IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
May 2020 Vol.:18, Issue:2

Effect of Azithromycin Concentration in O/W Macroemulsion on the Antibacterial Activity against Pathogenic Microorganisms

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Submission: 23 April 2020
Accepted: 30 April 2020
Published: 30 May 2020

Keywords: Azithromycin, macroemulsion, antibacterial activity, pathogenic microorganisms

ABSTRACT

The objective of the present study was to determine the effect of azithromycin concentrations in the macroemulsion on the antibacterial activity against pathogenic microorganisms. The emulsion was prepared with the help of mechanical stirrer by varying their concentration. The prepared emulsion was checked for compatible studies with the help of FT-IR. Then the emulsion was checked by centrifugation, and other characterizations like pH, viscosity, drug entrapment efficiency, particle size analysis, zeta potentials were also determined. The effect of azithromycin in the emulsion formulation was determined by time-release kinetics studies against some gram-positive and gram-negative microorganisms. FT-IR results showed there was no interaction between the drug and the polymers used in the formulations. The centrifugation results showed that no phase separation was seen when the preparation was centrifuged at 10,000 rpm at room temperature for 30 min. The pH of the preparations shows that the formulations are slightly basic. The viscosity for both the formulation was determined as 1.5 cps. The drug entrapment efficiency for F-1 formulation was found to be 85.0267±1.3606% and F-2 formulation was 88.1667±0.9052%. The particle size of the formulation was measured as 180.534 µm. The zeta potential of the formulation was measured as +6.72±0.36 for F-1 formulation and +7.31±0.21 for F-2 formulation. The azithromycin in emulsion formulation was effective against some gram-positive organisms like *Haemophilus influenza* (NCTC 8468) and *Staphylococcus aureus* (NCTC 6571) when compared to gram-negative organisms like *E. coli* (NCTC 10538) and *P. aeruginosa* (NCTC 10662). This study concluded that the increases in exposure time inhibit the bacterial growth, and the drug partitioning from oil droplet to the water phase of the emulsion determines/controls the bacterial inhibition capability of the formulation.

INTRODUCTION

An emulsion is a heterogeneous formulation consist of two immiscible liquids (by convention described as oil and water), one of which is dispersed as fine droplets equally throughout the other. Emulsions are thermodynamically unbalanced and revert to separate oil and water phases by combination or coalescence of droplets unless kinetically stabilized by a third constituent, the emulsifying agent. The phase present as tiny droplets is called the disperse, dispersed or internal phase and the supporting liquid is known as the continuous or external phase.[1] Azithromycin is semi-synthetic derivatives of erythromycin.[2] Azithromycin is an azalide antibiotic, a sub-class of the macrolides.[3] Azithromycin varies chemically from erythromycin in that a methyl replacement nitrogen atom is included in the lactone ring.[2,3] Azalides means new macrolides with the like spectrum of activity as erythromycin, but more resistant to acid hydrolysis, have been developed. They are most likely improved absorption and provide improved tissue levels. As of match up to erythromycin, it penetrates the tissue better and is concentrated in polymorphonuclear leucocytes.[4] Macrolides antibiotics is so-named for the reason that they have a macrocyclic lactone-ring being connected with amino sugars through glycosidic bonds.[5] The macrolides are broad-spectrum antibiotics to which resistance develops quickly. They hold back protein synthesis by binding to the ribosome and are bacteriostatic at usual doses but bactericidal in high doses.[6] The mechanism of action is the macrolides inhibit bacterial protein synthesis by an effect of translocation. Their action may be bactericidal or bacteriostatic, the outcome depending on the concentration and the type of microorganism. The drugs are bound to the 50S subunit of the bacterial ribosome.[7] Azithromycin has an action against gram-positive organisms also offers improved gram-negative coverage over erythromycin and clarithromycin. It also showed good action against *H. influenza*.[8] Azithromycin has been most effective against isolates of the following microorganisms, *Staphylococcus aureus*, *Streptococcus agalactiae*. *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Neisseria gonorrhoeae*, *Chlamydia pneumonia*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, *Helicobacter pylori*, *Salmonella typhi*, and *Mycobacterium avium intracellulare*.[9] The present work aimed to formulate emulsion by varying the concentration of azithromycin on the antibacterial activity against pathogenic microorganisms.

