

## Design and Development of Dual Drug Loaded Niosomes Containing Amoxicillin and Clavulanic acid

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### ABSTRACT

Niosomes formulation for the fixed dose combinations of amoxicillin-clavulanic acid were prepared using Span 60 and cholesterol by the ether injection method. Span 60 and cholesterol were added in various molar ratios to affect the vesicle membrane rigidity. Drugs combinations of amoxicillin-clavulanic acid entrapped in the bilayer of niosomes were quantified and the entrapment efficiency was found to be reaching up to 76%. The size and size distribution of niosomes were determined on light microscopy and transmission electron microscopy. The accelerated stability testing was used to predict the stability of the formulations. The test revealed changes in the characteristics of the liposomes. The *in-vitro* antibacterial assay of amoxicillin-clavulanic acid, revealed that niosomes formulations have stronger inhibitory activity.

**Keywords:** Niosomes, drug, amoxicillin, clavulanic acid

### Introduction

Development of newer drugs is very difficult, expensive and rather time consuming due to its tedious preclinical and clinical testing. Improving safety and efficacy of existing drugs has been attempted using different methods such as therapeutic drug monitoring and targeted drug delivery etc [1]. Drug delivery systems could provide an extended drug bioavailability so that less drug is required for therapeutic effectiveness and reduces its toxicity [2].

Recently, lipid and nonionic surfactant based drug delivery systems specifically niosomes have drawn much attention from researchers as potential carriers of various bioactive molecules that could be used for therapeutic applications. Several commercial niosome-based drugs have already been marketed with a great success such as estradiol and dithranol [3, 4].

Niosomes are vesicles composed mainly of hydrated non-ionic surfactants in addition to, in many cases, cholesterol or its derivatives. The unique structures of niosomes make it capable of encapsulating both hydrophilic and lipophilic substances. This can be achieved by entrapping hydrophilic in vesicular aqueous core or adsorbed on the bilayer surfaces while the lipophilic substances are encapsulated by their partitioning into the lipophilic domain of the bilayers [5]. Fixed-dose combination drugs (FDCs) are products that contain two or more active drugs in a fixed-dose ratio, and are useful for minimising pill burden and lowering cost. However, FDCs should ideally contain constituents that act via different mechanisms and do not cause additive toxic effects. At present, 118 antibiotic FDCs are available on the Indian market [6]. Antibiotic resistance is of increasing concern worldwide. Studies of several antibiotic combinations, such as amoxicillin-clavulanic acid, have reported additional advantage over their individual constituents, and have been reported to cause less toxic reactions and reduced resistance [7]. Keeping these things in mind, the present study is focused on the design and development of dual drug loaded niosomes containing amoxicillin-clavulanic acid (Fig. 1).

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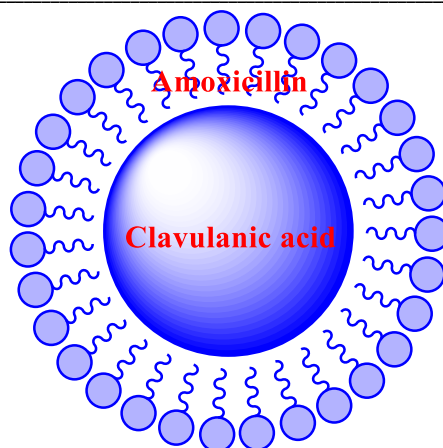


Fig 1: Design of dual drug loaded niosomes

## Materials and Methods

### Preparation of niosomes

Ether injection method: A mixture of Span 60 and cholesterol in different ratio was added to a beaker containing 10 ml diethyl ether organic solvent. To this mixture, 250 mg of amoxycillin was added portion wise with continuous stirring and maintaining a temperature of 30 °C. This heated solution was finally filled in a 20 mL syringe. A solution 125 mg clavulanic acid dissolved in 10 mL phosphate-buffered saline was prepared separately. The contents of the syringe were injected drop wise into a mixture with continuous stirring [8].

