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The modification of Cowpea Mosaic Virus (CPMV) with Aspirin and Its application as Anti- Infectious Drugs

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Abstract

Currently, Infectious diseases are of the most concern and threat to the entire world. They are caused by bacterial, fungal, parasitic and viral infections. Now days, nanotechnology-based drug delivery systems are in use to overcome these challenges. The current research focuses on the use of aspirin as an anti-inflammatory drug, both with and without conjugation to the outer surface of (CPMV) in order to enhance the efficacy of therapeutics and reduce or eliminate the drug resistance which is the biggest biological problem in the treatment of infectious diseases.

Due to the resistance to commercially available medications, the particle used as an Aspirin (AS) carrier. When compared to free Aspirin, which has limited anti-infectious effect, the CPMV-AS conjugate, in which 160 molecules are covalently linked to the exterior surface amines of the viral nanoparticle (VNP), showed the strongest suppression against both gram-positive and gram-negative bacteria.

Keywords

Aspirin, inflammatory disease, Viral nanoparticle.

Aspirin (AS), is often observed as a prototype of drug series designated non-steroidal anti-inflammatory acids.[1] The metabolite activity of (AS), is that has important effects on bacteria that might improve the efficiency of antibiotics treatment by prompting phenotypic resistance in bacteria. Recently, researchers have found the responsibility of AS for reducing the toxicity of bacteria which is associated with serious infections.[2] The effect of (AS) on the interaction between platelet and bacteria (*S. aureus*) have been considered by two distinct effects : first associated to main molecule, acetyl salicylic acid (AS), which essentially works on platelets, and another, associated with salicylic acid (SA), which is important metabolite, which works on the genetic regulation of the bacterium virulence factors.

Additionally, AS treatment was linked with decreasing in neutrophil recruitment and the producing of neutrophil platelet agglomeration. Thus, affecting aspirin through the metabolic pathway of fatty acid which has suggested to describe the decrease in mortality in studies of sepsis associated with *S. aureus*.[3,4]

Due to increasing antibiotic resistance and absence of new antibiotic development, looking for new technique becomes required.

Plant viruses could provide the best source for developing drug delivery vehicle. For example, They have evolved to carry cargos to host cells with remarkable efficiency, as predicted. The plant virus CPMV was used as a carrier in this investigation; it is an icosahedral virus with a diameter of 30 nm. The asymmetric unit is made up of sixty copies of two different types of coat proteins. Many copies of a particle can provide many additional attachment points, allowing binding and the production of a wide-range of moieties on the external surface.[5-7]

Additionally, CPMV is safe for humans and the products because, the culture of plant virus is not contaminated inside animal cells or viruses. [8,9]. CPMV is extremely stable in a range of (pH, temperature and a variety of organic solvents like DMSO). [10, 11]

In this study, Aspirin was tested as antimicrobial drug with and without conjugation with CPMV particle, then the effect of CPMV particle to enhance the activity of AS as an anti-infectious agent has been determined.

Experimental Part

Materials and Instrumentation

1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), N hydroxysuccinimide (NHS) were purchased from Sigma. Dimethyl sulfoxide, DNA loading dye 6X, agarose low melting molecular biology grade, standard Aspirin was provided from local pharmacy.

Electrophoresis Methods

A mixture of 20 µg of CPMV particles suspended in 10 mM sodium phosphate buffer pH 7.1 with 3 µl of loading dye were loaded on 0.6 % (w/v) agarose gel in an electric field of 60 V for 2 hours with coomassi stain. The Gene Genius Bio Imaging System and the software Gene Snap, have been used for imagining particles on a UV transilluminator at 302 nm (Syngene). For coat protein staining, the gel was stained for 5 hours with Coomassie staining solution (25 percent (v/v) methanol; 5 percent (v/v) acetic acid; 0.12 percent (w/v) Coomassie Brilliant Blue G-250), then destaining solution (25 percent (v/v) methanol; 10 percent (v/v) acetic acid in Milli-Q water) overnight.