MATERIALS AND METHODS

Materials

Azithromycin was obtained as a gift sample from Micro Labs laboratory, Bangalore. Castor oil (Dabur ERAND) was purchased from the local Market. Poloxamer was obtained as a gift sample from Lupin bioresearch center, Pune. *Pseudomonas aeruginosa*, *Haemophilus influenza*, *Staphylococcus aureus* and *Escherichia coli* were purchased from the Institute of Microbial Technology, Chandigarh. Tryptic Soy agar was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India. All other chemicals used were of analytical grades and utilized as received.

Methods

Preparation of macroemulsion

Azithromycin loaded microemulsion was prepared by slight modification according to the method described by Tamilvanan and Kaur.[10] Accurately 5 ml of castor oil was taken in a beaker and heated up to 70ºC. Azithromycin at two different concentrations (500 mg and 1000 mg) was added in the oil phase while maintaining the same temperature condition. 500 mg of poloxamer was dissolved in 50 ml of double-distilled water taken in another beaker and this aqueous phase was also heated up to 70°C. At this temperature condition of 70ºC, both oil and water phases were mixed initially for a few minutes using a magnetic stirrer to make a coarse emulsion, and then the emulsification was continued further for over 10 to 15 min. at a stirring speed of 800 rpm using an electric stirrer. The macroemulsion thus formed was cooled immediately in an ice bath. The formulation was shown in Table 1.

Table No. 1: Preparation of o/w type emulsion

Ingredients	Formulations		
	F-1	F-2	Without drug
Azithromycin	500 mg	1000 mg	-
Oil Phase			
Castor oil	5 ml	5 ml	5 ml
Aqueous phase			
Poloxamer-407	500 mg	500 mg	500 mg
Distilled water	50 ml	50 ml	50 ml

Characterization of the prepared emulsions formulations

Centrifugation

To check out the stability of the prepared formulations, the emulsions were centrifuged (Remi cooling centrifuge, Remi Electrotecnik Lmt., Vasai) at 10,000 rpm for 30 min.

pH

Both formulated macroemulsion pH was recorded using a pH meter under the identical storage temperatures[10] (pH meter L 1120, Elico Limited, Hyderabad).

Viscosity determination

The viscosity of the formulated macroemulsion was determined with the help of an Ubbelohde capillary viscometer[10] (Schott, Hofheim, Germany).

Particle Size Analysis

The mean droplet diameter was determined to utilize a Malvern Mastersizer 2000 Ver. 5.61 (Malvern Instrument Ltd, Malvern, UK) at 25°C. A laser beam of the He-Ne light source at 633-nm wavelength was used. The sensitivity range was 0.02–2000 μm. About 100–200 μl of the emulsion was mixed with 150 ml of dispersing water (Hydro S) before making the measurement.[10] Values reported were the mean droplet diameter of triplicate emulsion samples.

Zeta Potential

The zeta potential measurements were carried out using the Malvern Zetasizer 3000 (Malvern Instruments, Ltd, Malvern, UK). The emulsion formulations were diluted in double-distilled water and the measurements were carried out in 10 mM NaCl solution. Each sample was analyzed of three replicates.[10]

FT-IR studies

It was used to study the interactions between the drug and the polymer. The drug and polymer must be compatible with one another to produce a stable product. Drug and polymer interactions were studied by using FTIR (Shimadzu, Japan model – 8400S). IR spectral analysis of pure azithromycin, poloxamer, and emulsion formulations with drugs and without

drug was carried out. The peak and patterns produced by the pure drug were compared with a combination of pure drug and polymer.[11]

Drug Entrapment Efficiency

The process was carried out with a slight modification previously performed by Tamilvanan et.al.[10,12] The drug entrapment efficiency (EE) of the formulated emulsions was determined by the ultracentrifugation method. Centrifugation was performed with the help of the HITACHI ultracentrifugation apparatus, which was allowed to run at 50,000 rpm (~162,000×g) at 4°C for 2 h. After centrifugation, the bottom of the used polyallomer tubes was pricked with a syringe needle to collect the aqueous phase. The concentrations of azithromycin in both the aqueous layer and the whole macroemulsion were determined by Reverse Phase High-Performance Liquid Chromatographic technique (Jasco, Borwin Version No. 1.5, Japan). The mobile phase ratio (25:75 v/v) containing the mixture of acetonitrile: buffer (25 mM potassium dihydrogen phosphate with pH 4.0) with a flow rate of 1.0 mL/min. in Grace C_{18} (250 x 4.6) mm 5µ column with an injection volume of 20 µL and detected at 210 nm.

