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Formulation and Evaluation of Periodontitis Eugenol Loaded Nanoemulsion Gel

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ABSTRACT
The aim of this study was to develop and optimize a nanoemulsion gel loaded with eugenol for the treatment of periodontal disease. Periodontal disease is a common dental problem that affects the gums and bones supporting the teeth. Eugenol is a natural compound with antibacterial and anti-inflammatory properties, making it a promising candidate for treating periodontal disease. The study involved formulating the nanoemulsion gel using various ratios of oil, surfactant, and co-surfactant. The nanoemulsion gel was formulated using various ingredients and optimized using a statistical approach. The optimized gel was evaluated for its physicochemical properties, drug release profile, and antimicrobial activity against periodontal pathogens. It was observed that the eugenol NPs group shows significant difference in the continuity of epithelium of the interdental papilla in comparison to the untreated, pure eugenol and placebo groups. The eugenol NPs retained the continuity of epithelium of the interdental papilla up to much extent. The results showed that the developed eugenol-loaded nanoemulsion gel had a small particle size, good stability, and sustained drug release. The antimicrobial activity of the optimized gel was significantly higher than the control group. The study concluded that eugenol-loaded nanoemulsion gel could be a potential therapeutic option for the treatment of periodontal disease.
Keywords: eugenol, nanoemulsion gel, periodontal, eugenol-loaded nanoemulsion.

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INTRODUCTION

Periodontal disease, also known as gum disease, is a chronic inflammatory condition that affects the periodontal tissues, which include the gums, bone, and ligaments that support the teeth. It is caused by bacteria that form a sticky film called dental plaque on the teeth and gums.[1]

The early stage of periodontal disease is called gingivitis, which is characterized by red, swollen, and bleeding gums. If left untreated, gingivitis can progress to periodontitis, which is a more advanced stage of the disease. In periodontitis, the gums begin to pull away from the teeth, creating pockets that become infected with bacteria. Over time, the infection can damage the bone and other supporting tissues, leading to tooth loss. [2, 3]

Risk factors for periodontal disease include poor oral hygiene, smoking, diabetes, hormonal changes, certain medications, and genetics. Treatment for periodontal disease involves professional cleaning of the teeth and gums, as well as lifestyle changes such as improved oral hygiene and quitting smoking. In advanced cases, surgical intervention may be necessary to repair damage to the periodontal tissues. Regular dental check-ups and cleanings can help prevent periodontal disease or catch it early when it is easier to treat.[4,5] Periodontitis is a chronic inflammatory condition that affects the gums and surrounding tissues of the teeth. The condition is caused by the accumulation of plaque and bacteria on the teeth and gums, leading to the formation of pockets and eventual destruction of the gum tissue and bone. One of the treatments for periodontitis is the use of topical gels to deliver antimicrobial agents to the affected areas. However, conventional gels often have limited penetration and efficacy. Nanoemulsion gels, on the other hand, offer several advantages in terms of improved penetration, increased stability, and enhanced antimicrobial activity.[6,7]. This article will discuss the development and potential of nanoemulsion gels for the treatment of periodontitis

Nanoemulsion gels

Nanoemulsion gels are a type of formulation that combines the advantages of both nanoemulsions and gels. Nanoemulsions are thermodynamically stable, transparent or translucent dispersions of oil and water stabilized by an emulsifying agent. They have a droplet size in the nanometer range (typically less than 200 nm), which makes them highly stable and able to penetrate biological barriers. Nanoemulsions have been extensively studied for drug delivery applications due to their ability to improve the solubility, stability, and bioavailability of drugs. However, they are often difficult to apply topically due to their liquid nature.[8,9]

Gels, on the other hand, are semisolid formulations that are easy to apply topically and provide sustained release of active ingredients. They are often used in topical drug delivery applications due to their ability to adhere to the skin and mucous membranes, which allows for prolonged contact time and increased absorption. However, conventional gels often have limited penetration due to their high viscosity.

Nanoemulsion gels combine the advantages of both nanoemulsions and gels, resulting in a formulation that is easy to apply, highly stable, and able to penetrate biological barriers. The droplet size of the nanoemulsion in the gel allows for improved penetration of the active ingredient into the affected area, while the gel matrix provides sustained release and increased contact time.[10]

Nanoemulsion gels offer several advantages for the treatment of periodontitis.

- Firstly, the small droplet size of the nanoemulsion in the gel allows for improved penetration of the active ingredient into the affected area. This can lead to improved efficacy and faster healing of the affected tissues.
- Secondly, the gel matrix provides sustained release of the active ingredient, which allows for prolonged contact time and increased absorption. This can result in a longer-lasting effect and reduced dosing frequency.
- Thirdly, nanoemulsion gels are highly stable, which can improve their shelf-life and reduce the need for preservatives. This can be particularly important for periodontal applications, where prolonged exposure to saliva and other oral fluids can lead to degradation of the formulation.
- Fourthly, the use of nanoemulsion gels can potentially reduce the risk of systemic side effects associated with systemic administration of antimicrobial agents. Topical delivery of drugs directly to the site of infection can reduce the amount of drug that enters the systemic circulation, leading to a reduced risk of adverse effects.

Finally, the development of nanoemulsion gels for periodontitis can potentially improve patient compliance. The ease of application and sustained release properties of the gel can reduce the need for frequent applications, leading to improved patient comfort and adherence to treatment.[11,12]

MATERIAL AND METHODS

The specific ingredients and their proportions will depend on the desired properties and intended use of the nanoemulsion gel. It is essential to carefully consider the compatibility of the components and ensure that the formulation is stable and effective.

