

Research Article

In Vitro Anti-inflammatory and Antimicrobial Activities of Azithromycin After Loaded in Chitosan- and Tween 20-Based Oil-in-Water Macroemulsion for Acne Management

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Received 27 March 2015; accepted 19 August 2015; published online 28 August 2015

Abstract. The objectives of the current investigation are (1) to prepare and characterize (particle size, surface charge (potential zeta), surface morphology by transmission electron microscopy, drug content, and drug release) the azithromycin (AZM, 100 mg)-loaded oil-in-water (o/w) macroemulsion, (2) to assess the toxicity of macroemulsion with or without AZM using RBC lysis test in comparison with AZM in phosphate buffer solution of pH 7.4, (3) to compare the *in vitro* antimicrobial activity (in *Escherichia coli* using zone inhibition assay) of AZM-loaded macroemulsion with its aqueous solution, and (4) to assess the *in vitro* anti-inflammatory effect (using egg albumin denaturation bioassay) of the AZM-loaded macroemulsion in comparison with diclofenac sodium in phosphate buffer solution of pH 7.4. The AZM-loaded macroemulsion possessed the dispersed oil droplets with a mean diameter value of 52.40 ± 1.55 μm . A reversal in the zeta potential value from negative (-2.16 ± 0.75 mV) to positive ($+6.52 \pm 0.96$ mV) was noticed when AZM was added into the macroemulsion. At a 1:5 dilution ratio, 2.06 ± 0.03 mg of drug was released from macroemulsion followed by 1.01 ± 0.01 and 0.25 ± 0.08 mg, respectively, for 1:10 and 1:40 dilution ratios. Antimicrobial activity maintenance and significant reduction of RBC lysis property were noticed for AZM after loaded in the macroemulsion. However, an increment in the absorbance values for emulsion-treated samples in comparison to the control samples was noticed in the anti-inflammatory test. This speculates the potential of the AZM-loaded emulsion to manage inflammatory conditions produced at *Acne vulgaris*.

KEY WORDS: *Acne vulgaris*; inflammation; nano- vs. macroemulsion; protein denaturation bioassay; RBC breakdown.

INTRODUCTION

Acne vulgaris (simply acne) is a multifactorial disease that affects almost >80% of people and it comprises lesions of various skin morphologies, ranging from comedones, papules, and pustules to nodules and cysts (1). Its pathophysiology centers on the interplay of hyperkeratinization in pilosebaceous follicles, colonization with the anaerobic diphtheroid, notably *Propionibacterium acnes*, increased sebum production, and inflammation (2,3). Furthermore, current theories on the pathophysiology of acne hold that the inflammation of acne is due, in part, to an immune reaction to the bacterium or to extracellular products produced in response to the presence of the bacterium, rather than being due to presence of the bacterium itself (4). This indicates that treatments which are aimed solely at reduction in numbers of *P. acnes* organisms are generally not very effective in long-term management of acne. Hence, the acne armamentarium contains many different

therapeutic options, including topical benzoyl peroxide, topical and oral antibiotics, topical and oral retinoids, and hormonal agents (2,5). However, topical treatment with antibiotic alone (6) or in combination with other antiacne agents remains as the first-line therapy to manage mild and moderate acne (7). Furthermore, given the multifactorial pathogenesis of acne and the hurdles of adherence to treatment, it becomes always necessary to develop topically applied combination acne products blended with some innovative concept.

The use of oil-in-water (o/w) nanosized emulsions (with mean particle sizes ranging from 150 to 450 nm) having inherent antimicrobial and/ anti-inflammatory properties (8–12) to combat the antibiotic-resistant bacteria is a well-established strategy. Unlike that of antibiotics, the antimicrobial activity of nanosized emulsions is non-specific, thus allowing broad-spectrum activity while limiting the capacity for the generation of resistant bacterial strains (8). These features make nanosized emulsion a suitable candidate for wound treatment (10). In addition, incorporating the antibiotic molecule or antiacne agent into the emulsion having inherent antimicrobial activity should ideally produce a synergistic therapeutic efficacy for the efficient management of acne conditions. However, the current report explores the preparation of o/w macroemulsion (with mean particle sizes ranging from 1 to

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75 μm) using a polycationic polysaccharide biopolymer and non-ionic emulgator combination that possesses inherent anti-acne, antimicrobial, anti-inflammatory, immunological, and wound healing accelerant properties, and this macroemulsion is also incorporated with an antibiotic molecule. Azithromycin is selected as model antibiotic molecule while chitosan and Tween 20 are used as polycationic biopolymer and non-ionic emulgent molecules to make the o/w macroemulsion.

The objectives of the current investigation are, therefore, (1) to prepare and characterize (particle size, surface charge (potential zeta), surface morphology by transmission electron microscopy, drug content, and drug release) the azithromycin (AZM, 100 mg)-loaded o/w macroemulsion, (2) to assess the toxicity of macroemulsion with or without AZM using RBC lysis (hemolysis) test in comparison with AZM in phosphate buffer solution of pH 7.4, (3) to compare the *in vitro* antimicrobial activity (in *Escherichia coli* using zone inhibition assay) of AZM-loaded macroemulsion with its aqueous solution, and (4) to assess the *in vitro* anti-inflammatory effect (using egg albumin denaturation bioassay) of the AZM-loaded macroemulsion in comparison with diclofenac sodium in phosphate buffer solution of pH 7.4.

MATERIALS AND METHODS

Materials

Azithromycin dehydrate was a gift sample from Ranbaxy Laboratories, Gurgaon, India. Castor oil and coconut oil were purchased from S.K. Oil Industries, Jalgaon, Maharashtra, India. Chitosan (molecular weight, 22 kDa; deacetylation degree of ~81%) and Tween 20 were obtained from Sigma-Aldrich Chemicals Co., St. Louis, MO, USA. Potassium dihydrogen orthophosphate was purchased from CDH Pvt. Ltd. Laboratories, Mumbai, India. All other chemicals used were of analytical grade and used as received.