### Determination of niosome size

Niosomes, similar to liposomes, assume spherical shape and so their diameter can be determined using light microscopy, photon correlation microscopy and freeze fracture electron microscopy. Freeze thawing (keeping vesicles suspension at – 20°C for 24 hrs and then heating to ambient temperature) of Niosomes increases the vesicle diameter, which might be attributed to fusion of vesicles during the cycle.

### Morphology analysis of niosomes

Morphology analysis of niosomes was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM (Japan). A drop of the niosomal formulation was placed on a carbon coated copper grid, and the sample excess was removed with filter paper. Then a drop of 2% (w/v) PTA (phosphotungstic acid solution) was applied to the carbon grid and left to stand for 2 min. Once the excess staining agent was removed with filter paper, the sample was air-dried and the thin film of stained niosomes was observed with the transmission electron microscope.

### Drug entrapment efficiency of niosomes

After preparing niosomal dispersion, untrapped drug is separated by dialysis, centrifugation, or gel filtration and the drug remained entrapped in niosomes is determined by complete vesicle disruption using 50% n-propanol or 0.1% Triton X-100 and analysing the resultant solution by appropriate assay method for the drug. Where,

Entrapment efficiency (EF) = (Amount entrapped / total amount) x 100

### In-vitro release

A method of in-vitro release rate study includes the use of dialysis tubing. A dialysis sac is washed and soaked in distilled water. The vesicle suspension is pipetted into a bag made up of the tubing and sealed. The bag containing the vesicles is placed in 200 ml of buffer solution in a 250 ml beaker with constant shaking at 25°C or 37°C. At various time intervals, the buffer is analyzed for the drug content by an appropriate assay method.

### Antibacterial Assay

For the *in vitro* antibacterial assay of formulations were studied on bacteria *S. aureus*. The cultures were grown on nutrient agar at 37 °C for 18 h and the colonies were suspended in saline (0.85% NaCl) and its turbidity was adjusted to 0.5 Mac Farland standards (108 CFU/mL). This saline culture preparation was used to inoculate the plates. In the agar disc diffusion method the standard drugs and formulations were introduced into a disc 0.5 mm (hi-media) and then allowed to dry. Thus the disc was completely saturated with the test compound at concentration of 10 mg/mL. Then these discs were placed directly on the surface of Muller Hinton agar plates, swabbed with the test organism and the plates were incubated at 37 °C for 24 h [9].

## Results and Discussion

### Preparation of niosomes

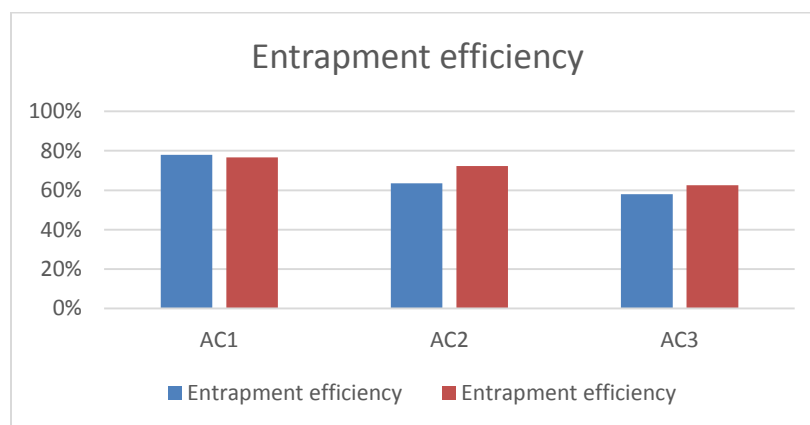
Niosomal formulations of amoxycillin- clavulanic acid were prepared with Span 60 as surfactants (Table 1).