The EE was calculated according to the following equation:

$$EE\ (\%) = \frac{\{(C_{total} \times V_{total}) - C_{water} \times V_{water})\}}{C_{total} \times V_{total}} \times 100$$

Where C_{total} is the concentration of azithromycin in the whole emulsion, V_{total} is the volume of the emulsion prepared, C_{water} is the concentration of azithromycin in the water/aqueous phase, and V_{water} is the volume of water phase collected after centrifugation.

Killing Kinetics

The killing kinetics study was done with slight modifications previously done by D'Arienzo et.al.[13] The 1% overnight microorganism's culture was added to the undiluted, 10 dilutions, and 100 dilutions of F-1 and F-2 formulations. Also, the inoculated emulsion was withdrawn at 0, 1, 5, 10, 15, 30, and 60 min. The samples were spread onto to tryptic soy agar plate. Then the plates were incubated at 37°C for 24 h to observe the number of surviving colony-forming units (CFU). 1 ml of pure 1% overnight microorganism's cultures (*E.coli*,

staphylococcus aureus, pseudomonas aeruginosa, H.influesza) was taken in a sterile test tube (separately). 9 ml of sterile distilled water was added to each test tube. Different concentration of emulsion that is undiluted, 10 dilutions and 100 dilutions of F-1 and F-2 formulations were added to the culture broth. Then, the test tubes were incubated at different time intervals at 0 min to 60 min. Immediately after the addition of macroemulsion to the bacterial culture at 0 min 0.2 ml of sample was withdrawn and it was spread on to the tryptic soy agar plate and the plate was incubated at 37°C for 24 h. Similarly at 1, 5, 10, 15, 30, and 60 min. 0.2 ml of sample was withdrawn and spread on to the tryptic soy agar plate and the plate was incubated at 37°C for 24 h. After 24 h the microorganisms were counted to observe the number of surviving CFU. The same procedure was performed for 10 dilutions, 100 dilutions for both formulations (F-1 and F-2). For W formulation (formulation without drug) only the undiluted formulation was performed.

RESULTS AND DISCUSSION

Manufacturing of emulsion

The formation of emulsion involves both traditional and modern size reduction techniques. Constant stirring of oil and water phases in hot conditions using a magnetic stirrer is the traditional size reduction technique however using the magnetic stirring alone cannot produce the emulsion with desired particle sizes but it will produce an only coarse emulsion with dispersed particles having medium sizes. Both oil and water phases were heated and mixed using a mechanical stirrer to form an emulsion. The emulsion formation was visualized the formation of a milky white color.

Centrifugation

The examination of the physical stability of the macroemulsion was performed by centrifugation at 10,000 rpm at room temperature for 30 min. No phase separation was seen, indicating that the prepared macroemulsion is stable.

pH and Viscosity

Developed macroemulsion formulation showed the pH values of 7.184±0.305 for F-1 formulation, 7.198±0.383 for F-2 formulation, and 6.946±0.321 for formulation prepared without the drug. There is no difference between the two formulated macroemulsion. The

viscosity of both the formulation shows 1.5 cps, close to the viscosity of water or other normal physiological fluids. This pH and viscosity value shows the prepared macroemulsion formulation is suitable for topical application onto the skin surface.

Particle size analysis

The mean droplet diameter size of the F-1 formulation prepared with 500 mg of azithromycin was measured as 180.534 µm in size. Macroemulsion prepared with 1000 mg and without azithromycin droplet diameter size was not measured. The droplet size distribution curve as shown in Fig.1.

