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Inhibitory effect of new ligand 2, 3, 5, 6, 0, 0, 0, 0- tetra acetic acid-L- ascorbic acid on pathogenic bacteria

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Abstract:
The inhibitory effect of new product (2, 3, 5, 6, 0, 0, 0, 0- tetra acetic acid-L- ascorbic acid)(V-C-4Ac) on growth of different types of pathogenic bacteria was investigated. Different concentrations were prepared from (V-C-4Ac) , (1%, 3%, 4% and 5%) nearly completely inhibited the growth of *Streptococcus pneumoniae* and *Streptococcus agalactiae* after 24 hrs incubation, and the same range of concentrations did not inhibit the growth of *Streptococcus mutans*, *Klebsiella pneumonia* and *Proteus mirabilis*. Also, this study shows low effect of new ligand in the growth of *Staphylococcus aureus* and *E. coli* in high concentration (5%) after 2 hrs incubation.
Keywords: L- ascorbic acid, New ligand synthesis (V-C,4Ac) , Antibacterial effect.

1. Introduction:

L- ascorbic acid is the water soluble form of vitamin C. it is considered a potent antioxidant. Besides its antioxidant benefits, ascorbic acid plays a primary role in collagen formation which is essential for the growth and repair of tissue cells, gum, blood vessels, bones and teeth. Because it is derived from glucose, many animals are able to produce it, but humans require it as a part of their nutrition (Lachapella and Drouin, 2010). L-ascorbic acid has been reported to act in a number of ways. It acts as a biological hydrogen carrier for redox enzyme systems in cell metabolism (Gilula *et al*., 1998), as a food preservative by oxidative rancidity fatty oily foods or to prevent discoloration of preserved fruits and vegetables (Weiss, 2007). Although ascorbic acid has a wide range of antibacterial effects, some of its, oxidative products are toxic (Halli, 1996). L-ascorbic acid was bactericidal for many types of bacteria like *Bacillus pertussis* and *Mycobacterium tuberculosis* that lead to inhibition of their growth in a medium containing ascorbic acid (Davies *et al*., 1991). Less sever deficiency of L-ascorbic acid produces alterations in combative tissue structure and may also cause decreased resistance to some infections (Hemila, 2006). It has multi-functional molecule in tissues. It usually act as antioxidant, free radical scavenger, neuroprotectant and plays an important physiological function in activating peptide hormone and regulating cell division and growth (Maryrat *et al*., 2007). In present study the reaction of L-ascorbic with the chloroacitic acid in presence of potassium hydroxide new product called (2, 3, 5, 6, 0, 0, 0, 0- tetra acetic acid-L-ascorbic acid) (V-C-4Ac) has been investigated as antibacterial against gram positive and gram negative bacteria.

Aims of study: The study examined the effect of new derivative of L- ascorbic acid (2, 3, 5, 6, 0, 0, 0, 0- tetra acetic acid-L- ascorbic acid) on growth of different types of gram positive and gram negative bacteria isolated from different sites of infections give some explanation of this effect.

2. Material and methods:

2.1 Organic synthesis of (V-C-4Ac)

All chemicals were purchased from BDH, and used without further purifications. Synthesis of (2, 3, 5, 6, 0, 0, 0, 0- tetra acetic acid-L- ascorbic acid), Admixture of solution L-ascorbic acid (0.001 mole, 0.176 gm.) in aqueous ethanol (15 ml ethanol+ 5ml water) and solution of potassium hydroxide (0.004 mole, 0.224 gm.) in 5ml ethanol was stirred for 30 minutes at room temperature and then chloroacitic acid (0.004 mole, 0.380 gm.) in 10 ml ethanol was added. The mixture was stirring for one hour then leaves it to evaporate slowly. The precipitate is taken up in ethanol/water (10:2) and filtered through a pad of sialic gel. The filtrate is evaporated slowly to give orange product, m.p 138-139^{o}C, soluble in water and DMSO.IR (KBr, cm^{-1}); 3421(br) 160 and (COOH); 1755 (Lacton CO). H N.M.R (ppm) (D_2O; 4.2 (1-H-CH-Lacton) 4.8 (2H, OCH_2); 8-9.5 (H (br) COOH) (Sultan and Musa, 2011).

2.2 Bacterial strains:
Clinical strains of *Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus mutans, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Proteus mirabilis* were obtained from Department of Microbiology-Collage of Medicine-Babylon University. All the bacterial strains were maintained on freshly prepared blood agar.