The modification of CPMV with AS

(4 mg/ml) CPMV suspended in 0.01 M sodium phosphate buffer (SPB) pH7.1 was reacted with freshly prepared EDC in 1000 molar excess (0.006 g, 0.0416 mmol,) and (2 ml) of 4000 molar excess of NHS (0.006g, 0.052 mmol,) in DMSO and (SPB) pH 7.1. The reaction was allowed to proceed for 3 hours while gently stirring. Then, in 6 ml (SPB) pH 7.1, a 5000 molar excess of AS (0. 4g, 0. 0056 mol) was added. Then the reaction was stirred overnight at room temperature. The final DMSO content was adjusted to 20% by volume. CPMV-AS was purified against 0.01 M (SPB) using 100 kDa molecular weight cut-off membranes (Float-A-Lyzer G2), while it was concentrated using 100 kDa cut-off columns (Millipore)[12] .Recovery of CPMV particle was 85%.

The conjugation of CPMV-AS with Alexa Flour Dye

A molar excess of 30000 dye molecules of Alexa Fluor 488 dye was used to identify the conjugated number of AS on the external surface of CPMV according to published procedure.[13] Recovery of virus was 78% according to initial concentration.

Antibacterial activity

Preparation of suspended bacterial

Stock cultures of two types of bacteria (*S. aureus* and *E. coli*) were sub cultured onto BA plates then incubated for 24h at 37 °C. Similar morphology of bacterial colonies were added to (10 ml) sterile Mueller Hinton broth (MHB) and left at 37°C for 24 h. The overnight bacterial suspensions were adjusted to (0.5) McFarland Standard with sterile broth of MHB.

Determination of Minimum Inhibitory Concentration (MIC) assay.

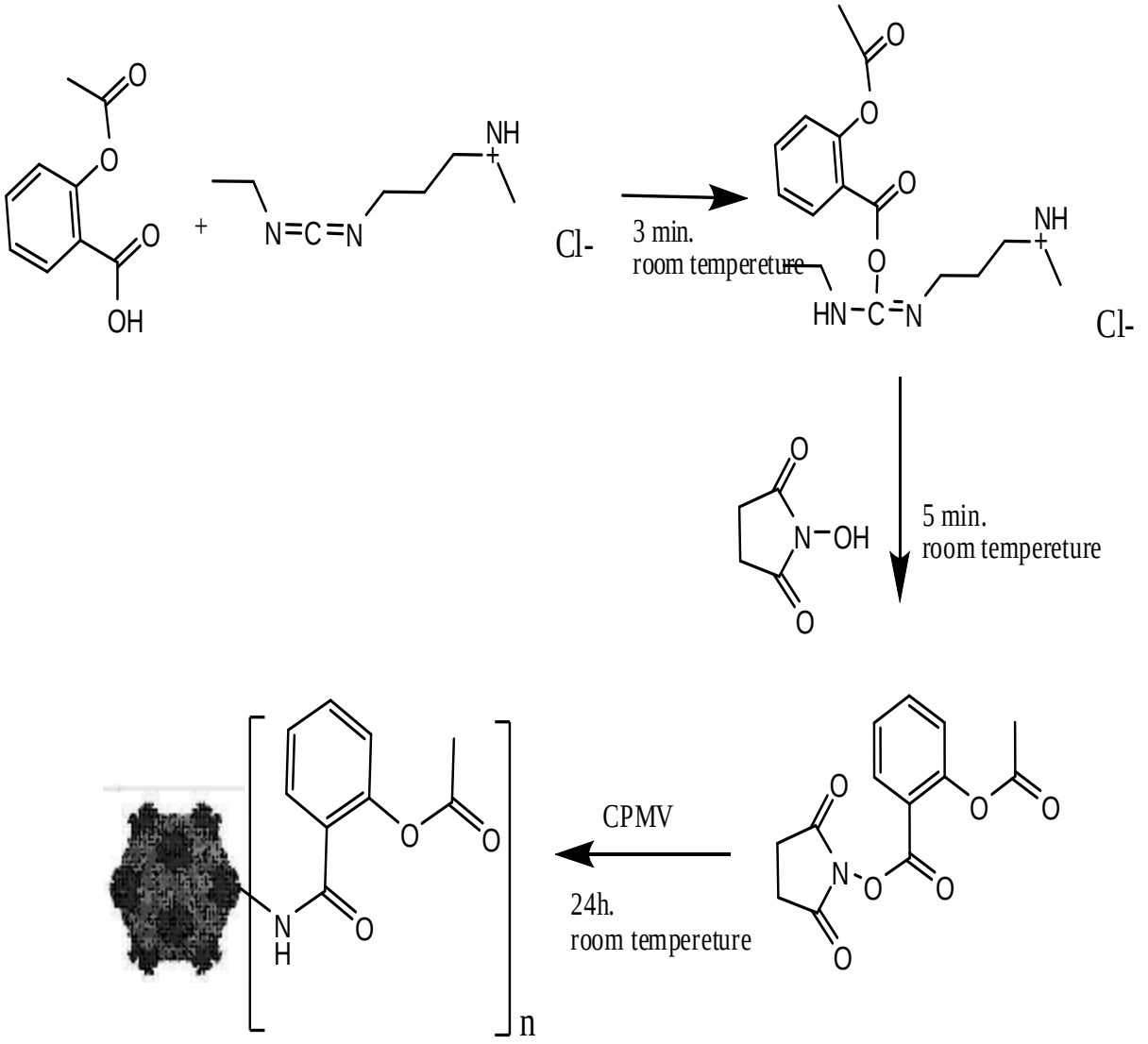
Minimum Inhibitory Concentration (MIC) has been evaluated according to published procedure. [14]