Table .1: List of materials

S. No.	Material	Manufacturer/ Supplier
1	Eugenol	Central Drug House, New Delhi, India
2	Carbopol 940	Noveon Corporation, Cleveland, OH, USA
3	Tween 80	S D Fine-Chem Ltd., Mumbai, India
4	Triethanolamine	S D Fine-Chem Ltd., Mumbai, India
5	Sodium hydroxide	S D Fine-Chem Ltd., Mumbai, India
6	Potassium dihydrogen orthophosphate	Qualigens fine chemicals, Mumbai, India
7	Ethanol (99.9%)	Jiangsu Huax Co. Ltd., Jiangsu, China
8	Poly-ε-caprolactone (MW – 14000)	Sigma-Aldrich Co., MO, USA
9	Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Co., MO, USA
10	Water (Ultrapurified)	Millipore, Molsheim, France

Selection of surfactant and co-surfactant

Three surfactants (Tween 80) and cosurfactants (ethanol, propylene glycol) were considered for use in the nanoemulsion formulation. All the possible nine combinations (S_{mix}) of the chosen surfactants and co-surfactants were studied in the surfactant:co-surfactant volume ratio of 2:1. In order to formulate eugenol nanoemulsions, eugenol itself was taken as the oil phase. For each phase diagram, eugenol and S_{mix} was mixed thoroughly at different volume ratios from 1:9 to 9:1 in different glass vials. Sixteen different combinations of eugenol and S_{mix}, 1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5

(1:1), 6:4 (1:0.7), 7:3(1:0.43), 8:2 (1:0.25), 9:1 (1:0.1), were made so that maximum ratios are covered for the study to delineate the boundaries of phases precisely formed in the phase diagrams [2, 3].

Selection of Smix ratio with Tween 80 as surfactant and ethanol as co- surfactant

The selected surfactant (Tween 80) and co-surfactant (ethanol) were mixed (Smix) in the volume ratios of 5:1, 4:1, 3:1, 2:1, 1:1 and 1:2. For each Smix ratio, eugenol and Smix was mixed thoroughly at different volume ratios from 1:9 to 9:1 in different glass vials. Sixteen different combinations of oil and Smix, 1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2 (1:0.25), 9:1 (1:0.1) were made. (Bali et al., 2010; Shafiq et al., 2007).

Slow titration with aqueous phase was performed for each ratio of eugenol and Smix and visual observations were made for transparent and easily flowing o/w nanoemulsions. Vortex mixer was used for mixing whenever required.

Stress testing of nanoemulsions

The selected nanoemulsion formulations were observed for phase separation, turbidity, creaming or cracking after each test. The centrifugation, heating–cooling cycle and freeze–thaw cycle tests [2, 9] were done to confirm that the nanoemulsions are stable and the instability of any formulation is not affecting their topical gels.

a) Centrifugation test: Nanoemulsion formulations were centrifuged at 4000 rpm for 30 min and were observed for any phase separation.
b) Heating–cooling cycle: Six cycles between refrigerator temperature (4°C) and 45°C with storage at each temperature of 48 h were conducted, and the nanoemulsion formulations were examined.
c) Freeze–thaw cycle: Nanoemulsion formulations were subjected to freeze-thaw cycle. Three freeze–thaw cycles between −20°C and +25°C, with storage of formulation at each temperature for 48 h, was performed and the samples were evaluated.

Characterization of the nanoemulsion formulations

Refractive index

Refractive index of selected formulations was determined using an Abbe type refractometer (Bausch & Lomb Optical Company, NY,USA). The refractive index was determined in triplicate from samples taken from three different regions (lower, middle and upper parts) of the nanoemulsion formulations.

Droplet size and polydispersity index

The droplet size and polydispersity index were determined using Zetasizer Nano ZS Particle Sizer (Malvern Instruments Ltd, Worcestershire,United Kingdom) at a temperature of 25°C. The nanoemulsions were suitably diluted with distilled water before taking the droplet size.

Zeta potential

The zeta potential of the nanoemulsions was determined using Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, United Kingdom) at 25°C after suitable dilution with distilled water.

Transmission electron microscopy

The TEM images of the nanoemulsions were obtained using Morgagni 268(D) Transmission Electron Microscope (FEI™ Company, Oregon, USA). In this method, a drop of the diluted nanoemulsion sample was placed on copper electron microscopy grid and then negative stained with phosphotungstic acid solution. After about 30 s, the sample was washed with ultra-purified water and the excess fluid was removed with a piece of filter paper. The dried sample was then examined and the resulting images were recorded.

Preparation of eugenol nanoemulsion gels

The gels were prepared by dispersing 1% w/w Carbopol 940 in the selected nanoemulsion formulations and subsequently neutralizing the Carbopol dispersion using triethanolamine. The final concentrations of eugenol in the nanoemulsion gels (NEGs) were maintained at 1, 2 and 5% w/w and were coded as NEG-1, NEG-2 and NEG-5 respectively.

Evaluation of eugenol nanoemulsion gels

The prepared eugenol nanoemulsion gels were evaluated for pH, spreadability, extrudability, in vitro drug release, ex vivo skin permeation and in vivo anti-inflammatory activity.

pH, spreadability and extrudability

pH, spreadability, and extrudability are important factors to consider when formulating a gel, including nanoemulsion gel

pH measurement

The pH of a gel formulation can affect the stability, efficacy, and safety of the product. For example, some active ingredients may degrade or lose their effectiveness at high or low pH values. Hundred milligram of the gel formulation was weighed in a 50 mL volumetric flask and then volume was made up with double

distilled water to 50 mL (0.2% strength)[3]. The pH of the dispersion was measured using pH meter (Mettler Instruments, Germany).

Spreadability

Spreadability refers to the ease with which a gel can be spread over the skin or other surfaces. A gel with good spreadability will allow for even and uniform application, which can improve the product's effectiveness and user experience. 0.5 g of gel was placed within a circle of 1cm diameter pre-marked on a glass plate, over which second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. The increase in the diameter due to gel spreading was noted [4].

Extrudability

Extrudability refers to the ease with which a gel can be dispensed from its packaging, such as a tube or pump. A gel with good extrudability will be easy to dispense without excessive force or clogging. Measuring the quantity of gel extruded from collapsible tube on application of constant weight. A closed collapsible tube containing 20 g of gel was pressed by applying constant load of 1 kg at the crimped end. When the cap was removed, gel extruded until pressure dissipated. The extruded gel was collected and weighed [4].