2.3 Preparation of different concentration of (V-C-4Ac):
Different concentrations of (V-C-4Ac) were prepared to study the antibacterial effect (1%, 3%, 4% and 5%) uses as stock for this detection (special communication of Prof.Dr. Mufeed Ewadh, 2013).

2.4 Antibacterial activity:
The agar diffusion method (Bauer *et al*., 1966) was followed for the antibacterial susceptibility test. A loopful bacteria was taken from the stock culture and dissolved in 0.1 ml of saline. All the tests were done by placing the disk (6mm diameter) impregnated with (200ml) extracts on the Mueller Hinton agar surface previously inoculated with 10 ml of MHA liquid medium with gram negative and gram positive bacteria.

2.5 The effect of (V-C-4Ac) on bacterial growth by using optical density:
Flasks containing 30 ml nutrient broth (pH 7.2), nutrient broth plus (V-C-4Ac) (1%, 3%, 4% and 5% separately) were inoculated with 0.5 ml of overnight grown bacterial culture. The pH of nutrient broth was adjusted to 5.9 (using HCL or NaOH) before addition of the L-ascorbic acid. Then, the L-ascorbic acid was added to flasks containing 30 ml of nutrient broth (pH 5.9) and treated as previously described. After insulation, the flasks were incubated at 37^{o}C for 24 hrs. and at suitable intervals, samples were withdrawn from each flask and growth was monitored by measuring absorption at 560 nm spectrophotometrically. During incubation, at least three reading were obtained from each flask. All experiments in this study were performed three times, and the absorption readings presented are the mean value.

3. Results:
In the present study investigation of antibacterial activity of the (V-C-4Ac) against different gram positive and gram negative bacteria was recorded. Table (1) summarizes the microbial growth inhibitory by different concentration of (V-C-4Ac) The maximum inhibition zone present in the high concentration (5%) against *Streptococcus agalactiae* (25mm) followed by *Streptococcus pneumoniae* at the same concentration (24mm), *Staphylococcus aureus* (8mm) and *E. coli* (6 mm), there was no effect on *Streptococcus mutans*, *Klebsiella pneumonia* and *Proteus mirabilis* as shown in Figure (1.a, b, c, d) and Figure (2). The absorption A_{560} reading of *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *E. coli* subjected to (V-C-4Ac) in different concentrations. The presence of (V-C-4Ac) in the growth medium of *Staphylococcus aureus* and *E. coli* did not cause substantial inhibition of growth (Figure 3). However, (V-C-4Ac) nearly completely inhibited growth of *Streptococcus pneumoniae* and *Streptococcus agalactiae* (Figure 4). (V-C-4Ac,1%, 3% and 4%) did not show an inhibitory effect on *Staphylococcus aureus* and *E. coli*.

4. Discussion:
Streptococcus pneumoniae is bacterial pathogen which causes pneumonia, bacteremia, meningitis, sinusitis, and otitis media in human worldwide, especially in neonates and children, and often leads to significant rates of mortality and morbidity. Also, *Streptococcus agalactiae* is the certain pathogen isolated from vaginitis cases. Streptococci secret hyaluronate lyase to catalyze the degradation of hyaluronan, one of the main connective tissues in animals, to expose tissue cells to bacterial toxins. Therefore, it is important to control growth of these bacteria that present with high resistance to different antibiotic (Songlin *et al.,* 2011). (V-C-4Ac) showed an inhibitory effect on *Streptococcus pneumoniae* and *Streptococcus agalactiae* but show low effect on *Staphylococcus aureus* and *E. coli*. This is consistent with results obtained by other investigators(Richter *et al*., 1988) reported that addition of ascorbate to aerobically growing cultures of *E. coli* caused only a short pause in growth and no subsequent change in rate of growth. Tabak *et al*., (2003) reported that 10-20 mg/ml ascorbic acid inhibited *Helicobacter pylori* growth under microaerophilic conditions. Also, it is possible that *E. coli* strains tested utilized this compound. It has been shown that L- ascorbic acid exerts no growth promoting action upon *E. coli*, *Brucella abortus, Klebsiella pneumonia, Proteus* spp., *Shegilla dysenteriae, S. albus, Staphylococcus aureus* and *St. pyogenes* (Mieda *et al*., 2004), these results nearly similar to our results. Menzel and Farr, (1988) reported that variety of organisms could destroy L-ascorbic acid, including strains of *E. coli, Enterococcus, Proteus, Klebsiella, Vibrio* and *St. pyogenes*. From our results addition of (V-C-4Ac) to the growth medium of