0.1 mg / ml of stock solutions of each tested materials (AS and CPMV-AS) have been prepared, all

samples were serially two-fold diluted starting from 10 µM, which is a 100-fold dilution from the stock solutions of each sample (10,5,2.5,1.25 and 0.63 µM).

Results and discussion

AS was conjugated to the external surface of CPMV as shown in bellow scheme



Scheme 1: The modification of CPMV-AS mechanism

EDC/ NHS protocol was used to improve the modification of CPMV with AS and recovery of modified particles was 85%, according to the initial concentration of CPMV particle. The integrity of the changed particle was confirmed using agarose gel electrophoresis and TEM.. In present study, the specific binding of AS to the external surface of CPMV allowed the particles to be recognised specially through the cell lines of bacteria.

After staining with Coomassie Blue, the modified particle can be seen on an agarose gel. Additionally, the modification of CPMV-AS was confirmed using UV-Vis spectroscopy in aqueous 20 % DMSO. It has two absorption maxima at 260 nm for CPMV and 268 nm for AS. Thus making easy identification of covalent binding. **Fig 1B**

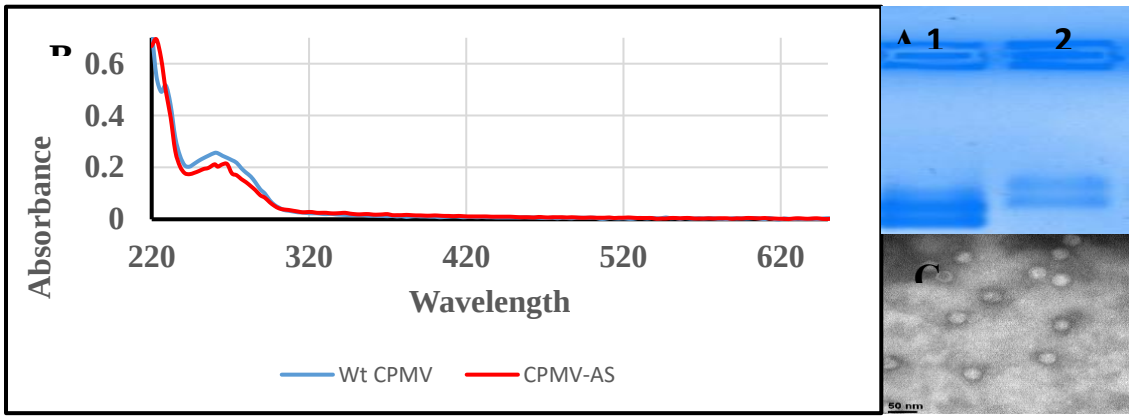


Figure 1: The modification of CPMV with Aspirin, A: 0.6 % (w/v) Commassie staining agarose gel: lane 1- Wt CPMV, lane 2- CPMV- AS, B: UV-Vis spectroscopy analysis, C: Uranyl acetate stained transmission electron micrographs of externally modification of CPMV- AS.

For CPMV-AS, **Fig 1A**, the mobility was slow compared to unmodified particle due to the effect the combination of charge and mass dependable on the modification of virus surface. Similarly, zeta potential for conjugation was shown differences, it being significantly more negative than Wt CPMV (CPMV-AS −35.4 mV, CPMV −12.1 mV).

(Uranyl acetate stained transmission electron micrographs) TEM shows that the virus particle remains its integrity with average diameter of (31 nm) after conjugation with AS. **Fig 1C**.

The coupling of modified and unmodified particle with Alexa Flour Dye 488 was confirmed using UV/Vis and electrophoresis. **Fig2**

The concentration of CPMV and Alexa Flour Dye 488 has been calculated using Beer-Lambert Law. UV/Vis spectroscopy indicated the absorbance of CPMV and dye at 260 nm, 290 nm respectively, the extinction coefficient of CPMV 8.1 $ml.mg^{-1}.cm^{-1}$ while, it was 70000 $M^{-1}.cm^{-1}$ for dye. 160 ± 3 molecules of AS per particle has been achieved. **Fig2B**