Span 60 yields very stable niosomes with a narrow particle size distribution but they exhibit rather low entrapment efficiency. By adding cholesterol to

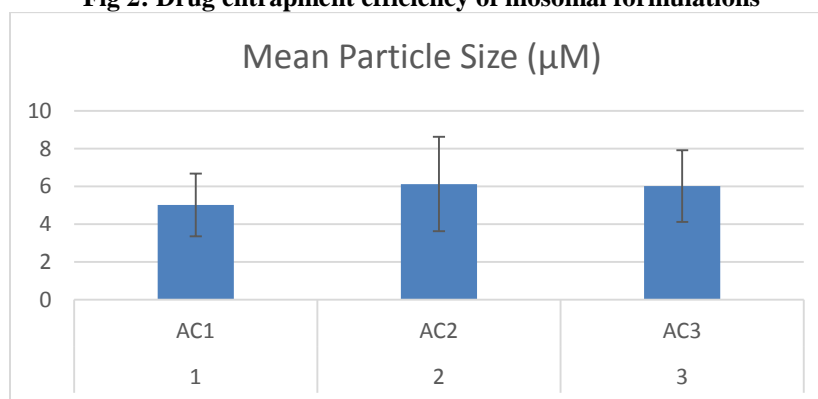
surfactant in a 1:1 weight ratio both stability and entrapment efficiency increased. In addition, surface of Span 60 niosomes is flexible due to their head group structure. The AC1niosomes have greater entrapment efficiency and least mean particle size (Fig. 2 and 3). The efficiency increases when low mechanical agitation speeds are used in the first stage of the preparation Protocol.

**Table 1: Formulation of Niosomes**

S. No.	Formulation Code	Span 60	Cholesterol	Solvent	Drugs
1.	AC1	50 mg	50 mg	10 mL DEA	250 mg amoxycillin + 125 mg clavulanic acid
2	AC2	50 mg	100 mg	10 mL DEA	250 mg amoxycillin + 125 mg clavulanic acid
3	AC3	100 mg	50 mg	10 mL DEA	250 mg amoxycillin + 125 mg clavulanic acid



**Fig 2: Drug entrapment efficiency of niosomal formulations**

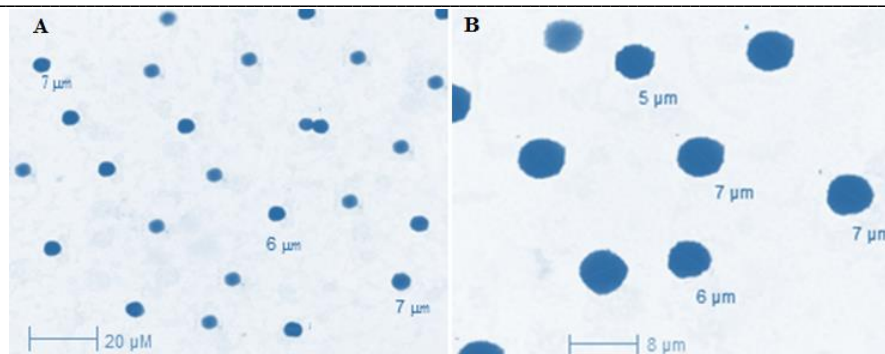


**Fig 3: Mean particle size of niosomal formulations**

### Transmission Electron Microscopy (TEM)

Fig 4 shows the transmission electron microphotographs of formula F8, as a representative sample, that was composed of Span 60 with cholesterol in equimolar ratio. TEM employed here to picture

directly the shape and size of TMC loaded niosomes. As occur in the images the niosomes appears as numerous scattered spherical dark stained micron vesicles with well-identified outline and core.