Streptococcus pneumoniae and *Streptococcus agalactiae* nearly completely inhibited growth of tested strains. Similar results were obtained by other investigations. Sonlin *et al.,* (2001) which reported that number of total aerobic bacteria *Streptococcus pneumoniae* and *Streptococcus agalactiae* were inhibited after treatment with L-ascorbic acid. There are many evident ways in which ascorbic acid could inhibit the growth of bacteria. Valpuesta and Botella (2004) tested the ability of ascorbic acid destroying bacteria to utilize the vitamin as a sole source of carbon using a synthetic basal medium, they found that only one species, *A. aerogenes* could grow on repeated sub cultivation. Also, Mieda *et al*., (2004) reported that at least three ways for ascorbic acid to inhibit the growth of bacteria, acidophobic organisms, by increasing the acidity of medium or with aerobic organisms by reducing the oxidation / reduction potential, particularly in liquid media and lastly by becoming oxidized with the formation of hydrogen peroxide. L- ascorbic acid had been reported to have certain inhibitory effect on the enzyme activity of hyaluronidaseses (spreading factors). Moreover, hyaluronidase inhibitors could be useful as drugs like in the treatment of arthritis, or combined with antibiotics, in antibacterial therapy of hyaluronate lyase producing bacteria like *Streptococcus pneumoniae* and *Streptococcus agalactiae* a major human gram positive bacteria (Botzki *et al*., 2004; Li and Jedrzejas, 2000).

5. Conclusions:

In this study , the uses of new ligand 2, 3, 5, 6, 0, 0, 0, 0- tetra acetic acid (V-C-4Ac) exhibited antibacterial activity and shows nearly completely inhibited or greatly reduced the growth of *Streptococcus pneumoniae*, *Streptococcus agalactiae* strain tested in all prepared concentrations while *E. coli* and *Staphylococcus aureus* shows low effect of their growth in 5% only. *Streptococcus mutans*, *Klebsiella pneumonia* and *Proteus mirabilis* present with no effect on their growth in different concentration of (V-C-4Ac).

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Table (1) effect of (V-C-4Ac) in different bacterial isolates

Bacterial isolates	Inhibitory effect of (V-C-4Ac) (mm)			
	1%	3%	4%	5%
Streptococcus pneumoniae	*16 mm	18 mm	22 mm	24 mm
Streptococcus agalactiae	18 mm	21 mm	22 mm	25 mm
Streptococcus mutans	-ve	-ve	-ve	-ve
Staphylococcus aureus	-ve	-ve	-ve	8 mm
Escherichia. coli	-ve	-ve	-ve	6 mm
Klebsiella pneumonia	-ve	-ve	-ve	-ve
Proteus mirabilis	-ve	-ve	-ve	-ve