Methods

Formulation Development

AZM-loaded macroemulsion was prepared with a slight modification according to the method described elsewhere (13). In brief, oil phase containing castor and coconut oils (1:1) and drug (100 mg) were taken in a beaker and heated up to 70°C. Chitosan (50 mg) was dissolved in 0.05 M of acetic acid and heated up to 70°C. Water phase containing Tween 20 (1500 mg), glycerin (1.25 ml), and distilled water (to 50 ml) was taken in a separate beaker and heated up to 70°C. The oil phase was mixed initially with the chitosan solution and then the water phase was added and stirred well by means of a magnetic stirrer while further heated to a temperature of 85°C. At this temperature, the obtained crude emulsion was subjected to a droplet size reduction by means of ultrasonication (LOBA CHEMIE, Mumbai, India) for 5 min and rapidly cools to room temperature. The emulsion was packed in siliconized glass bottles. Three replicate emulsion samples were prepared freshly and were used for further analyses as described below. In addition, AZM solution (0.1% w/v) was prepared using a phosphate buffer saline

(PBS) solution of pH 7.4 and the AZM solution in PBS was the control.

Particle Size Analysis

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

Zeta Potential, pH, and Viscosity Measurements

The zeta potential measurements were carried out using the Malvern Zetasizer 3000 (Malvern Instruments, Ltd, Malvern, UK). The samples were diluted in double distilled water and the measurements were carried out in 10 mM NaCl solution. Each sample was analyzed twice, and each analysis consisting of three replicates. The pH was recorded at given time intervals using a pH meter under the identical storage temperatures (MP220 pH meter, Mettler Toledo, UK). An Ubbelohde capillary viscometer (Schott, Hofheim, Germany) was used to measure the viscosity of the emulsion samples.

Transmission Electron Microscopy

In order to evaluate the emulsification efficiency of the two different size reduction steps (initial mixing of oil and water phases by mechanical stirrer followed by high-speed mixing by an ultrasonicator) used during the preparation process, the freshly prepared AZM-loaded emulsion was studied using a transmission electron microscope (CM 12, Philips, Eindhoven, The Netherlands). A drop of diluted emulsion (1:5 dilution with double distilled water (DDW)) was placed on a carbon-coated copper grid and underwent a negative staining technique using 1% solution of phosphotungstic acid (PTA) sodium salt at pH 7.4. The excess emulsion was sipped by the filter paper and then air dried at room temperature before being observed under transmission electron microscopy (TEM).

Quantitative Drug Analysis

AZM content was analyzed using a HPLC system. In the present study, the already reported method (14) was fully validated and was adapted to meet the requirements for the AZM stability-indicating test. In brief, a reversed-phase 250 \times 4.6-mm LunaTM C₁₈ (5 μm) column furnished by PhenomenexTM (USA) and a 2 \times 8-mm precolumn of the same material were used. The mobile phase, at 1 ml/min flow rate, consisted of a mixture (15:47, v/v) of acetonitrile and potassium dihydrogen phosphate buffer (0.067 mol/l) at pH 4.0, which was adjusted with phosphoric acid. AZM was monitored with a UV-visible absorbance detector at 210 nm (Kontron HPLC system, Kontron Instruments, Zurich, Switzerland). Six standard solutions of drug in acetonitrile were first prepared and appropriately diluted with acetonitrile to final AZM concentrations ranging from 12.5 (minimum

detectable concentration) to 2000 µg/ml. The AZM-loaded emulsions were directly dissolved in acetonitrile to appropriate dilutions (1: 10–1: 100); AZM was monitored with a UV-visible absorbance detector at 210 nm; under these experimental conditions, the run time was 7.2 min. All the experiments were duplicated and the deviation ranged from 0 to 3%, indicating that the various experimental conditions were well controlled.

Entrapment Efficiency

The entrapment efficiency (EE) of the emulsions was determined by measuring the concentration of AZM in the aqueous layer obtained by ultracentrifugation (UC) (15). Centrifugation was carried out using a HITACHI ultracentrifugation apparatus, operated at 50,000 rpm (~162,000×g) at 4°C for 2 h. Polyallomer tubes were used and their bottoms were pricked with a syringe needle after centrifugation to collect the aqueous phase. Concentrations of AZM in both the aqueous layer and the whole emulsion were determined by HPLC. The EE was calculated according to the following equation (16):

$$EE(\%) = \frac{\{(C_{\text{total}} \times V_{\text{total}}) - (C_{\text{water}} \times V_{\text{water}})\}}{C_{\text{total}} \times V_{\text{total}}} \times 100 \quad (1)$$

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

Thermodegradation Experiment

About 500 µl of AZM-loaded emulsion (equivalent to 1000 µg of AZM) was taken in a vial, and 50 ml of pH 7.4

phosphate buffer was added into it. In another vial, 1 ml of AZM solution (1 mg/ml) in pH 7.4 phosphate buffer was taken and diluted with 50 ml buffer. These two vials were kept in (1) an autoclave at 121°C and 15 lb/in pressure for 15 min, (2) a hot air oven at 70°C for 30 min, and (3) a room temperature at 25°C for 30 min. The drug leakage (in percentage) from both emulsion and solution at these three different storage conditions were calculated according to the following formula.

$$\text{Drug leakage \%} = \frac{\text{Drug amount leached}}{\text{Initial drug amount added}} \times 100 \quad (2)$$

In Vitro Dissolution

A membrane free dissolution model as shown previously for o/w nanosized emulsion (17) was adopted in the current investigation in order to study the *in vitro* release of AZM from o/w macroemulsion. The *in vitro* release studies were performed in a water bath maintained at 37°C using 3×50-ml vials in which 8 ml of DDW was served as medium; 1600, 800, and 200 µl of emulsion (entrapping 3.2, 1.6, and 0.4 mg of drug) were added to the first, second, and third vials, respectively, to denote/represent the corresponding dilution ratios of 1:5, 1:10, and 1:40. Periodically, 500 µl of sample was withdrawn from each vials at a time interval of 1, 5, 10, 15, and 20 min with replenishment using 500 µl fresh DDW. The withdrawn samples were analyzed using the validated HPLC method to calculate the amount AZM released from macroemulsion on these three different dilution ratios. The actual amount of AZM released was calculated using the following formula:

$$\text{AZM amount released} = (\text{Drug amount present at each of the dilution ratio}) - (\text{Mean drug amount released at particular time point over 20 min}) \quad (3)$$