**Fig 4: Representative TEM of AC1 niosome (A) (scale bar = 20  $\mu$ m); (B) (scale bar = 8  $\mu$ m)**

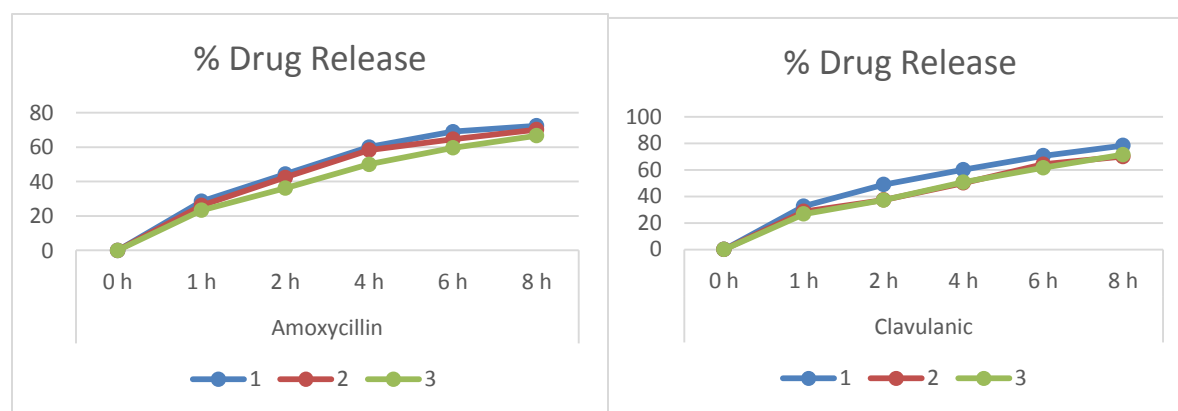
#### ***In-vitro* release study**

Release data expressed as percent drug released over 8 hours determined for three formulations are shown in Fig. 5. The release profile of the free untrapped amoxicillin and pot clavulanate showed that 75% of the drug was released within one hour. In comparison, the release profile of amoxicillin from the niosomal formulations showed that 72.4% of the drug was released in 8 hours, reflecting the sustained release of

amoxicillin from the niosomal formulations. Similarly, the release profile of pot. clavulanate from the niosomal formulations showed that 78.4% of the drug was released in 8 hours. The results of release profiles of the binary drugs indicate the effect on release of the changes in proportion of free to niosome-entrapped drug in the prepared formulations.

**Table 2: Release profiles of niosome formulations**

S.No.	Formulation	Amoxycillin(% Drug Release)						Clavulanic(% Drug Release)					
		0 h	1 h	2 h	4 h	6 h	8 h	0h	1 h	2 h	4 h	6 h	8 h
1	AC1	0	28.6	44.4	60.12	69.01	72.4	0	32.6	48.96	60.11	70.6	78.4
2	AC2	0	26.18	42.44	58.3	64.6	70.22	0	28.66	37.3	50.12	64.2	70.02
3	AC3	0	23.4	36.2	50.04	59.6	66.7	0	26.7	37.2	50.7	61.6	71.5