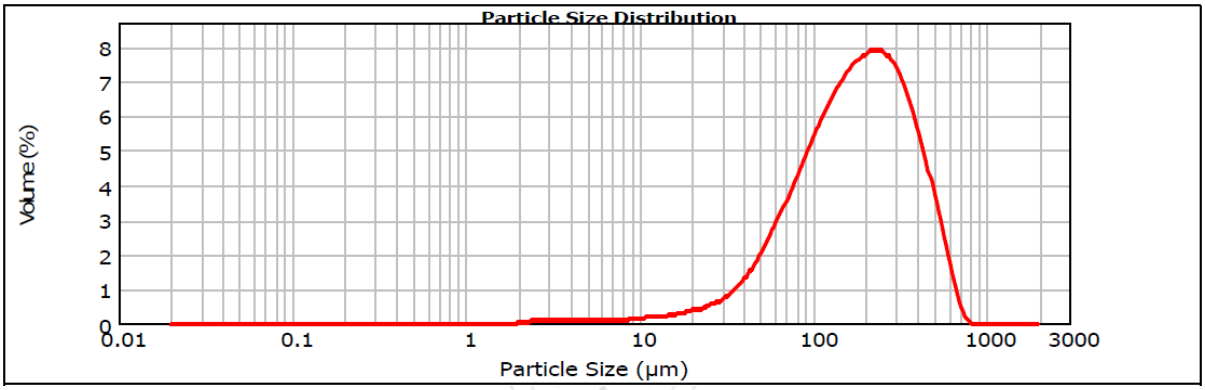

Figure No. 1: Mean droplet size for F-1 formulation

Zeta potential

The zeta potential value was measured as +6.72±0.36 mV for F-1 formulation and +7.31 ±0.21 mV for F-2 formulation. Both the formulated emulsions have low zeta potentials, it may tend to coagulate or flocculate, possibly leading to poor physical stability.

FT-IR studies

The IR study was performed to determine drug and excipients compatible. The IR spectrum for pure Azithromycin, poloxamer, and the F-1 and F-2 formulations were performed. The IR spectrum shows that there is no interaction between the drug and the other excipients used in the formulations.

Drug Entrapment Efficiency

The linearity was found in the concentration range 50-500 µg/mL with correlation coefficients calculated as 0.9997. The retention time of drugs was found 6.2 mins and with a run time of 10 mins. For F-1 formulation the drug entrapment efficiency was determined as 85.0267±1.3606 % and for F-2 formulation it was 88.1667±0.9052 %. The drug entrapment efficiency was slightly more for the F-2 formulation; it may be due to the high drug concentration when compared to the F-1 formulation.

Killing Kinetics

Effect of initial drug amount, emulsion dilution and exposure time on microbial growth

Fig. 2 to 9 present the effect of the initial drug amount, emulsion dilution, and exposure time on the growth of different microorganisms. When the undiluted emulsion having 500 mg drug was exposed to *H. influenza*, the too numerable to (bacterial) count (TNTC) observed at 1 min post-exposure time was reduced after 5 min post-exposure time. A similar trend of TNTC at 1 min post-exposure time was noticed in all of the tested microorganisms for both F-1 and F-2 emulsions formulations even with and without dilutions. This indicates that the one minute short exposure time is not enough to limit/control the bacterial growth. This means the drug is not partitioned from the oil droplets of the emulsion to the water continuous medium for exerting the bacterial inhibition activity. At 5 min post-exposure time, there was a massive inhibition in bacterial growth except for some organisms which were tested with few diluted emulsion samples. For example, emulsion having 500 mg drug showed the TNTC trend for *S. aureus* and *E. coli* at undiluted, 10, and 100 dilutions, respectively. However, emulsion having 1000 mg drug exhibited the TNTC trend when *S. aureus* and *E. coli* were tested using 100 dilutions only. From 10 min post-exposure time onwards, there was no appearance of the TNTC trend and the number of bacterial counts were also reduced progressively at all of the tested microorganisms and the dilution levels, irrespective of the initial drug amounts in the emulsions. It was worthwhile to point out that at 30 min post-exposure time, the undiluted emulsion having 1000 mg drug displayed no bacterial counts for *H. influenza* and *S. aureus*. However, no such a (no bacterial count) trend was observed for emulsion having 500 mg drug. Interestingly, the growth of the two bacteria, *H. influenza*, and *S. aureus*, were found to be completely inhibited at 60 min post-exposure time when tested using undiluted and 10 dilution level emulsions having both 500 and 1000

mg drug. Azithromycin emulsion can kill different bacterial cultures, at different concentrations. Considerable, azithromycin was much effective against gram-positive organisms likely *H. influenza* and also for *S. aureus*. Because it's able to cleave the peptidoglycan layer of bacteria cell wall easily. At the same time, the severe emulsion is active against gram-negative organisms likely *E. coli* and *P. aeruginosa*. It shows that the emulsion is cleaving the lipopolysaccharide layer.

