known as "red eye" is an inflammation of the thin protective membrane that lines the inside of eyelids and covers the outer part of eyeball (the conjunctiva) of the eye [1]. Mostly, primary eye care providers start the treatment of external ocular infection before the causative microorganisms have been identified, or submitted to antibiotic susceptibility tests. As antibiotics are naturally occurring or synthetic organic substances which inhibit or destroy selective bacteria or other microorganisms, consequently broad-spectrum antibiotics are routinely used in the treatment of bacterial conjunctivitis but recently these bacteria showed high resistant to most these antibiotics, therefore most of peoples tend to use alternative medications [2].

Ascorbic acid, water-soluble vitamin (vitamin C), with oxygen containing ring, is a significant antioxidant component of the aqueous humor of the eye[3].

The synthesis of aza-sugars, sugar mimics in which the ring, oxygen has been substituted by a nitrogen atom, have been the subject of much continued interest over the last years[4]. The often potent inhibitory activity of many of these compounds toward carbohydrate-processing enzymes has suggested their use in a wide range of potential therapeutic strategies including the treatment of viral infections[5], cancer[6], diabetes[7], tuberculosis[8], lysosomal storage diseases[9], and parasitic protozoa[10].

Most synthesis of aza-sugars including vitamin C aza sugar have focused on logical designs based on the stereochemistry of the functional groups around the heterocyclic ring of the putative carbohydrate mimic, and this approach has yielded potent Inhibitors[11].

Further more , deoxynojirimycin (compound that responsible for antibacterial activity), the direct configurational modulator, is a potent glucosidase inhibitor[12,13]. Interestingly, however, this type of approach does not always result in high inhibition. An exception was reported [14].

In addition, in certain cases, five-member ring aza-sugars have been shown to give rise to higher inhibition than their six-member ring counterparts [15] and subtle selectivity may be observed for five- versus six member ring systems. Furthermore , the five-member ring isomer is a potent mannosidase inhibitor[15] Therefore, it is not generally straight forward to predict by an entirely logical design based upon configurationally analogy the structure of the best inhibitor for a given carbohydrate-processing enzyme[16].

Furthermore, aldehyde is known as a chemical compound s containing carbonyl group (a carbon atom attached to an oxygen atom by double bound, and this group attached to the carbon atom in the original compound) with high bactericidal activity, so the aldehyde derivatives were considered as compounds with high antimicrobial activity[17,18]. Their predominant mechanism of action for controlling microorganisms is to react with peptides and proteins. As this reaction progresses throughout organisms its biochemical processes become increasingly impaired and the organism dies[17].

Materials and method :

Bacterial isolates:

A total of 36 bacterial isolates from patients with conjunctivitis who attended to Al-Diwaniyah teaching hospital from October 2010 to April 2011 were studied. Haemophilus influenza, Klebsiella pneumonia and Pseudomonas auerogenosa were identified and confirmed according to proper procedure[19], bacteria were inoculated for further assessment.

Chemical compounds:

All chemicals are obtained from (BDH, Fluka and Merck companies). The solvents were dried and distilled before use, and according to standard procedure modulation of the structure of vitamin C done by substitution of oxygen atom by nitrogen atom which lead to formation of aza ascorbic acid, and after a series of chemical reactions, formation of new aza sugar will result [Synthesis of 2-3-O-diacetyl -N-(isobutyl, isopentyl), aza ascorbic acid derivatives (A1,A2)] which prepared by treating (5.5 gm, 0.014 mole) 2-3-O-diacetyl 5-6-O-Isopropylidene- N-(isobutyl, isopentyl) aza ascorbic acid, that is primarily prepared after a series of chemical reactions from ascorbic acid, with a solution of iodine (1.82 gm, 0.0146 mole) in (30 ml) methanol is added with continuous stirring in (40 ml) of chloroform. The mixture was refluxed for (1hr), cooled and filtered. Recrystallization from ethanol gives brown crystals of (A1, A2). After that the newly formed aza ascorbic acid(A1,A2) treated with periodic acid at 0 C° and lastly we add ethylene glycol with extraction (with ethyl acetate) resulting in formation of new compound (aldehyde derivatives) containing carbonyl group (B1= [N-isobutyl 5-formyl-3,4dihydroxy- 2-N-pyroline], B2=[N-isopentyl 5-formyl-3,4dihydroxy-2-N-pyroline]) [20]. Furthermore, the stock solution was prepared with concentration 1000µg/ml by dissolving 0.01g of each compounds in 10ml of distilled water to have the concentrations with µg/ml: (0.8, 1.6), tetracycline antibiotic were prepared with the same way of aza sugars solutions.