In Vitro Hemolysis Test

A normal healthy human volunteer was given a written consent to donate blood for this test. Freshly collected blood from the healthy human volunteer was mixed with an anticoagulant solution (74.88 mM sodium citrate v/v) and centrifuged at 650×g for 10 min. The supernatant was discarded and the erythrocytes were resuspended in PBS (5 mM phosphate, 150 mM NaCl, pH=7.4). Next, the erythrocytes were washed three times with an isotonic buffer (PBS), and the upper phase with a buffy coat containing precipitated debris and serum proteins was carefully removed at each wash step. After the last washing, the packed cells were suspended in a buffer to a hematocrit of 50%. All the erythrocyte suspensions used in the experiments were prepared daily. To determine the hemolytic effect, 100 µl of each emulsion containing 1000 µg of AZM was diluted with 10 ml of PBS, and 1 ml from this diluted emulsion (containing 100 µg of AZM) was added to 20 µl of erythrocyte suspension (50% hematocrit)

and adjusted to a 4-ml volume with PBS. The samples were stirred and incubated for 30 min at 37°C. Debris and intact erythrocytes were removed by centrifugation at 650×g for 10 min. The hemoglobin released into the supernatant was detected spectrophotometrically at 540 nm against a corresponding blank sample. The hemolytic effect, measured as the percentage of hemolysis (H), was determined on the basis of released hemoglobin, according to the following formula (18)

$$H(\%) = \frac{A_s - A_{c1}}{A_{c2} - A_{c1}} \times 100 \quad (4)$$

where A is the absorbance, s is for the sample, c_1 is for the mechanical hemolysis (erythrocytes in PBS), and c_2 is for 100% hemolysis (erythrocytes in double distilled water). Control emulsions were prepared under identical experimental conditions, but without AZM, and were tested for hemolysis.

In Vitro Anti-inflammatory Experiment

To study the effect of o/w macroemulsion on the anti-inflammatory activity of AZM, an *in vitro* anti-inflammatory activity as shown previously for plant extracts by Chandra *et al.* (19) was adopted with a slight modification in the current investigation. In brief, the AZM (100 mg)-loaded macroemulsion (50 ml) was prepared freshly and 1000–5000 μ l of emulsion (equivalent to 2000–10,000 μ g AZM) was mixed individually with 100 ml of pH 7.4 phosphate buffer. From these, 2000 μ l (equivalent to 40–200 μ g of AZM) was mixed individually with 200 μ g of egg albumin powder and 2800 μ l of pH 7.4 phosphate buffer. Hence, the final concentrations of AZM in each one of the reaction mixtures were ranged from 8 to 48 μ g/ml. The corresponding control solutions were also prepared using an emulsion without drug and pH 7.4 phosphate buffer. Similarly, reference standard solutions were made by dissolving 100 mg of diclofenac sodium in 50 ml of pH 7.4 phosphate buffer and all other steps were also followed to get the final concentration of diclofenac in each one of the reaction mixtures ranging from 8 to 48 μ g/ml. All the reaction mixtures were incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in an incubator (NSW Ltd, New Delhi, India) for 15 min followed by heating to 70°C for 5 min in a water bath. The absorbance for all the reaction mixtures was measured at 660 nm in a spectrophotometer (SHIMADZU UV-1800, Japan). The percentage inhibition of protein denaturation was calculated using the following formula:

$$\% \text{ inhibition} = 100 \left[\frac{V_t}{V_c} - 1 \right] \quad (5)$$

Where, V_t =absorbance of test sample and V_c =absorbance of control.

Fifty percent inhibition (IC_{50}) values were determined for the AZM-loaded emulsion and diclofenac-containing phosphate buffer solution. The drug concentration for 50% inhibition (IC_{50}) was determined by plotting percentage inhibition with respect to control against treatment concentration.

In Vitro Antimicrobial Activity

The gram-negative facultative anaerobic bacterial strain of *E. coli* (MTCC No. 3221) was obtained from the Institute of Microbial technology, Chandigarh, Punjab, India. The bacterial strain which was obtained in lyophilized forms was further activated according to their manufacturer's protocol. At first, their stock samples that contained 20% w/w glycerin were prepared from activated bacteria and kept at a freezer for further uses. The activated *E. coli* was cultured in MacConkey broth incubated for 24 h at 37°C in anaerobic conditions. The antibacterial activity of the negative and positive controls, AZM (250 μ g) solution, and macroemulsion with AZM (250 μ g) was evaluated using agar-well diffusion method according to the standards approved by the National Committee for Clinical Laboratory Standards (20). Bacterial inoculums treated with macroemulsion without AZM and treated with respective mediums only were applied as negative controls. Two stock solutions (AZM powder and AZM-loaded macroemulsion) were prepared in phosphate buffer solution of pH 7.4. After suitable dilution of these two stock solutions

with the same buffer solution of pH 7.4, 100 μ l from each solutions containing about or equivalent to 250 μ g of AZM was used as test samples.

In the agar-well diffusion method, firstly, an appropriate amount of prepared inoculum (1 ml of the cell suspension containing 10^6 to 10^7 CFU/ml, approximately) of each bacterium (*E. coli*) was transferred into the medium. Wells with 8-mm diameters were punched on the surface of agar media using a sterile cork borer. Aliquots of 100 μ l of each samples (negative and positive controls, AZM (250 μ g) solution, and macroemulsion with AZM (250 μ g)) were transferred into the wells. After incubation at 37°C for 30 min, the diameter (in millimeter) of growth inhibition zones around the wells were carefully measured using a caliper (Mitutoyo, Japan) with a precision of at least 0.1 mm.