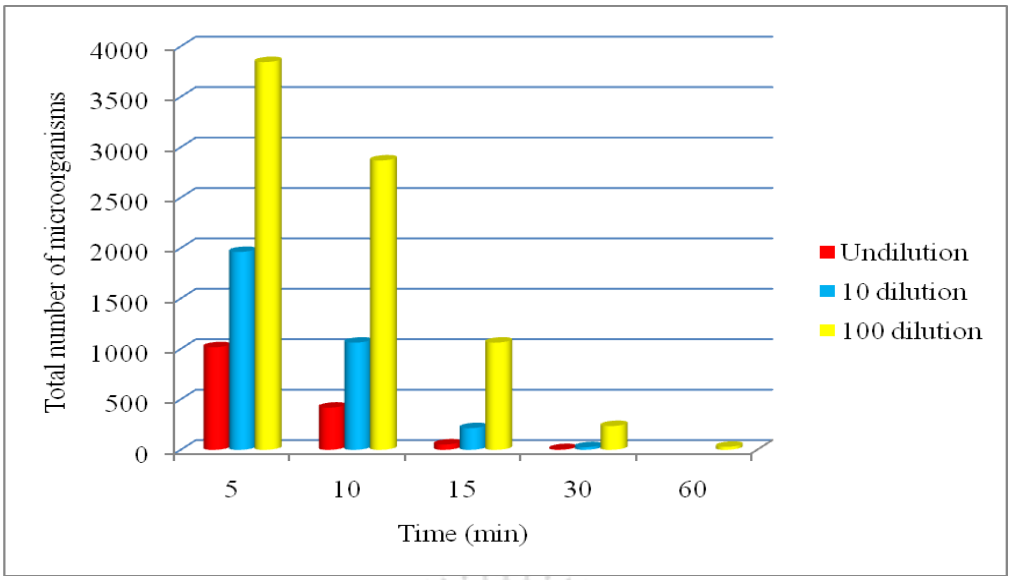

Figure No. 2: F-1 Formulation antibacterial activity against *Haemophilus influenza*

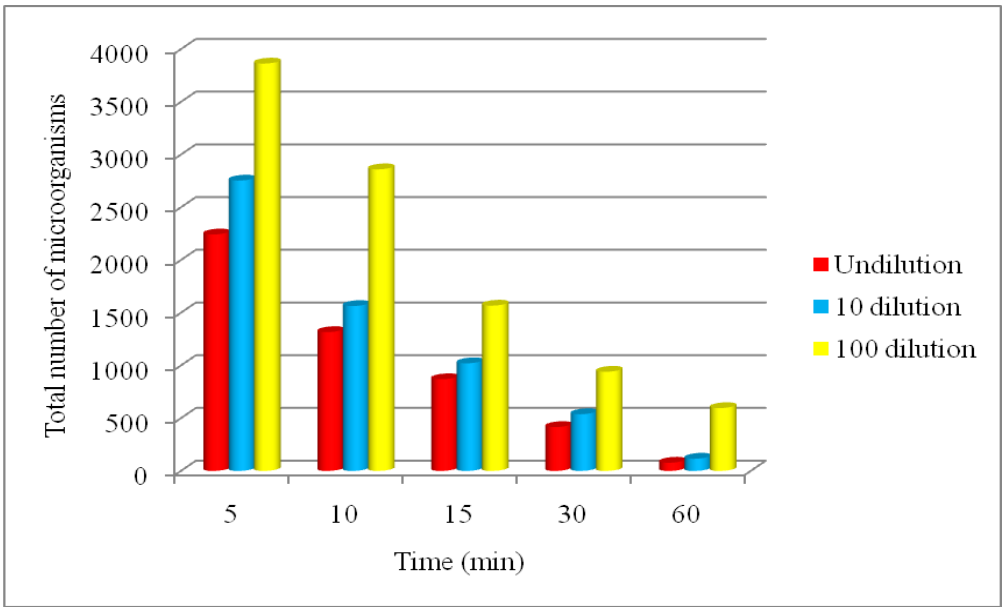

Figure No. 3: F-1 Formulation antibacterial activity against *Pseudomonas aeruginosa*