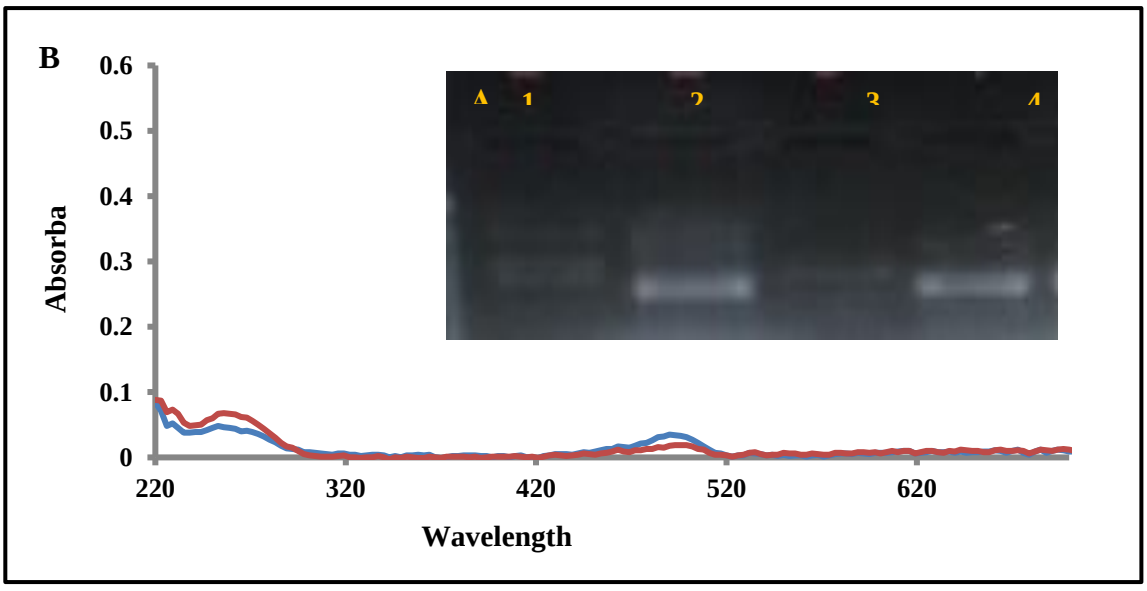


Fig 2. Modified and unmodified CPMV and CPMV-AS with Alexa Flour dye A: agarose gel electrophoresis using 0.6 % (w/v) agarose gel with Et Br staining:, lane 1 CPMV-AS, 2 Wt CPMV, 3 CPMV-AS- dye after 8h dialysis, 4 CPMV-dye after 8h dialysis , B: UV/Vis spectrum. Blue line confirm the modification of CPMV-AS-dye and Red one is CPMV- dye.

The present study suggested that CPMV has ability to redirect successfully by surface modification with AS allowing exact uptake into bacteria surface resulting in an excellent growth elimination of bacteria while, there is no significant inhibition when AS tested a lone against the same bacteria.