Statistical Analysis

The mean and standard deviation of measurements were computed. All statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software, La Jolla, CA). Continuous variables were analyzed using an unpaired two-tailed Student's *t* test and/or one-way analysis of variance (ANOVA) followed by Tukey post-test comparisons. The Kruskal-Wallis test with the Dunn multiple comparison test was used to evaluate differences in medians for data with a non-parametric distribution. Difference was considered significant when $p < 0.05$ for all experiments with an exception of *in vitro* antimicrobial activity where the p values of < 0.01 were considered significant.

RESULTS**Screening of Polysorbate (Tween)**

Polysorbates are available in both liquid and solid forms depending on their grades that are being segregated based on the number of oxyethylene units attached (21). Whereas polysorbate grades 20–60, 80, and 120 are yellow oily liquid at 25°C , grades 61–65 look tan solid and grades 81–85 appear amber-colored liquid. Among the different liquid and solid grades of polysorbates tested, polysorbate 20 (Tween 20) in conjunction with chitosan allowed the formation of a mono- or multilayered emulsifier film around the dispersed oil droplets, and therefore, this combination was selected as emulsifying agent to design o/w macroemulsion. Moreover, preliminary screening experiments indicate that using below 1.5 g of polysorbate 20 and macroemulsion made without chitosan always showed a tendency to form coalescence of dispersed oil droplets and thus leads to the breakdown of o/w macroemulsion.

Physicochemical Characteristics of Macroemulsion

Table I shows the physicochemical characteristics (mean droplet diameter, zeta potential, and drug entrapment efficiency) of o/w macroemulsion with or without AZM. Although no measurement was made to determine the mean droplet diameter value of the macroemulsion prepared without AZM, the macroemulsion made with AZM exhibited the mean diameter value of 52.40 ± 1.55 μm with a polydispersity of 0.899 and presented a normal droplet size distribution curve

Table I. Physicochemical Characteristics of Azithromycin-Loaded Oil-in-Water Macroemulsion

Formulation	Mean droplet diameter $d_{(0.5)}$ ($\mu\text{m} \pm \text{SD}$, $n=3$)	Zeta potential ($\text{mV} \pm \text{SD}$, $n=3$)	Drug entrapment efficiency (%)
o/w macroemulsion without AZM	ND	-2.60 ± 0.75	NA
o/w macroemulsion with AZM	52.40 ± 1.55	$+6.52 \pm 0.96$	91.54 ± 5.03

ND not determined, NA not applicable, SD standard deviation, AZM azithromycin, o/w oil-in-water

(Fig. 1). In addition, TEM images (Fig. 2) illustrate that mixing of oil and water phases using the mechanical stirrer followed by the sonicator resulted in forming homogeneously dispersed macroemulsion. The data presented in Table I also shows that the zeta potential of o/w macroemulsion was changed depending on the presence and absence of AZM inside it. It should be noted that though polycationic polysaccharide biopolymer (chitosan) was included as one of the emulsifying agents to stabilize the o/w macroemulsion, the macroemulsion exhibited a negative zeta potential value (-2.16 ± 0.75 mV). On the other hand, upon addition of AZM into the emulsion, a reversal in the zeta potential value was noticed ($+6.52 \pm 0.96$ mV). The drug entrapment efficiency of AZM-loaded macroemulsion was found to be $91.54\% \pm 5.03\%$.

In Vitro Dissolution

Figure 3 depicts the actual amount of AZM released from macroemulsion in DDW over 20-min time as a function of increasing dilution ratio. At 1 min post-dissolution time period, the amount of drug released from 1:5 dilution ratio was found to be 1.92 ± 0.06 mg followed by 1.08 ± 0.06 and 0.39 ± 0.01 mg, respectively, when the dilution ratios were kept at 1:10 and 1:40. The Student's *t* test showed a statistical difference in the amount of AZM released observed for these three different dilution ratios studied. At 5, 10, 15, and 20 min post-dissolution time periods, a slight but steady decrease in the amount of drug released at all of the studied dilution ratios was noticed.

Thermodegradation Study

Figure 4 depicts the thermodegradation behavior of AZM-loaded macroemulsion in three different storage conditions. It should be added that keeping the 10-ml AZM

solution (2 mg/ml) in pH 7.4 phosphate buffer at all the three studied storage conditions led to the formation of drug precipitation at the bottom of the vials. In contrast, the macroemulsion allowed the AZM leakage from the inner oil phase or the oil/water interface of the emulsion that depended on the storage conditions used. The autoclave condition showed the drug leakage percentage value of 0.972 ± 0.02 followed by hot air oven with the drug leakage percentage value of 1.46 ± 0.72 , and only the drug leakage percentage value of 1.18 ± 0.54 was noticed for room temperature.

RBC Breakdown Study

At a similar concentration of 100 μg of AZM, the free drug induced $99.68\% \pm 0.05\%$ hemolysis while the blank o/w macroemulsion and AZM-loaded macroemulsion showed only the RBC destruction in the range of $58.35 \pm 2.72\%$ – $64.00 \pm 4.12\%$ (Fig. 5) following incubation for 30 min. According to Student's *t*-test, no significant difference (*p* value at 0.05) was noticed in the percentage hemolysis values shown by macroemulsion with or without AZM. However, there was a significant difference between the hemolysis percentage values obtained by free drug and macroemulsion with or without AZM.

In Vitro Antimicrobial Activity

Table II shows zone inhibition diameter values obtained for medium only-treated, AZM solution, AZM-loaded macroemulsion, and macroemulsion without AZM following the incubation time of 30 min with *E. coli*. The AZM solution showed a zone inhibition diameter value of 5 mm. However, the macroemulsion with AZM and macroemulsion without AZM exhibited the zone inhibition diameter values of 4 and 3.4 mm, respectively. The two negative controls tested produced only minimal or no zone inhibition diameter values.