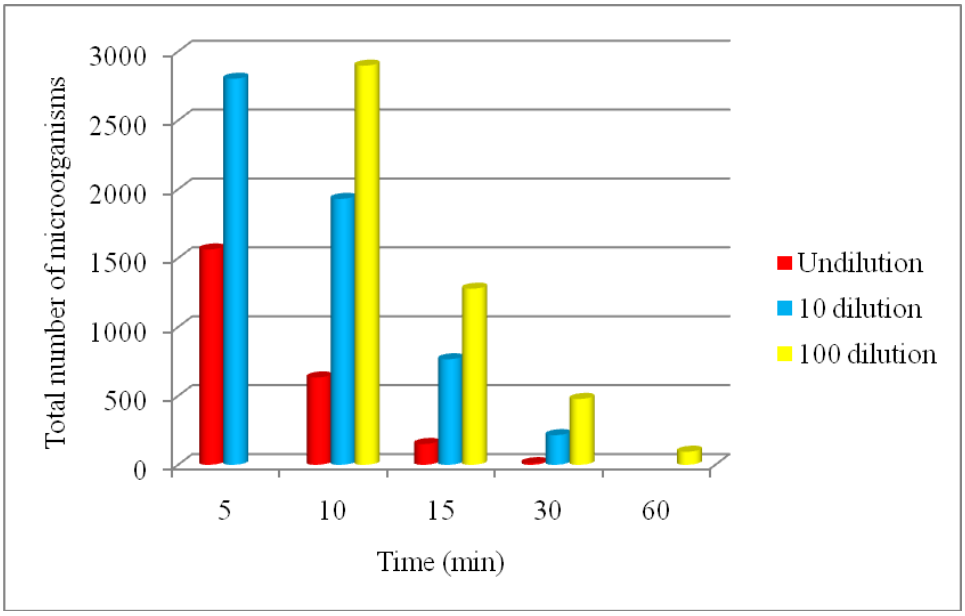

Figure No. 4: F-1 Formulation antibacterial activity against *Staphylococcus aureus*