In vitro drug release studies

The paddle over disc apparatus (USP Apparatus 5; type II apparatus (Hanson Research SR8PLUS dissolution test station, Hanson Research Corporation, CA, USA), with the addition of a small stainless steel disk assembly designed for holding the gel at the bottom of the vessel) was used for studying the in vitro drug release from the nanoemulsion gels [5]. The temperature was maintained at 32±0.5oC [1, 6]. Phosphate buffer solution (pH 7.4) with 0.5% w/v Tween 80 was used as the dissolution medium and 900 mL of this medium was placed in the dissolution vessel and maintained at 32±0.5oC (Arora and Mukherjee, 2002; Joshi and Patravale, 2008). One gram of the nanoemulsion gel was applied on the disk assembly, assuring that the release surface was as flat as possible. The disk assembly was gently inserted at the bottom of the dissolution vessel. The vessel was covered during the test to minimize evaporation of the medium. The speed of rotation of the paddle was maintained 25 rpm [6]. The drug was protected from light by use of amber colored dissolution vessels to prevent any possible photodegradation. Samples of 5 mL were withdrawn at predetermined time intervals of 0, 0.5, 1, 2, 3, 4, 5 and 6h and were replaced with same volume of fresh medium. The samples were analyzed spectrophotometrically using UV– Vis spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) at 282 nm. Baseline correction was done using the placebo gel which underwent similar treatment to determine in vitro release to nullify any possible absorbance arising from the formulation excipients. All the experiments were performed in triplicate. The amount of drug release at different intervals was determined from the standard plot prepared in the dissolution medium. A graph of cumulative percentage drug release against time (h) was plotted. The dissolution profiles of the nanoemulsion gels were compared using similarity factor [4, 3].

To describe the kinetics of the eugenol release from the gel, mathematical models namely zero-order, first-order, and Higuchi's model were used. The data obtained from the in vitro drug release studies were plotted in various kinetic models: Zero order (Eqn.4.9) as cumulative percentage of drug released versus time, first order (Eqn. 4.10) as log cumulative percentage of drug remaining versus time, and Higuchi's model (Eqn. 4.11) as cumulative percentage of drug released versus square root of time (Merchant et al., 2006). The criterion for selecting the most appropriate model for the release kinetics of the nanoemulsion gels was the goodness-of-fit test using correlation coefficient.

$C = K0\ t$ (4.9)

Where C is the concentration of drug, K0 is the zero-order rate constant expressed in units of concentration/time and t is the time in hours. The slope of a graph of concentration versus time would yield a straight line with a slope equal to K0.

$\log C = \log C0 - k\ t / 2.303$ (4.10)

Where C0 is the initial concentration of drug, k is the first order constant, and t is the time.

$Q = K\ t1/2$ (4.11)

Where K is the constant reflecting the design variables of the system and t is the time in hours. Q is the drug release.

Ex vivo skin permeation studies

Ex vivo skin permeation experiments were carried out using vertical Franz-type diffusion cells having a receptor compartment capacity of 11.0 mL. Phosphate buffered saline (PBS) (pH 7.4) containing 0.5% w/v Tween 80 was used as the receptor phase. The area for diffusion was 2.404 cm2. The abdominal skin of rat was excised, the hair of which had been previously removed with an electric clipper. The subcutaneous tissues were surgically removed and dermis side was wiped with isopropyl alcohol to remove residual adhering fat. The skin was washed with phosphate buffer saline (PBS) (pH 7.4), wrapped in aluminium foil and stored in a deep freezer at -20°C till further use. The excised skin was equilibrated

in the receptor phase for 1 h before being mounted on the diffusion cell. On the donor side accurately weighed quantities of the nanoemulsion gels equivalent to 5 mg of eugenol was applied uniformly over the diffusion area. The receptor phase was maintained at 32±2°C by circulating water jacket and was stirred at 500 rpm using a magnetic stirrer (Remi instruments Ltd., Mumbai, India) [1, 8, 9]. At predetermined time intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8h, 0.5 mL samples were taken from the receiver compartment and replaced by the same volume of fresh receptor phase. All the experiments were performed in triplicate. The amount of eugenol released into the receptor phase from the NDGs was determined by spectrophotometrically after suitable dilution using UV–Vis spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) at 282 nm. Baseline correction was done using the placebo gel which underwent similar treatment to determine ex vivo permeation to nullify any possible absorbance arising from the formulation excipients. By determining the amount of eugenol permeated at various time intervals, the cumulative amount of eugenol permeated (µg cm-2) versus time (h) graph was plotted. The linearity of the steady state in the plot was checked using statistical testing of linear regression. After the linearity is confirmed, the mean flux (µg cm-2 h-1) was determined by calculating the slope at the steady state of the plot[6]. From the value of flux, mean permeability coefficient (cm h-1) was calculated using the drug load [1, 10].

In vivo studies by ligature-induced periodontitis in rats

Animal models have been used in periodontology, especially to study the influence of risk factors on the progression of periodontitis and to test the effect of different therapeutic approaches. It has also been reported that the ligature-induced periodontitis model in rats presents various bacterial species that hybridize to probes of periodontal bacterial species commonly observed in humans [3]. Ligature-induced periodontitis in rats have been reported for the evaluation of some active agents against periodontitis [7-9]. Thus the aim of the study was to evaluate the in vivo performance of the developed eugenol loaded polycaprolactone nanoparticles using ligature-induced periodontitis model in rats.

The activity of the developed eugenol nanoparticles against periodontal infections was evaluated by ligature-induced periodontitis in Wistar albino rats. The protocol for the study was approved (Proposal No. 706, dated 05.01.2011) by the Institutional Animal Ethics Committee (IAEC) of Jamia Hamdard, New Delhi (173/CPCSEA, 28th Jan 2000).