Preparation of culture media (Muller- Hinton agar):

It has been prepared according to the manufacturer instructions, by dissolving 38 gm of Muller –Hinton agar in 1 liter of distilled water with mixing and heating, then skirled the culture media in autoclave for 15 minute, finally put in plastic Petri dish to be used in sensitivity test of bacteria.

Determination of Minimal Inhibitory Concentrations (MIC_s) by using wells methods:

Two fold agar dilution methods [21] were used as concentrations of chemical compounds solution (0.8, 1.6 µg/ml) were prepared from the original working compounds solution (1000µg/ml), then the bacterial inoculums (to be used in this test) was prepared by adding growth from 5 isolated colonies grown on blood agar plate to 5 ml of Nutrient broth. This culture was then incubated for 2 hours to produce bacterial suspension of moderate turbidity. Then, the turbidity was compared to that of the recommended turbidity of standard (McFarland) Tube No. (0.5). A sterile swab was used to obtain an inoculum from the standardized culture. This inoculum then was streaked on a Muller-Hinton plate, after that the inoculated plates were left undisturbed at room temperature to permit the inocula to be absorbed in to the medium surface. By using cork, 2 wells were made in Petri dishes which had bacterial growth prepared previously. Added (0.5ml) from each concentration of compounds which prepared previously to each well. The inoculated plates were incubating at (37C°) for 24h. Then the MIC was measured as the lowest concentration from compound showing no bacterial growth.

The results were expressed as mean ± SEM unless otherwise stated. Statistical analysis was carried out using paired t-test and ANOVA. P value less than (0.05) level of significance was considered statistically significant.

Results

In comparison to tetracycline, some aza sugars were used to show their effect on *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Pseudomonas auerogenosa* isolates and The results of sensitivity test are shown in Table (1).

It was found that aza sugars compounds were had significant effects on bacterial isolates ($p<0.05$) and these effects different according to type of compound and genus of bacteria, where all bacterial isolates showed high sensitivity (100%) to B1 and B2 compounds while sensitivity percent to A1 and A2 was 72.72% and 45.45% compared with 27.27 % for tetracycline **(table 1)**. While in **table (2)** *Haemophilus influenzae* do not appear resistant to all studied aza sugars with high resistance (100%) to Tetracycline, While *Klebsiella pneumoniae* isolates were showed significant I ($p<0.05$) high sensitivity to all aza sugar (A1,A2,B1,B2) and Tetracycline, on the other hand *Pseudomonas auerogenosa* appear high resistance (100%) to Tetracycline but did not appear any resistance (0 %) for A1, A2, B1 and B2 compounds .

Table (1) Growth zone inhibition (mm) of bacteria isolates against the chemical the tested compounds as compared to tetracycline.