*: Inhibition zone in mm

-ve: no effect

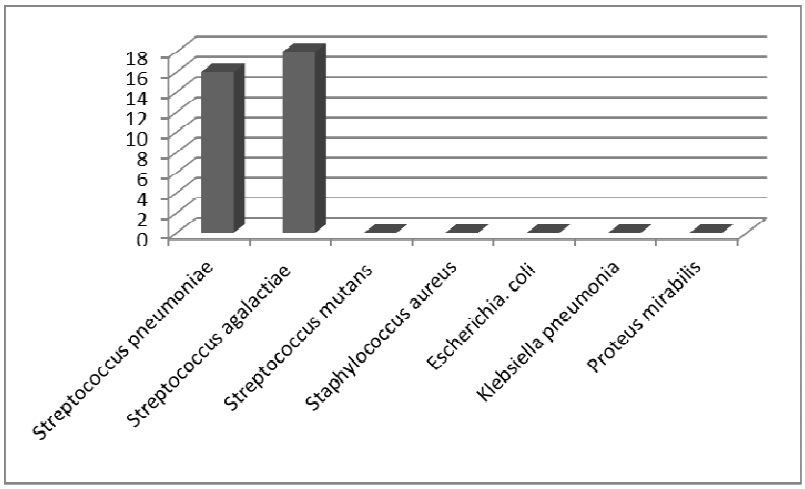

Figure (1.a) effect of 1% (V-C-4Ac) in different bacterial isolates

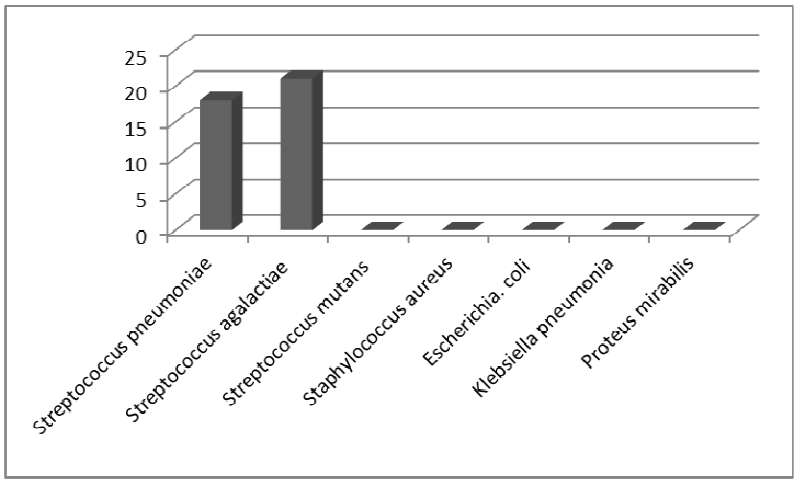

Figure (1b) effect of 3% (V-C-4Ac) in different bacterial isolates

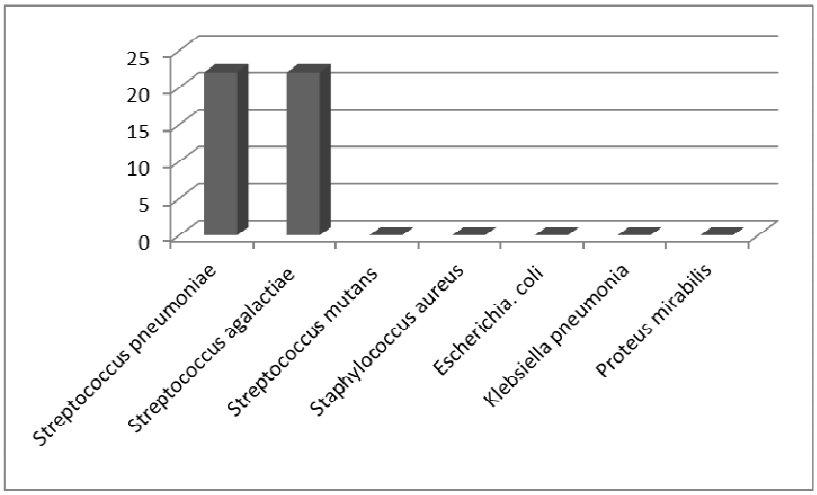

Figure (1c) effect of 4% (V-C-4Ac) in different bacterial isolates

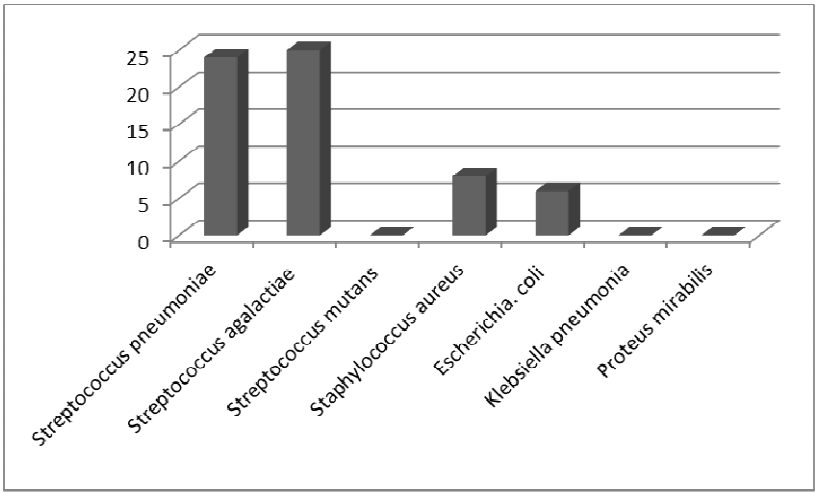

Figure (1d) effect of 5% (V-C-4Ac) in different bacterial isolates