Species: Adult Wistar albino rats

Weight: 150-200 g

Gender: Either sex

Dose calculation for the rats

The dose of eugenol in human being for the treatment of periodontal diseases has been reported to be 10 mg [5]. Dose for the rats was calculated after taking into consideration surface area ratio of a rat to that of a human being [10, 11]. The dose to be given to a 200 g rat on the basis of surface area ratio was determined by multiplying the human dose by a factor of 0.018. Since 10 mg is the dose of eugenol for adult human being, the eugenol dose for 200 g rat will be (10 × 0.018) = 0.18 mg. Thus eugenol dose for rats was calculated to be 0.90 mg/kg body weight.

Procedure of the study

The animals were kept under standard laboratory conditions of temperature at 25±2ºC and relative humidity of 55±5%. The animals were housed in polypropylene cages, six animals per cage with free access to standard laboratory diet (Lipton feed, Mumbai, India; providing 3630 kcal gm-1 energy and containing 22.10% crude protein, 4.10% crude oil, 4.05% crude fiber, 10.05% ash, 0.75% sand silica) and water ad libitum. Dose for the rats was calculated as described in section.

Table .2: Treatment plan for the ligature-induced periodontitis study in rats

Group code	No. of rats	Treatment
A	5	No treatment (Model)
B	5	Eugenol
C	5	Minocycline (5 mg kg-1 day-1 orally)
D	5	Placebo nanoparticles
E	5	Eugenol loaded nanoparticles

The animals were treated as per the plan given in Table 2. The animals were anesthetized and a non-absorbable suture was placed in the cervical area of the maxillary rat molars to allow plaque accumulation, inducing changes in the periodontal tissues similar to those observed in human periodontitis. Administration of each treatment commenced on the second day after ligature [12]. The nanoparticles were applied to the site as an aqueous dispersion using a syringe. At the end of the

experimental period of 2 weeks the rats were sacrificed. The maxillae were dissected and fixed in 10% freshly prepared formalin in phosphate buffered saline (PBS) for 24 h, decalcified with 10% EDTA and embedded in paraffin. Serial parasagittal sections of 7 µm thickness were cut from paraffin-embedded tissue blocks, mounted on microsope slides and stained with haematoxylin and eosin for general tissue survey [2, 8, 10].

Table 3: Grading system for semi-quantitative analysis of the interdental papilla

Observation	Grade
Continuity of epithelium	
Intact (continuity of the epithelium along the whole interdental papilla)	0
Partly absent (small or large disruptions of the epithelium)	1
Absent (complete absence of epithelium on the interdental papilla, but no exudates of inflammatory cells)	2
Absent + exudate (same as grade 2, but presence of an exudate)	3
Continuity of transseptal fibers	
Intact (no disruption of transseptal fibers)	0
Partly absent (some transseptal fibers still present)	1
Absent (complete disruption of transseptal fiber system)	2

The continuity of the epithelium and the transseptal fibers of the interdental papilla were evaluated separately using a semi-quantitative grading (Table 3). These parameters were tested for statistical significance by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test using GraphPad Instat software (GraphPad Software Inc., CA, USA).

To study the bone loss, two parameters viz. cement enamel junction (CEJ)-bone distance and septal bone resorption (Xie et al., 2011) were examined.

Accelerated stability studies of nanoparticles

The accelerated stability study was carried out according to both International Conference on Harmonisation (ICH) guidelines and Arrhenius method.

Accelerated stability testing according to ICH guidelines

Controlled temperature and humidity condition

The accelerated stability study under controlled temperature and humidity conditions was carried out according to International Conference on Harmonisation (ICH) Q1A(R2) guidelines.

Photostability testing

The accelerated photostability study was carried out according to International Conference on Harmonisation (ICH) Q1B guidelines.

After exposure the absorbances of the sample (AT) and the control (Ao) at 400 nm were determined. The change in absorbance,

$$\Delta A = AT - Ao$$

was calculated. The length of exposure was confirmed to be sufficient to ensure a change in absorbance as indicated by its value of 0.58 (a value greater than 0.5).

RESULT AND DISCUSSION

Stress testing of nanoemulsions

All the selected nanoemulsion formulations were found to be stable against the stress tests and no phase separation, turbidity, creaming or cracking were observed in the samples. The results of the tests are presented in Table 3. A tick mark indicates that the nanoemulsion sample passed the test. The positive results of centrifugation, heatingcooling cycle and freeze thaw cycle indicated that optimized formulation passed the stress testing of eugenol nanoemulsion.

Table .4: Stress testing of eugenol nanoemulsion samples

Nanoemulsion code	Percentage content (v/v)		
	Eugenol	S_{mix}	Water
NE1	1	45	54
NE2	2	45	53
NE5	5	45	50

Characterization of the nanoemulsion formulations

The characterization parameters of the nanoemulsions are reported in Table 5. The nanoemulsions could be considered to be isotropic as the refractive indices of the nanoemulsion samples taken from three different regions of the sample are almost same as indicated by low standard deviations. The mean droplet size was well in the nanometric range and that too below 250 nm for all the samples. Low polydispersity index values indicated a uniform droplet size distribution in all the nanoemulsion samples. The zeta potential values were in the acceptable range for sufficient droplet stability. In case of gels the zeta potential values does not have much importance as the gel rigidity itself confers stability to the droplet gels against possible coalescence.