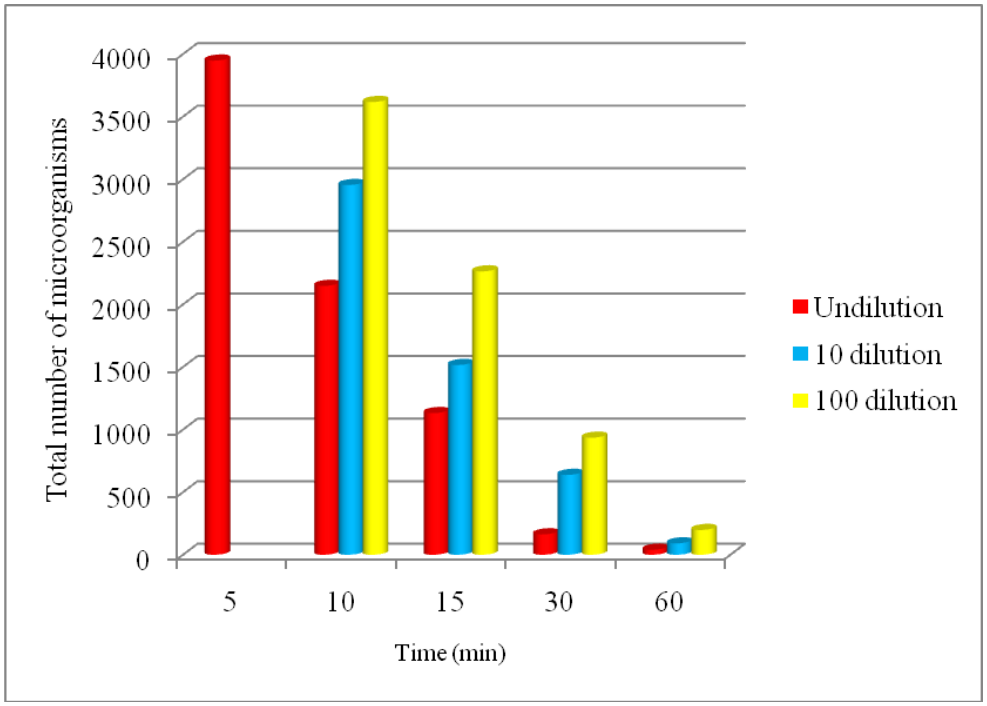

Figure No. 5: F-1 Formulation antibacterial activity against *Escherichia coli*

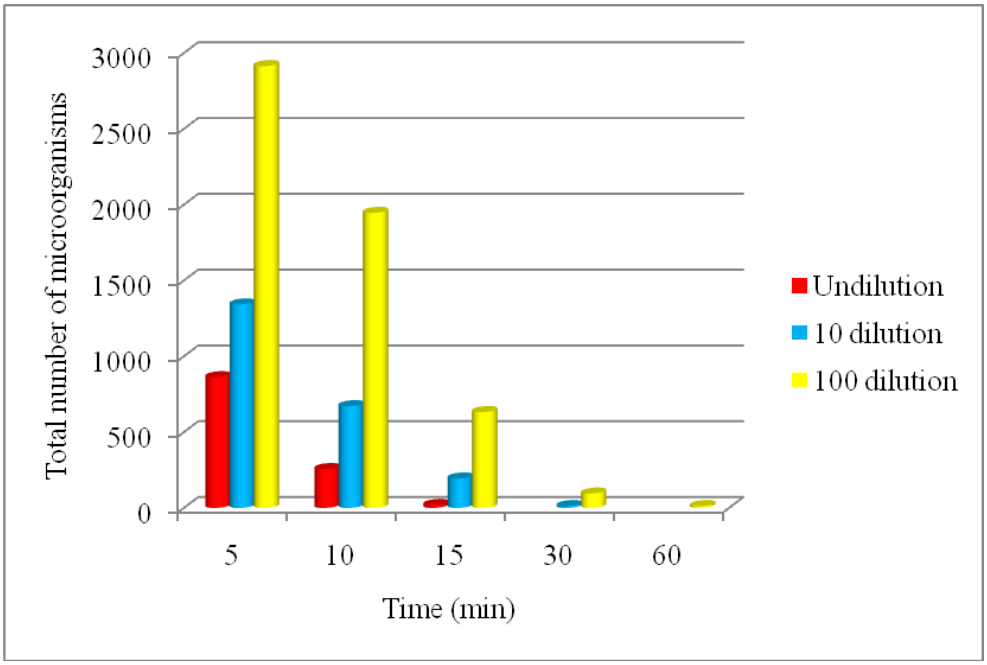

Figure No. 6: F-2 Formulation antibacterial activity against *Haemophilus influenza*

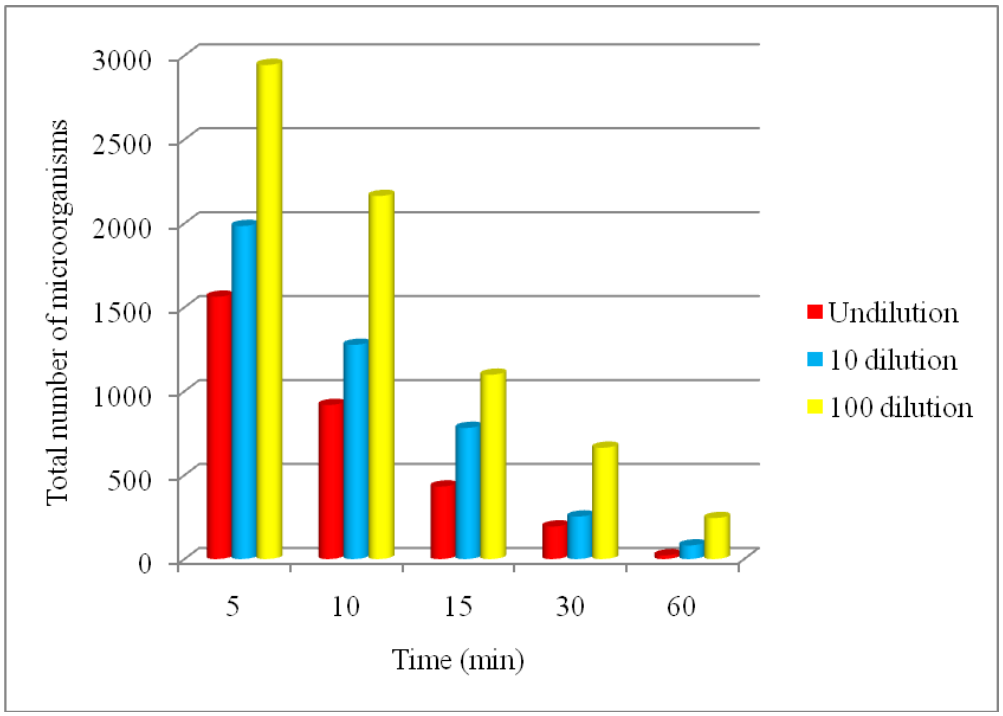

Figure No. 7: F-2 Formulation antibacterial activity against *Pseudomonas aeruginosa*