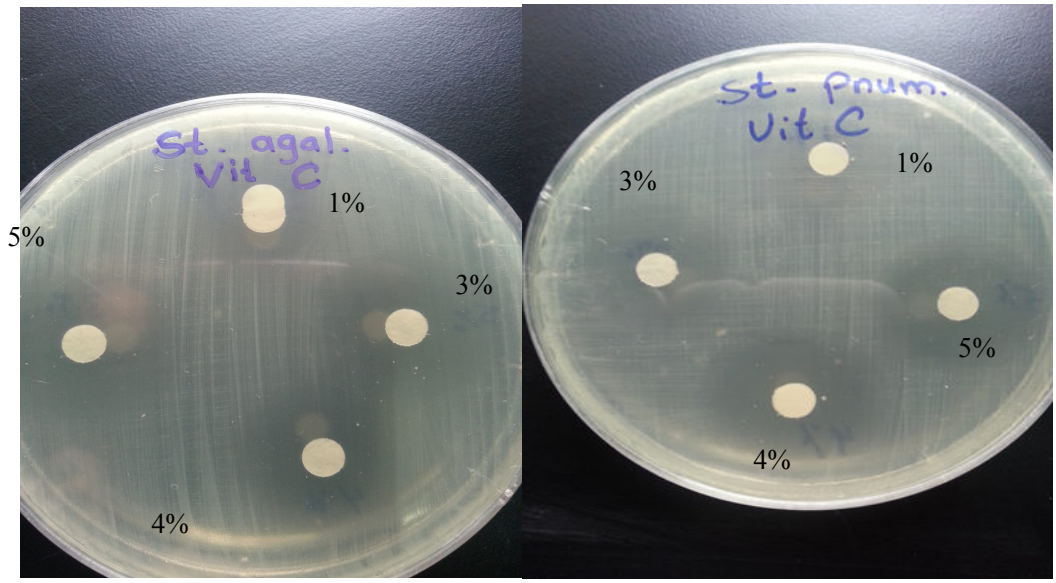

Figure (2) effect of (V-C-4Ac) on *Streptococcus pneumoniae* and *Streptococcus agalactiae* (disk method)

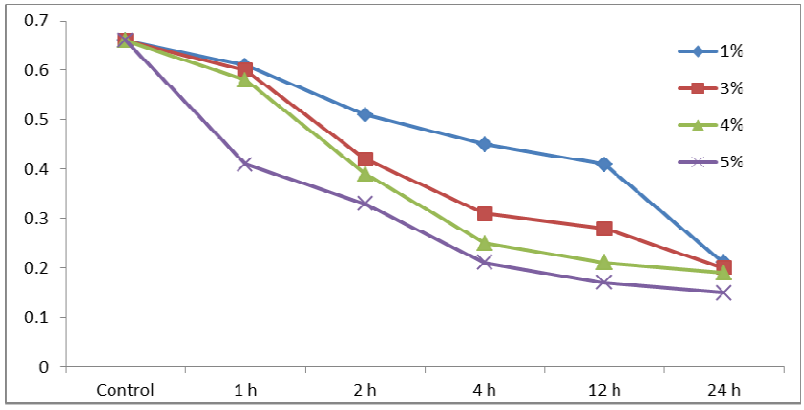

Figure (3) effect of (V-C-4Ac) on *Streptococcus pneumoniae* A_{560}

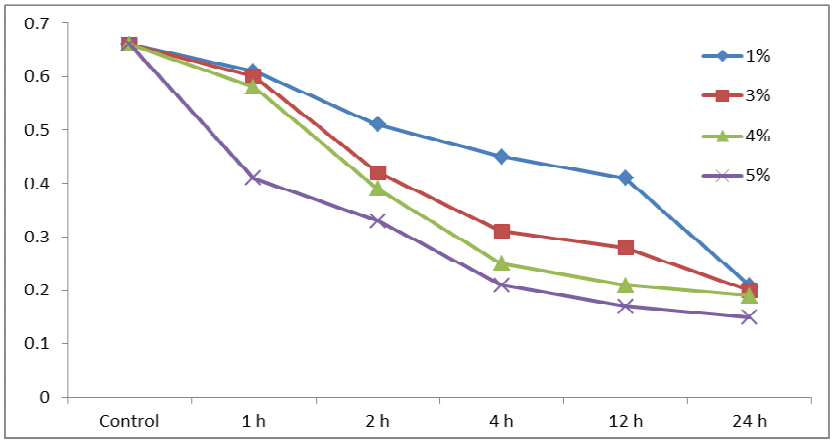

Figure (4) effect of (V-C-4Ac) on *Streptococcus agalactiae* A_{560}

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