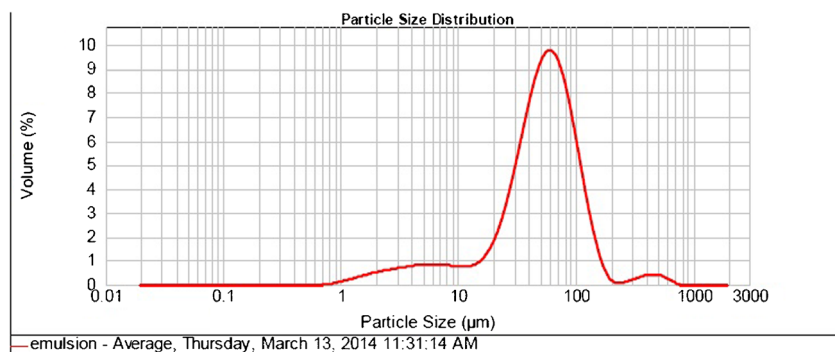


Fig. 1. Distribution of (dispersed oil) droplet size of azithromycin (AZM)-loaded oil-in-water macroemulsion analyzed utilizing a Malvern Mastersizer

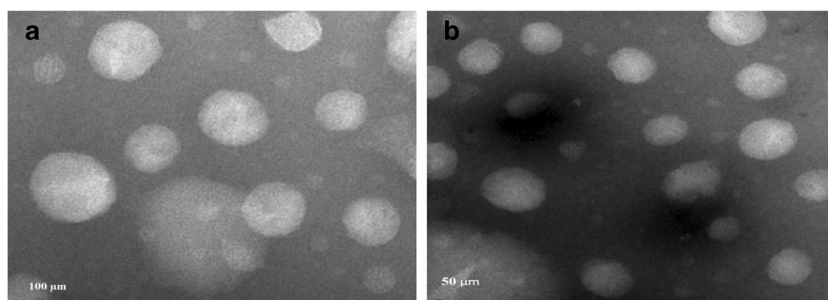


Fig. 2. Transmission electron microscopic images, following negative staining with sodium phosphotungstate, showing the population droplet size of azithromycin (AZM)-loaded emulsion immediately after mixing of oil and water phases with mild constant mechanical stirring (a) and after ultrasonication (b)

In Vitro Anti-inflammatory Studies

Figure 6 shows the percentage inhibition values for the protein (albumin) denaturation phenomenon obtained with AZM-loaded macroemulsion (test) and diclofenac sodium solution (reference). When looking at the bar graphs individually, it can be concluded that both the test and reference produced the percentage inhibition values for the protein denaturation phenomenon that was concentration-dependent (8, 16, 32, and 40 $\mu\text{g/ml}$). Both the test and reference exhibited the IC_{50} value at or around the same concentration level ($\sim 16 \mu\text{g/ml}$). From Fig. 6, it is evident that there was an occurrence of two different trends in the percentage inhibition values for the protein denaturation phenomenon, i.e., before IC_{50} and after IC_{50} . Before IC_{50} , the AZM-loaded macroemulsion possessed the percentage inhibition value at 8 $\mu\text{g/ml}$ concentration level ($44.96\% \pm 1.25\%$) for the protein denaturation that was higher than the percentage inhibition value at the same concentration level ($10.42\% \pm 2.37\%$) for the protein denaturation obtained with diclofenac sodium reference solution. But after IC_{50} , there was a change in the trend of percentage inhibition values for the protein denaturation obtained with test and reference. At 32 $\mu\text{g/ml}$, the percentage inhibition values for the protein denaturation phenomenon

observed with test and reference were $54.65\% \pm 3.15\%$ and $72.51\% \pm 1.08\%$, respectively. Similarly, $86.06\% \pm 0.96\%$ and $90.04\% \pm 2.95\%$ inhibition values for the protein denaturation were observed, respectively, with test and reference at 40 $\mu\text{g/ml}$.

DISCUSSION

The skin at times is afflicted with a variety of inflammatory and non-inflammatory disorders. One such disorder is acne, a common disease characterized by various types of lesions. The lesions associated with acne are usually categorized as either non-inflammatory or inflammatory. While the non-inflammatory lesions include comedones, the inflammatory lesion consists of pustule, papule, nodule, or cyst depending on the depth in the dermis and degree of inflammation.

Azithromycin (AZM), a broad-spectrum azalide antibiotic, has been administered successfully through oral, topical, and intravenous (iv) routes in order to treat gram-negative, gram-positive, and atypical infections of the ear, eye, skin, and upper respiratory tract (22). In addition, several scientific articles have published studies establishing the efficacy of azithromycin in treating inflammatory lesions (papules and pustules) of acne (23), and thus, the AZM is rated as a

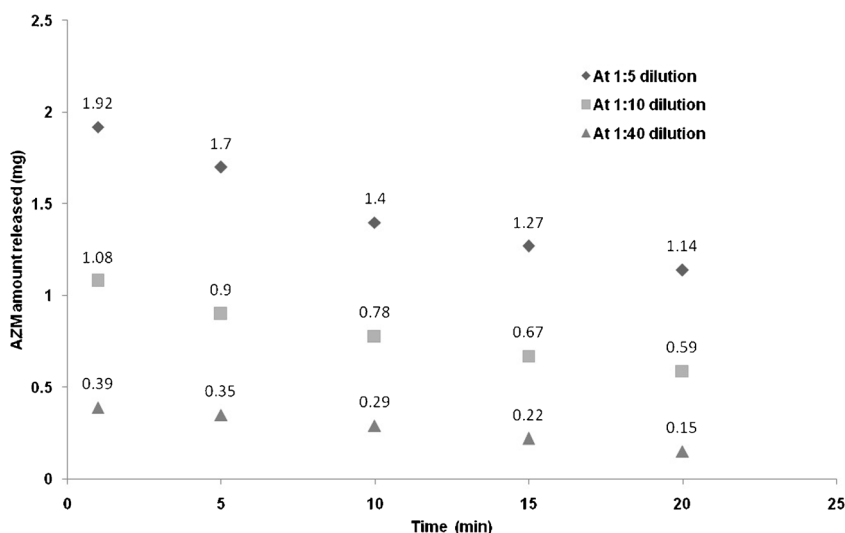


Fig. 3. Amount of AZM released from oil-in-water macroemulsion in double distilled water over 20-min dissolution time period as a function of increasing dilution ratio