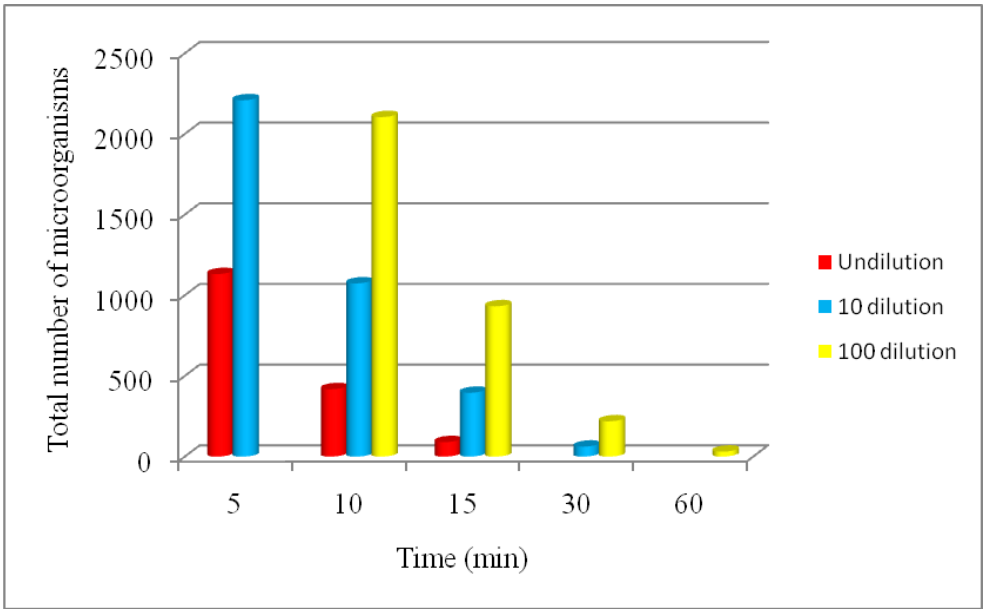

Figure No. 8: F-2 Formulation antibacterial activity against *Staphylococcus aureus*

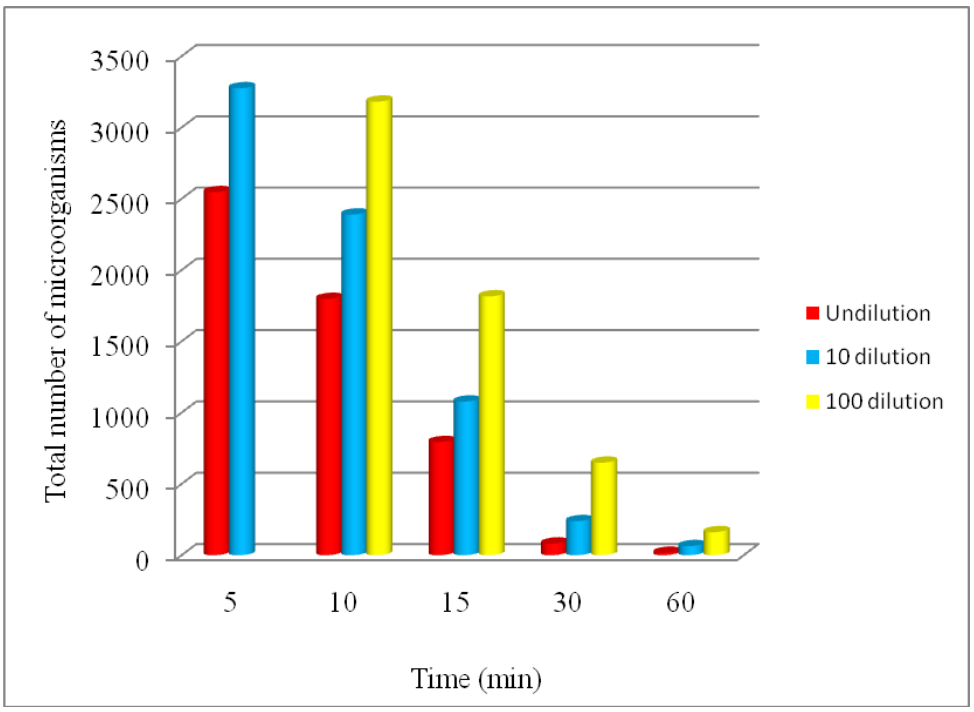

Figure No. 9: F-2 Formulation antibacterial activity against *Escherichia coli*

CONCLUSION

The conclusion from this study was that the increases in exposure time inhibit the bacterial growth, and the drug partitioning from oil droplet to the water phase of the emulsion determines/controls the bacterial inhibition capability of the formulation. The increase in

concentration also showed an increase in drug effects against the microorganisms. In the future, we are planned to perform in Azithromycin nanosized emulsion with the same formulation with some other microorganisms also. The macroemulsion of azithromycin could be used as an antibacterial agent in the medical field.

ACKNOWLEDGMENTS

The authors are grateful to Mr. S. Sriram Ashok, the correspondent of Sankaralingam Bhuvaneswari College of Pharmacy, Sivakasi for his support during the present research work.

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