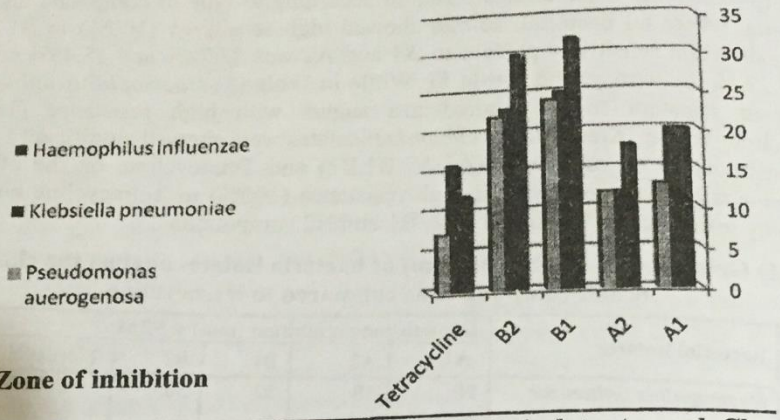
Bacterial isolates	Growth zone inhibition (mm) ± SEM				
	A1	A2	B1	B2	Tetracycline
Haemophilus influenzae	20 ± 0.07	18 ± 0.06	32 ± 0.06	30 ± 0.08	12 ± 0.06
Klebsiella pneumoniae	20 ± 0.03	12 ± 0.04	25 ± 0.04	23 ± 0.03	16 ± 0.05
Pseudomonas auerogenosa	13 ± 0.06	12 ± 0.02	24 ± 0.04	22 ± 0.04	7.2 ± 0.02
Percent of sensitivity	%72.72	45.45%	100%	100%	%27.27

A1= 2-3-O-diacetyl –N-(isobutyl),aza ascorbic acid derivatives ,A2=2-3-O-diacetyl –N-(isopentyl),aza ascorbic acid derivatives ,B1=N-isobutyl 5-formyl-3,4dihydroxy- 2-N-pyroline., B2 = N-isopentyl 5-formyl-3,4dihydroxy- 2-N-pyroline.

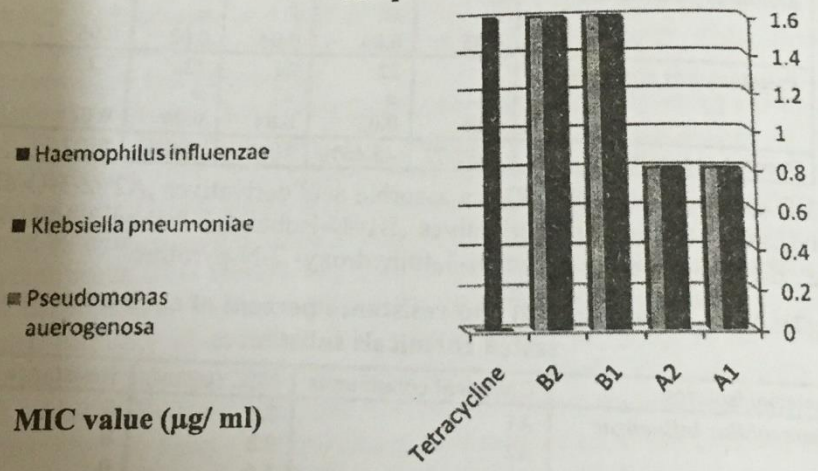
Table (2): MIC_s value (µg/ ml) and resistance percent of each bacteria against the tested chemicals substances.

Bacterial isolates	Chemical compounds	MIC (µg/ml)	Resistance percent
Haemophilus influenzae	A1	0.8	0
	A2	0.8	0
	B1	1.6	0
	B2	1.6	0
	Tetracycline	.	%100
Klebsiella pneumoniae	A1	0.8	0
	A2	0.8	0
	B1	1.6	0
	B2	1.6	0
	Tetracycline	1.6	0
Pseudomonas auerogenosa	A1	0.8	0
	A2	0.8	0
	B1	1.6	0
	B2	1.6	0
	Tetracyclin	.	100