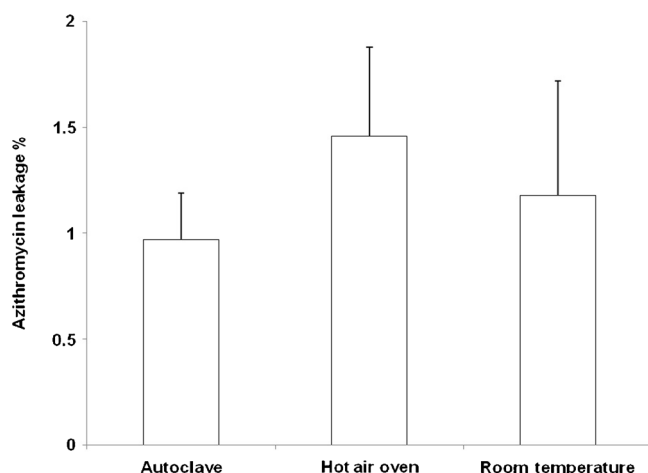


Fig. 4. Azithromycin (AZM) leakage percentage observed following the storage of AZM-loaded oil-in-water macroemulsion at three different storage conditions

mediocre treatment for acne. Moreover, a recent patent by Dow *et al.* (4) disclosed that following low-dose (sub-antibiotic dose level) systemic administration, the AZM retains its anti-inflammatory activity and is therefore effective in treating especially inflammatory lesions (papules and pustules) of acne and other skin disorders while showing virtually no or minimal unwanted antibiotic side effects such as abdominal cramping, nausea, vomiting, diarrhea, or vaginitis. In addition, at each low-dose systemic administration of AZM, the plasma concentration never reached the minimum inhibitory concentration (MIC) of 150 ng/ml against *P. acnes* and thus minimizes the serious problem of establishing drug-resistant populations of bacteria. To treat the inflammations of the skin, it would be a better idea to develop a formulation which could be applied topically onto the inflamed areas of the skin. However, developing an aqueous-based topical formulation for AZM to manage the symptoms of acne is limited partly due to its low water solubility at neutral pH (0.003 mg/ml) (24). Nevertheless a 1% aqueous solution of AZM can always lead to the formation of its major degradant product, decladinosyl azithromycin

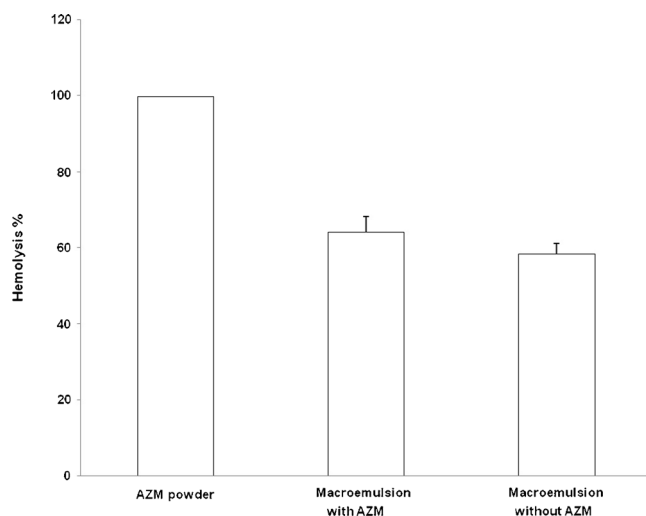


Fig. 5. Percentage hemolysis values obtained with azithromycin (AZM) powder and oil-in-water macroemulsion with or without AZM

(DES), and thus prevents the long-term therapeutic use of the aqueous-based solution (25).

When compared to the other already reported different AZM-containing solutions and suspensions, the o/w emulsion itself would offer better formulation or drug stability as the AZM is expected to entrap into either oil phase of the emulsion or o/w interface of the emulsion. Keeping into consideration the upcoming issues like the bio- and immune-toxicity problems associated with the use of inorganic- and organic-based nanomedicines, the current investigation, however, deals with the potential of o/w macroemulsion rather than nanosized emulsion for topical application onto the skin to manage acne conditions. The previously screened castor and coconut oil combination to make the o/w nanosized emulsion was simply chosen to prepare the o/w macroemulsion (13). The chitosan and Tween 20 emulgator combination was selected for the following two purposes: (1) to prepare the o/w macroemulsion having the inherent antimicrobial activity and (2) to incorporate the AZM into the oil droplets or oil-water interface of the macroemulsion. Furthermore, the developed macroemulsion showed the viscosity and pH values of 1.5 cps and 6.8–7.2, respectively, close to the viscosity and pH values of water or other normal physiological fluids. This indicates the suitability of the macroemulsion for topical application onto the skin.