(MICs) was used to identify the inhibition of visible growth of bacteria and its resistance after overnight incubation, but exactly, to determine the in vitro activity of new CPMV-AS modified particle as antimicrobials agent against *Staphylococcus aureus* and *Escherichia coli* to improve the activity of CPMV as a carrier vehicle for AS. The results shown an excellent inhibition and illumination of bacterial growth with CPMV-AS modified particle in comprising with free AS. **Table1**

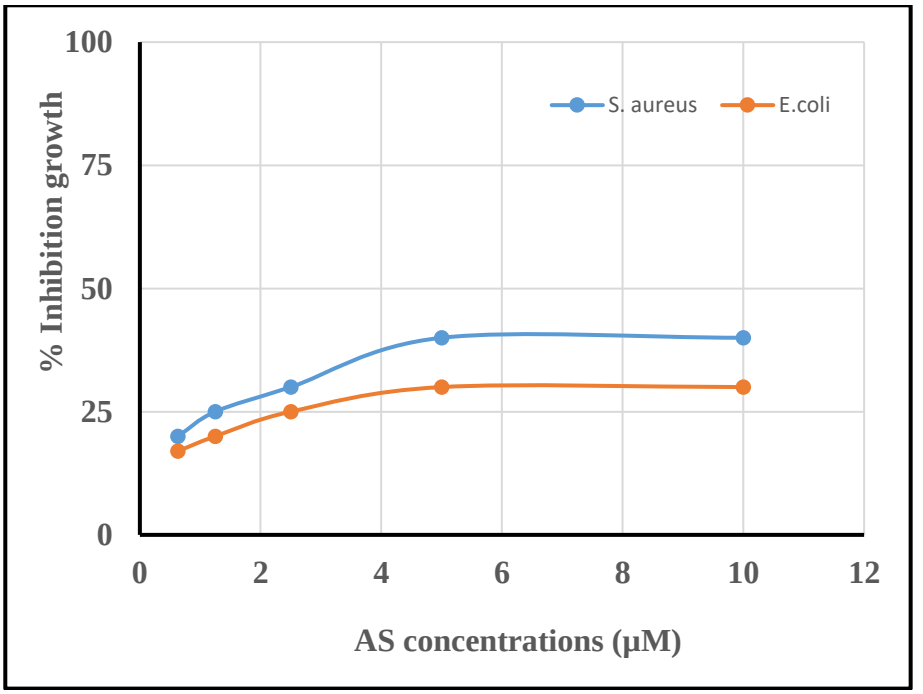
Table 1: minimum inhibitory concentrations for tested materials

bacteria	Aspirin (0. 63 µM) MIC	CPMV-AS (0. 63 µM) MIC	The inhibitor concentration of modified AS
S. aureus	20 %	80% inhibition	**1.1 nM**
E. coli	17 %	70% inhibition	1.1 nM