**Fig 5: % Drug released of drugs in niosome over 8 hours**

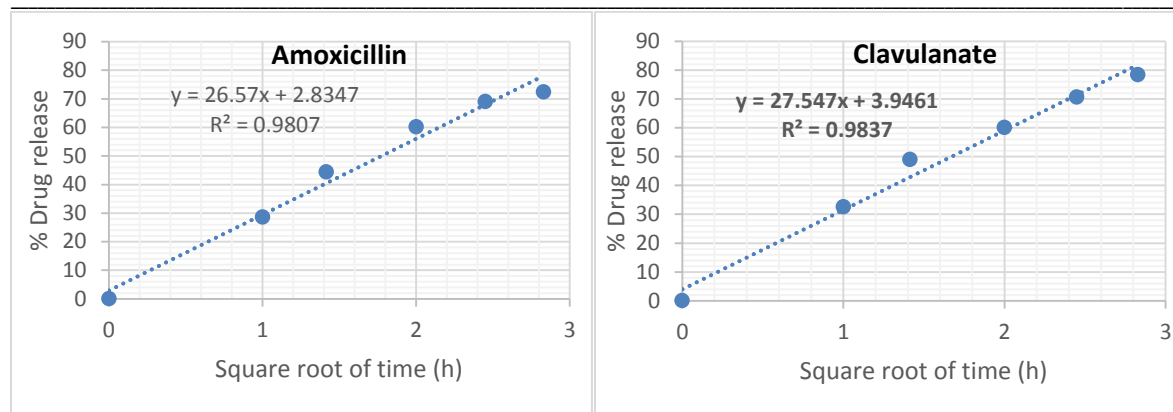


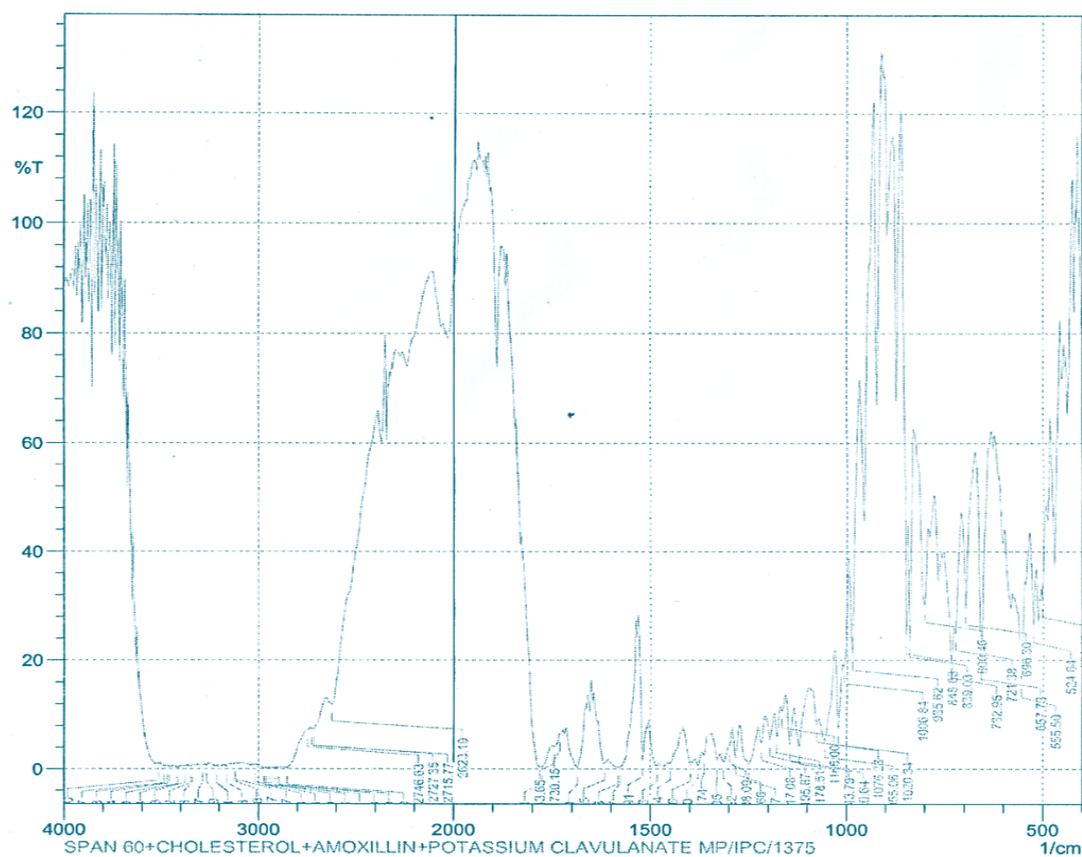
Fig 6: Best fitted Higuchi model of amoxicillin and clavulanic acid release in AC1

**Compatibility studies:** Excipients compatibility studies the results depicts there was no change in color, no lump formation occurred in any of the mixture at different temperature & humidity conditions, when observed on different days (7th, 15, 30th days) interval in comparison to initial observation on 0th day. This confirmed that both the drugs were compatible with each other as well as with excipients.  $R_f$  values obtained from TLC studies on (7th, 15, 30th days) were approximately similar to  $R_f$  values of pure drugs and niosomes obtained on 0th day, predicting the compatibility of both drugs with gel excipients.