Although the mean droplet diameter of the macroemulsion prepared without the addition of AZM was not determined, the mean droplet diameter of the AZM-loaded macroemulsion was found to be $52.40 \pm 1.55 \mu\text{m}$ (Table I). It should be reasonably understood that the macroemulsion without AZM might also possessed the mean droplet diameter value similar to the value observed with AZM-loaded macroemulsion. However, a very surprising result was noticed by seeing the zeta potential values of the macroemulsion with or without AZM. The macroemulsion without AZM did show the zeta potential value in negative side ($-2.60 \pm 0.75 \text{ mV}$) while the AZM-loaded macroemulsion possessed the zeta potential value that was in positive side ($+6.52 \pm 0.96 \text{ mV}$). It indicates that there was a reversal in zeta potential value from negative to positive side when 100 mg of AZM was incorporated into the macroemulsion. It should be noted that the macroemulsion without AZM was prepared based on the emulgator combination that consisted of 50 mg of chitosan and 1500 mg of Tween 20. Naturally, the presence of chitosan in the emulgator combination should confer/provide a positive charge to the macroemulsion as the chitosan is polycationic biopolymer having pKa values of ~ 6.5 , which can become protonated in weakly acidic conditions. It is this polycationic character that confers chitosan's antimicrobial properties, which favors interaction with negatively charged microbial cell walls and cytoplasmic membranes. This electrostatic interaction between protonated chitosan and microbial cell walls results in decreased osmotic stability, membrane disruption and eventual leakage of intracellular elements (26). Since the macroemulsion without AZM showed a negative zeta potential value, it is clear that the formation of protonated chitosan is very low. But the question remains how the macroemulsion without AZM was able to be physically stable at room temperature. It should be remembered that there are two stabilizing forces acting inside the o/w emulsion system to disperse/stabilize the oil droplets in the water namely, cationization and steric hindrance. Indeed, the Tween 20 is known for its steric hindrance effect and thus it basically stabilizes the o/w macroemulsion. By weight amount comparison, the ratio

Table II. Zone Inhibition Diameter Values Obtained for Medium-Only Treated, Azithromycin Solution, AZM-Loaded Macroemulsion, and Macroemulsion Without AZM Following an Incubation Time Period of 30 min With *Escherichia coli*

Incubation time (min)	Zone inhibition diameter (mm) values obtained with			
	Medium only	AZM solution	AZM-loaded macroemulsion	Macroemulsion without AZM
30	0.0	5.0	4.0	3.4

AZM azithromycin

between chitosan and Tween 20 (as the current emulgator combination) is 1:30. So, naturally, the o/w macroemulsion without AZM was found to be stable because of the steric hindrance effect produced by the Tween 20, which, in turn, prevented oil droplet coalescence upon random collisions. This explanation seems to be a plausible reason why the o/w macroemulsion having the negative zeta potential value was able to maintain its physical stability at the room temperature condition. However, a long-term stability study is underway in our laboratory to see the shelf life of the o/w macroemulsion having chitosan and Tween 20 emulgator combination.

When looking for the reason why the chitosan (and Tween 20)-based macroemulsion exhibited a negative zeta potential value, the dispersed oil droplets are basically stabilized by a mono- or multimolecular emulgator film comprising of an intercalated two different emulgator molecules. In the current macroemulsion, such type of intercalation networking occurs between the two emulgator molecules by engulfing of smaller amount emulgator molecules (chitosan) with the high amount emulgator molecules (Tween 20). In other words, the direct exposure of chitosan to surrounding water medium is somewhat masked by the Tween 20 which in turn prevented the protonated chitosan form to occur and therefore no positive charge is conferred over the emulsified oil droplets of the macroemulsion.

On the other hand, the AZM-loaded o/w macroemulsion was also found to be stable at room temperature condition and this emulsion possessed the positive zeta potential value of

$+6.52 \pm 0.96$ mV. The addition of a fixed amount (100 mg) of basic drug like AZM into the macroemulsion should contribute a positive charge to the o/w interface of the emulsion by intercalating into the emulgator combination network. This is why the reversal in zeta potential value was noticed upon addition of AZM into the macroemulsion. A similar reversal in zeta potential value of o/w nanosized emulsion was noticed after inclusion of either AZM or cyclosporine A (14,27). Collectively, the stability of macroemulsion without AZM was primarily due to the steric hindrance effect of Tween 20, whereas for AZM-loaded macroemulsion, the physical stability of the macroemulsion was because of positive zeta potential value conferred by the AZM along with the Tween 20's steric hindrance effect.

Keeping the AZM molecule in phosphate buffer solution (pH 7.4) always led to the precipitation of the drug molecules in all of the studied three different (stipulated) storage conditions. According to Bowman *et al.* (25), the AZM molecule is prone to undergo hydrolytic degradation in aqueous-based solution that lead to the formation of its major degradant product, DES. It is our belief that the total precipitation of AZM molecule in pH 7.4 phosphate buffer should be corroborated with the complete drug degradation product formation. On the other hand, the AZM molecule after its incorporation into chitosan- and PEG-based o/w macroemulsion exhibited the drug leakage percentage value of 0.972 ± 0.02 in the autoclave storage condition followed by $1.46\% \pm 0.72\%$ drug leakage in hot air oven and $1.18\% \pm 0.54\%$ in room temperature

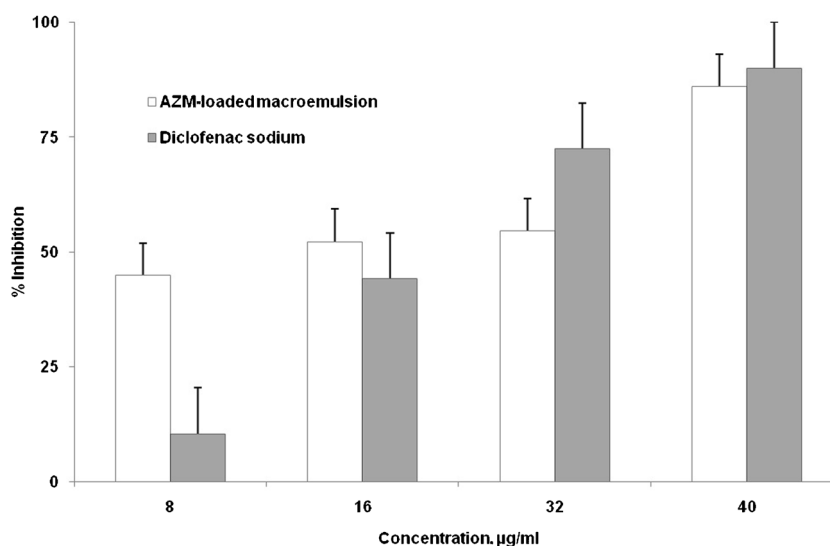


Fig. 6. Percentage inhibition values for the protein (albumin) denaturation phenomenon obtained with AZM-loaded macroemulsion (test) and diclofenac sodium solution (reference)