Table 5: Stress testing of eugenol nanoemulsion samples

Nanoemulsion code	Refractive index ± SD(n=3)	Droplet size ± SD (nm)(n=3)	Poly dispersity index ± SD (n=3)	Zeta potential ± SD (mV) (n=3)
NE1	1.387±0.002	78.37±4.98	0.183±0.009	22.1±2.5
NE2	1.393±0.002	111.34±6.82	0.265±0.020	21.9±2.6
NE5	1.410±0.001	208.51±5.96	0.207±0.006	27.4±3.6

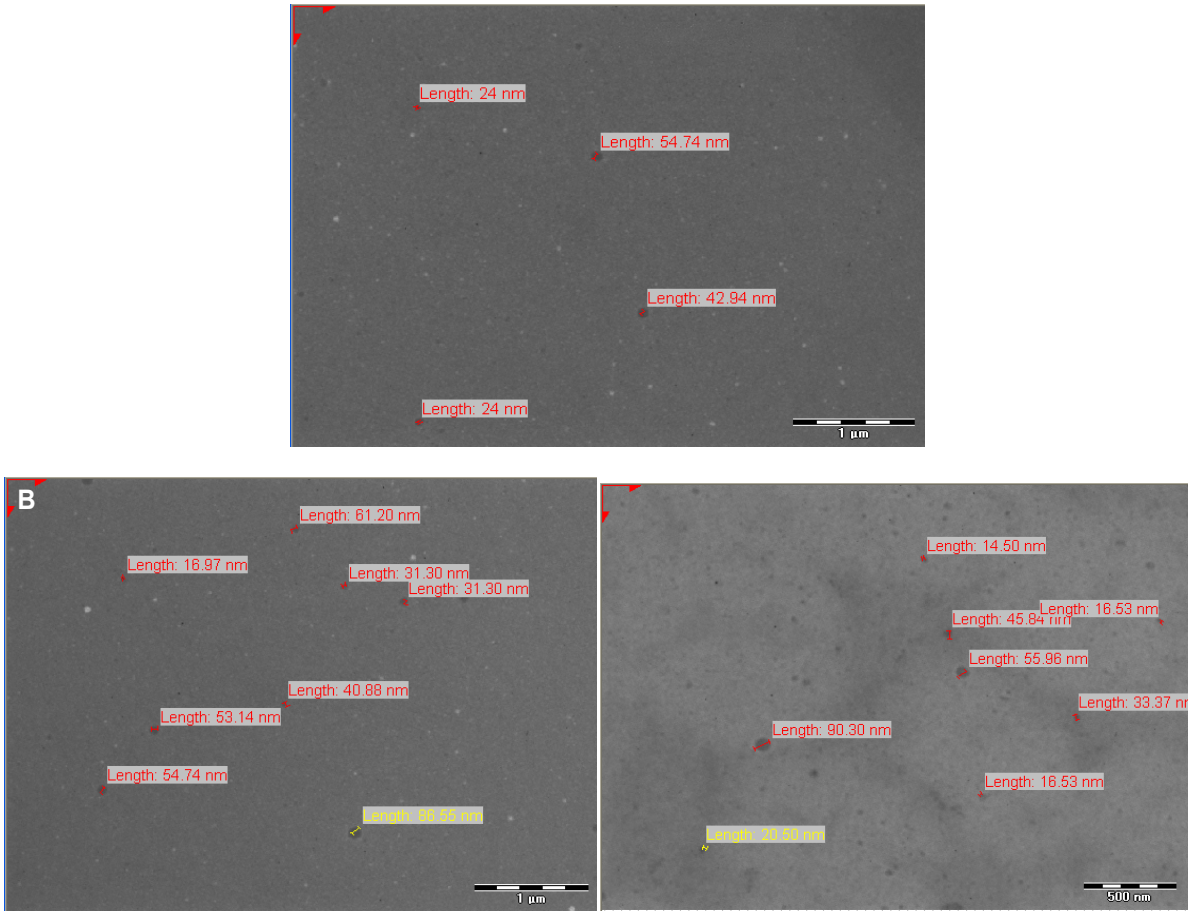

Fig. 1. TEM photomicrographs of the diluted nanoemulsion formulations. (A) NE1 (B) NE2 and (C) NE5

The TEM photomicrographs (Fig.1) further confirmed the nanometric size of the nanoemulsions supported the results for droplet size analysis.

Evaluation of eugenol nanoemulsion gels

The prepared and characterized nanoemulsion gels were evaluated for their critical performance. The results of the studies are as follows.

pH, spreadability and extrudability

The prepared nanoemulsion gels were evaluated in terms of pH, spreadability and extrudability and the results are displayed in Table 6.

Table 6: pH, spreadability and extrudability of the nanoemulsion gel samples

Sample	Ph ± SD (n=3)	Spreadability ± SD (cm) (n=3)	Extrudability ± SD (g) (n=3)
NEG1	6.87 ± 0.25	5.83 ± 0.06	1.76 ± 0.15
NEG2	6.75 ± 0.18	5.87 ± 0.06	1.75 ± 0.12
NEG5	6.72 ± 0.21	6.37 ± 0.06	1.97 ± 0.12

There was no statistically significant difference (p>005) in the pH values of the nanoemulsion gels. In the case of spreadability and extrudability, the values for NEG5 were significantly higher (p<0.05) than those for NEG1 and NEG2. There was no significant difference (p>005) between NEG1 and NEG2 in terms of spreadability and extrudability. Thus, it could be inferred that higher eugenol content causes a decreased spreadability and extrudability for the nanoemulsion gels.

In vitro drug release studies

The results of the in vitro drug release studies are displayed in Table 7. From the release profiles in Fig. 5.5.19, it could be seen that there is an initial burst release of eugenol. This is mainly due to the nanodroplets near the periphery of the gel samples. As the gel samples are applied as a thin layer it is possible to have increased surface area for drug release. The mechanism of drug release is possibly due to both diffusion of the eugenol nanodroplets and partially due to the erosion of the gel matrix by the uptake of dissolution medium. The illustrations provided in the inserts of Fig. 2 show the different stages and mechanism of eugenol release from the nanoemulsion gels. Fig. 3 shows the initial stage where the nanodroplets near the surface gets released into the medium whereas the medium gets into the gel. The eugenol nanodroplets gets solubilized in the medium due to the presence of the interfacial Smix. The Tween 80 present in the dissolution medium prevents the demulsification, if any, caused due to dilution with the medium. Fig. 3b explains the second stage of drug release where a major part of eugenol nanodroplets is released in the medium while the rest of the nanodroplets get diffused in the gel. This diffusion is dependent on the gel consistency and the rate of uptake of dissolution medium by the sample. This step can also involve erosion of the gel matrix as it was observed during the experiment that some portion of gel is getting eroded and separated from the matrix applied to the stainless steel disc. Fig. 3c illustrates significant erosion of the matrix of nanoemulsion gels and almost complete release of eugenol into the medium. This represents the final stage of drug release where the drug release from the matrix is almost complete. The gel matrix is also eroded to much extent as was observed during the experiment.