**FT IR:** Drug excipients interaction was also checked out by comparing the FTIR spectra of pure drug amoxycillin trihydrate, clavulanate potassium and FTIR spectra of the physical mixture of drugs with excipients; span 60 and cholesterol. The FTIR spectra in this region of amoxicillin-clavulanic acid binary system, of molar ratio 1:1, are shown in Fig. Frequencies assigned to aromatic C=C, to  $\nu$ NH, to amidic  $\nu$ C=O, to  $\beta$ -lactamic C=O ( $\nu$ C=O), and to carboxylate ( $\nu$ sCOO<sup>-</sup>) ( $\nu$ asCOO<sup>-</sup>), have all been identified in free amoxicillin-clavulanic acid and are reported in Table. IR spectra indicate no significant difference in characteristic peak at wave numbers of the drug in presence of the excipient. Thus, IR spectra indicated no drug-excipient interaction.

Table 3: Assignment of relevant IR absorption bands of amoxicillin-clavulanic acid and niosome formulation

Assignment	Amoxycillin( $\text{cm}^{-1}$ )	Pot. clavulanate( $\text{cm}^{-1}$ )	Formulation AC1 ( $\text{cm}^{-1}$ )
$\nu_{\text{C=O}}$ ( $\beta$ -lactamic)	1774	1751	1774, 1743
$\nu_{\text{C=O}}$ (amide)	1685	-	1685
C=C (Aromatic)	1585	-	1581
$\nu(\text{COO}^-)$	1577	1519	1577, 1519
$\nu_{\text{NH}}$ ( $\beta$ -lactamic)	1519	-	1519



**Fig 7: IR spectrum of formulation AC1**

**HPLC:** The evaluation of the stability and compatibility of amoxicillin and pot. clavulanate binary mixtures with the span 60 and cholesterol after one month at 40 °C and 75% RH was conducted using the HPLC-UV method described in the relevant USP monograph. The response was linear within the range studied ( $R^2 = 0.9882$ ). The HPLC-UV method for quantitation of amoxicillin and pot. clavulanate can

detect thermal degradation and chemical interactions with excipients, which decrease the concentration of drugs. The chromatograms of binary mixtures are shown in Figure. No alteration in peak area was detected, indicating that these excipients have sufficient compatibility for use in some formulation of amoxicillin and pot. clavulanate.