conditions (Fig. 4). In our previous experiment, the AZM molecule was incorporated into PEG-based microparticles wherein about 13% drug leakage was noticed after storing of the microparticles in autoclave conditions followed by $8.65\% \pm 0.72\%$ drug leakage in hot air oven and $4.15\% \pm 1.54\%$ in room temperature conditions (28). Switching from microparticles to macroemulsion, the AZM molecule appears to be more tightly bound to the inner castor oil core or oil-in-water interface of the macroemulsion. Therefore, the chitosan- and PEG-based o/w macroemulsion shows a lesser AZM leakage percentage value than the drug leakage percentage value observed with PEG-based microparticles at all of the studied three different storage conditions. Furthermore, the observed low drug leakage percentage values following even in the autoclave storage condition of chitosan- and PEG-based o/w macroemulsion (when compared to the precipitation of drug in phosphate buffer solution of pH 7.4) could provide an impetus to work further with the macroemulsion.

A very interesting insight found in the RBC breakdown study wherein the free drug produced almost 100% hemolysis while macroemulsion with or without AZM showed the RBC destruction with an average value of about 60% (Fig. 5) following 30 min post-incubation time period. Significant amount of AZM, like other hemolytic hydrophobic/lipophilic compounds, is probably incorporated inside the oil droplets of the macroemulsion. Consequently, AZM has only a limited direct contact with the tested blood cells and is not easily partitioned from oil phase to water phase of the macroemulsion, resulting in the loss of its hemolytic activity. Such a mechanism has been suggested for other lytic agents (29). Similar observations were also seen previously for AZM when incorporating it into the non-phospholipid-based cationic oil-in-water nanosized emulsion (13) or/and into PEG-based microparticles (28).

When looking at *in vitro* dissolution results, the AZM-loaded macroemulsion were shown retardation in drug release depending on the dilution ratios tested (Fig. 3). Upon keeping the drug-loaded macroemulsion in DDW at three different dilution ratios, the chitosan and Tween 20 mono- or multimolecular emulgator film which was formed at the vicinity of dispersed oil droplets was destabilized by dissolution medium (water) leading to the complete collapse in the emulsion structure. That is why the AZM was released from macroemulsion at 1 min post-dissolution time period at all of the studied dilution ratios. The DDW was selected as dissolution medium in the current investigation with an adamant belief that the macroemulsion would encounter the similar environment following its topical skin application over the inflamed area produced due to acne skin disorders. Furthermore, being a topically applicable formulation, the macroemulsion might be mixed with a few drops of water before its application onto the affected skin area by the patient. Hence, to see this possible dilution circumstance before its topical application onto the skin, the *in vitro* dissolution of drug-loaded macroemulsion in DDW at three different dilution ratios was tested. However, a question might arise regarding the influence of size of o/w emulsion (whether nano or macro) to prevent inflammation following topical application onto the skin areas that contained the inflammatory lesions

of acne. Some reports point out that it would be preferable to have a particle size range in submicron or even nanometer level for better therapeutic activity following topical application onto skin. Another question that might arise after looking at this work is that whether or not any synergistic antimicrobial activity will be observed following the antibiotic molecule (AZM) incorporation into the o/w macroemulsion having inherent antimicrobial activity. To substantiate this important question, macroemulsion with or without AZM was tested with the model microorganism (*E. coli*). At 30 min post-incubation time with *E. coli*, a slight increment in the zone inhibition diameter value was observed for AZM-loaded macroemulsion in comparison to the zone inhibition diameter value of macroemulsion without AZM (4 vs. 3.4 mm, Table II). A pilot study is going on at our laboratory to see the synergistic antimicrobial activity of AZM-loaded macroemulsion against the real microorganism using *P. acnes*.

Since there are certain limitations to use laboratory animals to induce acne and its associated inflammations, the anti-inflammatory potential of AZM-loaded coarse emulsion should be investigated in an indirect way. Hence, in the current investigation, the protein denaturation bioassay was selected for the *in vitro* anti-inflammatory assessment of drug-loaded macroemulsion. From the literature survey, it was found that production of auto-antigens in certain inflammatory or arthritis diseases may be due to the denaturation of proteins *in vivo* (30,31). In a previous report, Chandra *et al.* (19) also demonstrated that the prevention of protein denaturation by medicinal agents under an *in vitro* experimental condition is an indication of anti-inflammatory effect, and therefore, this test would be worthwhile to use routinely for the preliminary screening of anti-inflammatory effect during the drug development process. Using this reported fact, higher percent inhibition value at a lowest possible concentration level would be the best formulation. When looking at the bar graphs individually (Fig. 6), it can be concluded that both the test and reference produced the percentage inhibition values for the protein denaturation phenomenon that was concentration-dependent (8, 16, 32, and 40 $\mu\text{g/ml}$). Therefore, it can plausibly be concluded that upon topical application of AZM-loaded emulsion onto the inflamed skin surfaces produced due to *Acne vulgaris*, an increased anti-inflammatory effect is likely to occur.

CONCLUSION

The AZM-loaded o/w macroemulsion prepared based on chitosan and Tween-20 emulgator combination showed physical stability, reduced drug thermodegradation, diminished RBC breakdown, and sustained antimicrobial activity in comparison to AZM solution counterpart. Furthermore, improved *in vitro* anti-inflammatory property was also observed compared to control diclofenac sodium solution. The observed overall enhanced performances of AZM after its incorporation into o/w macroemulsion when compared to AZM solution should further be utilized for the judicious management of multifactorial acne conditions. However, thorough *in vivo* studies need to be conducted in real acne skin conditions.

ACKNOWLEDGMENTS

The encouragement and support given by the management of Lovely Professional University, India, to perform this research work is acknowledged.

Conflict of interest The authors declare that they have no competing interests.

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