Covalent bound of CPMV-AS conjugates provide an improvement over free AS. At low dosage

(0.63 µM) as a minimum inhibitory concentration, CPMV-AS was found to be more active in comparison to over free AS. **Fig 3**

Competition of (CPMV-AS $)_{160}$ was achieved with increasing amounts of AS. when, the concentration of virus particle 10 µM thus, giving increase to the concentration of displayed AS. (CPMV-AS $)_{160}$ = 1.1 nM AS.



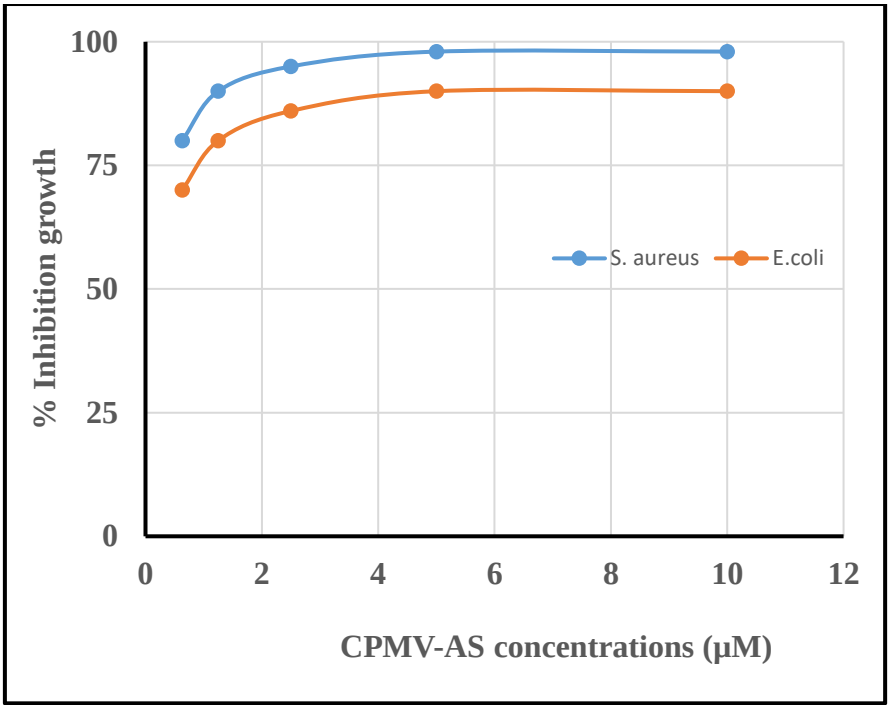


Figure 3: The effect of AS as anti -bacterial agent before and after conjugation with CPMV against Staphylococcus aureus and Escherichia coli

Additionally, the results shown good antibacterial activity against gram-positive than gram- negative bacterial because of the difference in the structure of the cell wall. **Table 1**

CPMV provided 160 molecules instead of one molecule can diffuse through the cell wall of bacteria, thus allowing higher doses of AS to accumulate at the target and destroy the resistance of bacteria.

Conclusions

At low doses concentration, the CPMV-AS was more effectiveness as an antibacterial agent against *S. aureus* and *E. coli* than free AS. In compared to AS modified particles, the harmful effect of AS is delayed. Furthermore, when compared to free AS, conjugated AS has a considerable effect on gram-positive and gram-negative bacteria suppression. Using CPMV nanoparticles as carrier vehicle provided 160 AS instead of one molecule can diffuse through the bacteria's membranes, allowing greater concentrations of AS to concentrate at the target and kill the resistant of bacteria. However, there is potential to extend the platform technology further to bring targeting drugs that can be utilized to reduce the negative effects of existing anti-infectious treatments.

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