Similarity factor (f2) is a simple approach to compare dissolution profiles [2, 4]. A pair-wise model independent method is used in calculation of similarity factor to assess the similarities between two dissolution profiles. Similarity factor values of 50-100 assures the sameness or equivalence of the two dissolution profiles (FDA, 1997; Moore and Flanner, 1996). The similarity factor values calculated for the dissolution profiles were f2 (NEG1 and NEG2) = 72.03; f2 (NEG1 and NEG5) = 56.81; f2 (NEG2 and NEG5) = 61.55. The similartiy factors between the three developed formulations indicated that the formulations are similar in their cumulative percentage drug release profile. This assures that the nanodroplet gel matrix developed is sufficiently robust for content of eugenol in the formulation.

Table 7: shows the in vitro drug release profile from the nanoemulsion gel formulations

Time (h)	Cumulative % drug release, Mean ± SD (%) (n=3)		
	NEG1	NEG2	NEG5
0.5	47.56±10.38	50.04±2.48	56.89±1.20
1	67.07±4.99	66.75±3.37	74.03±5.43
2	92.28±2.92	86.79±1.94	87.61±3.71
3	95.74±1.41	96.36±1.79	93.78±0.37
4	95.76±1.09	99.76±1.05	98.13±2.71
5	96.87±1.38	100.06±1.67	99.70±0.55
6	100.41±1.10	99.16±1.28	98.97±0.60

Similarity factor (f2): f2 (NEG1 and NEG2) = 72.03; f2 (NEG1 and NEG5) = 56.81; f2 (NEG2 and NEG5) = 61.55

From the analysis of the drug release kinetics it can be observed from the Table 8 that the correlation coefficient (r^2) value is highest for first order drug release model for all the nanoemulsion gel samples. Thus it can be considered that the eugenol release from the nanoemulsion gels follow first order kinetics. This implied that the amount of eugenol release is directly proportional to its initial concentration. This was supported by the observation that the amount of eugenol release is directly proportional to the initial eugenol load in the formulation. This result is best understood from the illustration in Fig.2A. This revealed that increased eugenol content will result in increased surface availability of the eugenol

nanodroplets in the gel at all time points which will eventually be released into the dissolution medium. Fig. 3 displays the plots for determining the kinetics of drug release.

Table 8: Kinetics of drug release from nanoemulsion gels

Nanoemulsion gel	Zero order		First order		Higuchi	
	K_0 (h^{-1})	r^2	K_1 (h^{-1})	r^2	K_H ($h^{-1/2}$)	r^2
NEG1	8.0457	0.6841	0.6292	**0.8555**	27.9014	0.8088
NEG2	10.6998	0.8234	1.4537	**0.9367**	33.4876	0.9176
NEG5	8.6932	0.8309	1.0360	**0.9621**	27.1479	0.9220

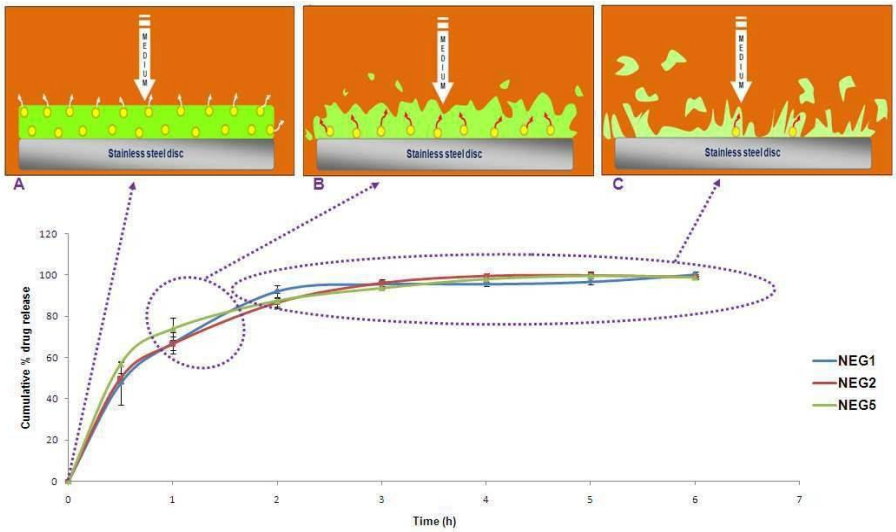

Fig. 2. *In vitro* release profiles of eugenol from the nanoemulsion gels. Inserts show the illustrations of the stages and mechanism of eugenol release from the NEGs. (A) Initial stage where the nanodroplets near the surface gets released into the medium whereas the medium gets into the gel. (B) Major part of eugenol nanodroplets get released in the medium while the rest of the nanodroplets gets diffused in the gel. (C) Significant erosion of the matrix of NEGs and almost complete release of eugenol into the medium.