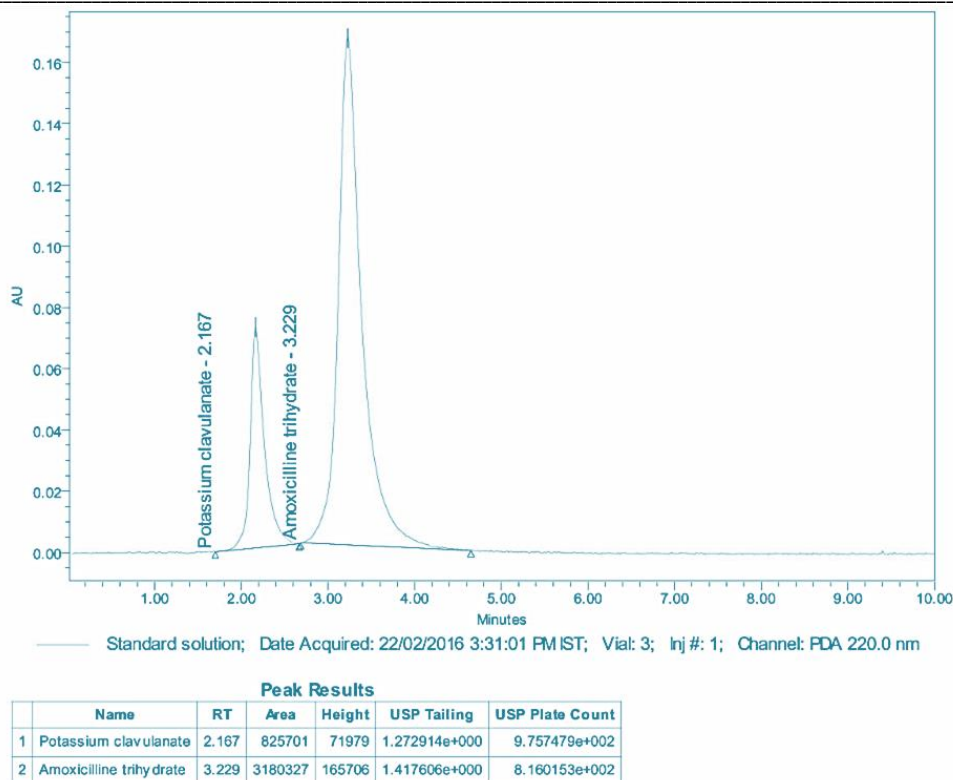


Fig 8: HPLC chromatogram of standard of amoxycillin and pot. clavulanate binary mixtures

**Antibacterial assay:** The antibacterial effects of the formulations AC1, AC2 and AC3 were studied against the *S. aureus* and compared with the standard drug augmentin at the same concentration level. The results clearly showed that the formulation AC1 inhibited *S.*

*aureus* 22.00 mm, whereas AC2 and AC3 inhibited 10.00 mm and 11.00 mm, respectively (Fig. 9). The standard drug augmentin inhibited 18.00 mm. These results indicate the promising effects of formulation AC1 against the bacteria *S. aureus*.

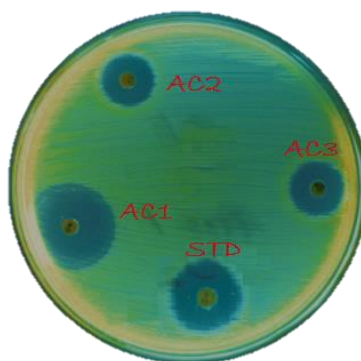


Fig 9: Zone of inhibition of niosomal formulations

## Conclusion

Niosomes prepared from Span 60-cholesterol were able to encapsulate amoxycillin and clavulanic acid. Average size, stability and RSV entrapment efficiency of niosomes are linked not only to their composition

but also to the protocol employed in preparing the niosomes. Optimized concentration of span 60 and cholesterol was found to be 1:1. In the *in-vitro* study, niosomes formulation of AC1 showed high percentage of drug release, 72.4 to 78.4% for about 8 hrs. This indicated that this batch of niosomes formulation

exhibit sustained drug release pattern as the niosomes act as reservoir system for continuous delivery of drug. Niosomes formed which were observed under transmission electron microphotographs were mostly spherical and in medium to slightly large size. In stability studies, the optimized formulation, AC1 Stability started to deteriorate from 2<sup>nd</sup> week where the niosomes vesicles are seen in non-spherical shape. On 21<sup>st</sup> and 28<sup>th</sup> day the niosomes formulation were examined and seen in non-spherical shape and are clumped for both storage condition. This is mainly due to disruption or aggregation of vesicles since it is exposed to chemical degradation like hydrolysis and oxidation. As for the drug release, niosomes formulation stored in room temperature and refrigerated condition showed 88.29% which mainly due to membrane-stabilizing effect of cholesterol. Thus, from the prepared niosomes formulation, it can be concluded that the vesicular system was more stable at 2°C-8°C. The released data of optimized niosomes formulation AC1 of amoxicillin were analysed mathematically according to zero order, first order, and Higuchi equations. As for the Higuchi's model ( $r^2=0.980$ ), First order ( $r^2=0.938$ ), and zero order ( $r^2=0.833$ ) regression co-efficient obtained. The amoxicillin drug release from niosomes does not obey first order kinetics, which means that the release of amoxicillin from the niosomes vesicle is independent to concentration gradient. Similarly, for clavulanic acid regression co-efficient obtained as Higuchi's model ( $r^2=0.983$ ), First order ( $r^2=0.962$ ), and zero order ( $r^2=0.835$ ). Best fitted Higuchi's model indicates that the drug release by diffusion.

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**Conflict of Interest:** None

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