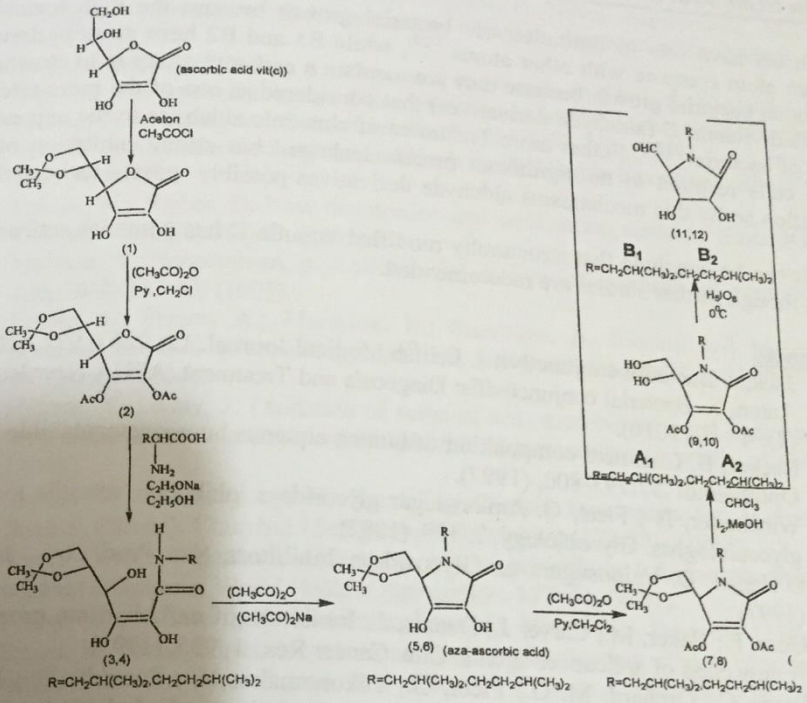
A1= 2-3-O-diacetyl –N-(isobutyl),aza ascorbic acid derivatives ,A2=2-3-O-diacetyl –N-(isopentyl),aza ascorbic acid derivatives ,B1=N-isobutyl 5-formyl-3,4dihydroxy- 2-N-pyroline.,B2=N-isopentyl 5-formyl-3,4dihydroxy- 2-N-pyroline,T= Tetracycline. . = no inhibition zone .



Fig(1): Growth zone inhibition (mm) of bacteria isolates toward Chemical compounds.



Fig(2): MICs value (µg/ ml) and sensitivity percent of each bacteria toward Different chemicals substances.



Discussion:

Haemophilus influenzae and *Pseudomonas auerogenosa* showed high resistant against tetracycline. This resistant may be due to miss use of tetracycline by the population[22] as compared to structurally modified vitamin C that relatively new and not use. The resistance of *Haemophilus influenzae* and *Pseudomonas auerogenosa* could be due to plasmid-dependent resistance to amino glycosides produce adenylylating, phosphorylating or acetylating enzymes that destroy the drugs[23]. In addition, the resistance to tetracycline results from changes in permeability of the bacterial cell envelope[24]. Also, this drug not actively transported into the cell or leaves it so rapidly that inhibitory concentration is not maintained. On the other hand many have pointed that all *Haemophilus influenzae* and *Pseudomonas auerogenosa* isolates from neonates with Ophthalmic Neonatorum are resistant to tetracycline[25,26].

The percent of resistance of the studied bacteria against vitamin C is zero with high percent of sensitivity, compared to tetracycline which is highly resisted by the studied bacteria, this could be due that the structurally modified vitamin C is new and not used.

The mechanisms of action of structurally modified vitamin C sensitivity in bacteria may be due to interferes with the synthesis of cell wall mucopeptide during active multiplication, resulting in bactericidal activity against bacteria or may be due to blocking the attachment of amino acids to the nascent peptide chain on the 50s unit of ribosome's by interfering with the action of peptidyl transferase[27].

In this study we noted that A1 and A2 compounds have less effect than B1 and B2 compounds ,this may be return to that A1 and A2 compounds contain an acetyl group

which act have role in limitation of bacterial growth because the high toxicity of oxygen atom compare with other atoms [28], while B1 and B2 have more bactericidal effects on bacterial growth because they are contain a carbonyl group in in structurally modified vitamin C (aldehyde derivatives) that considered as one of the more effective killer of bacteria[29,30]. Futher more Treatment of cinnamic aldehyde to the exponential phase cells resulted in no significant protein leakage but strong inhibition of cell separation so by this mechanisms aldehyde derivatives possibly posses its bactericidal effect[31].

Thus it can be conclude that structurally modified vitamin C has potential antibacterial effect, though further studies are recommended.

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