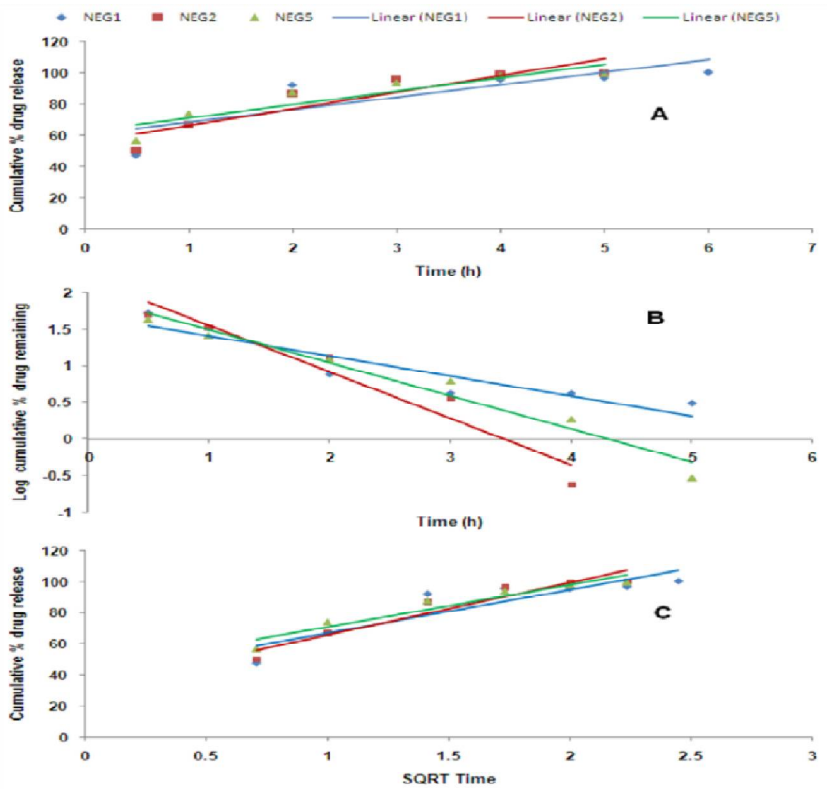

Fig. 3. Eugenol release kinetics from the NEGs (A) Zero order; (B) First order; (C) Higuchi model

Ex vivo skin permeation studies

Eugenol has been used and studied as a permeation enhancer in the transdermal drug delivery systems [1-7]. These studies have demonstrated the ability of eugenol to enhance permeation of drugs by altering the resistance afforded by the stratum corneum. The skin permeation is much dependent on the physicochemical properties of the drug and the nature of drug delivery system [1, 8]. Table 9 displays the data of ex vivo skin permeation studies and Fig. 4. shows the plot for permeation of eugenol from the nanoemulsion gels. The ex vivo skin permeation studies revealed that there were some difference in the permeation behavior of the nanoemulsion gels. The permeation of eugenol from NEG5 was higher when compared to NEG1 and NEG2. The permeation profiles of NEG1 and NEG2 were more or less comparable which could be attributed to the closeness of the eugenol content. Since the eugenol load applied in the donor compartment was same for all the samples, it can be explained that the higher concentration of eugenol, which outcomes in less amount of excipients which could impede drug permeation, results in higher amount of drug permeation. This is important as the area of the diffusion is fixed irrespective of the sample. Thus the thickness of the applied gel sample would be more for the NEG1 (with 1% eugenol) and least for the NEG5 (5% eugenol). On the other hand we can say that more number of nanodroplets per unit surface area in the case of NEG5 could be the most important reason for the study results.

Table 9: Ex vivo skin permeation data of eugenol from the NEGs

Time(h)	Cumulative amount of drug permeated, Mean ± SD (µg cm^{-2})(n=3)		
	NEG1	NEG2	NEG5
0.5	72.04±33.78	65.71± 36.66	35.32± 31.98
1	182.57±40.48	155.61± 38.11	178.93± 47.93
2	307.84±55.30	206.33± 47.59	324.92± 53.21
3	409.10±77.29	334.47± 55.56	456.34± 55.55
4	443.35± 66.22	383.99± 54.59	590.70± 65.61
5	465.47± 45.20	446.06± 65.25	669.37± 57.12
6	494.87± 52.32	504.95± 63.81	714.20± 65.06
7	514.82± 48.69	536.83± 61.55	765.32± 63.68
8	560.68± 58.29	566.85± 58.28	827.33± 71.13

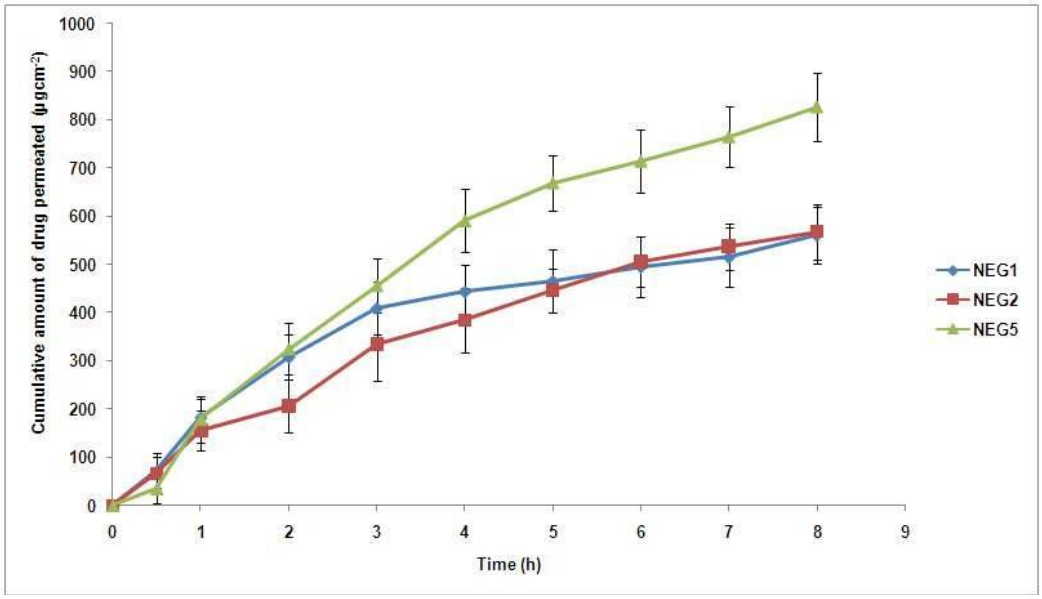

Fig. 4. Ex vivo skin permeation profiles of eugenol from the NEGs

The permeation parameters for the nanoemulsion gels are displayed in Table.10 The linearity of the steady state for the samples were confirmed by correlation coefficient (r2) values. The flux was found to increase with the eugenol concentration. The mechanism of action of eugenol in skin permeation has been widely studied and reported. The major mechanism include extraction of the stratum corneum lipids [11]. Though not much proposed for skin, promotion of paracellular transport by loosening the tight junction of the epithelium could also be a mechanism of eugenol transport permeation [12].

Table 10: Permeation parameters for the NEGs

Formulationcode	Correlation coefficient (r^2)	Mean flux (μg cm^{-2} h^{-1})	Permeabilitycoefficient (× 10^{-4} cm h^{-1})
NEG1	0.8714±0.0596	47.16±1.24	94.31±2.48
NEG2	0.9626±0.0161	60.77±3.05	121.54±6.10
NEG5	0.9482±0.0023	90.39±3.16	180.78±6.32

In vivo studies by ligature-induced periodontitis in rats

The results of the study to evaluate the tissue disruption in the interdental papilla is given in Table 11.

Table 11: Graded evaluation of the tissue disruption in the interdental papilla

Group Code	Group	†Epithelium Mean ± SD (n=5)	‡Transseptal fibersMean ± SD (n=5)
A	No treatment (Model)	1.60 ± 0.55	0.80 ± 0.45
B	Eugenol	1.80 ± 0.45	0.40 ± 0.55
C	Minocycline (5 mg kg^{-1} day^{-1} orally)	0.20 ± 0.45	0.20 ± 0.45
D	Placebo nanoparticles	1.40 ± 0.55	0.60 ± 0.55
E	Eugenol loaded nanoparticles	0.20 ± 0.45	0.40 ± 0.55

($p<0.05$ = Significant; $p>0.05$ = Not significant)

† p value (A & B >0.05; A & C <0.05; A & D >0.05; A & E<0.05; B & C <0.05; B & D >0.05; B & E <0.05; C & D <0.05; C & E >0.05; D & E <0.05)

‡ p value ($p>0.05$ between all groups)

The Fig. 5. gives the graded response for continuity of epithelium and Fig. 6. gives the graded response for continuity of transseptal fibers of the interdental papilla. It was observed that the eugenol NPs group shows significant difference in the continuity of epithelium of the interdental papilla in comparison to the untreated, pure eugenol and placebo groups ($p<0.05$). The eugenol NPs retained the continuity of epithelium of the interdental papilla up to much extent. The response of the eugenol NPs group in terms of the continuity of epithelium of the interdental papilla was similar to that observed for the minocycline group ($p>0.05$). With regard to the continuity of transseptal fibers of the interdental papilla, there was no significant difference in response between any of the groups.

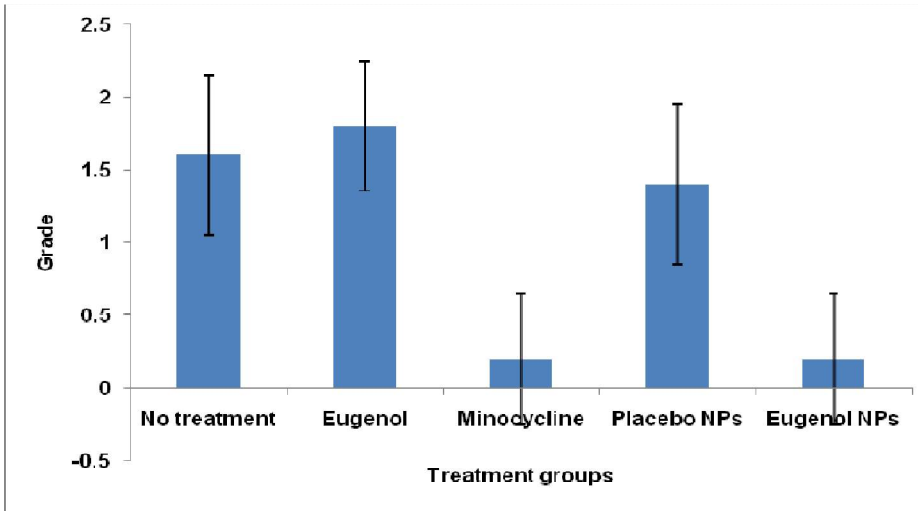

Fig. 6. Graded response for continuity of epithelium in the interdental papilla

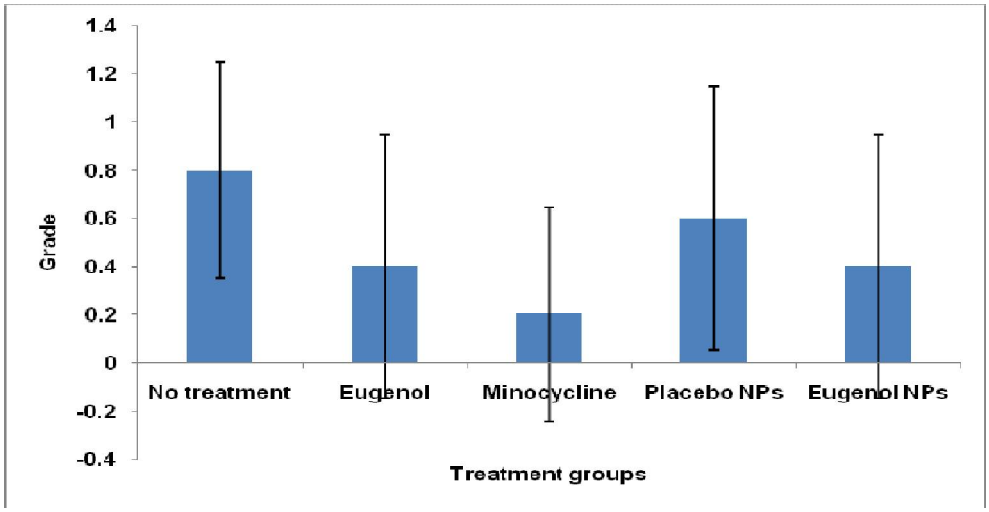

Fig. 7 Graded response for continuity of transseptal fibers in the interdental papilla

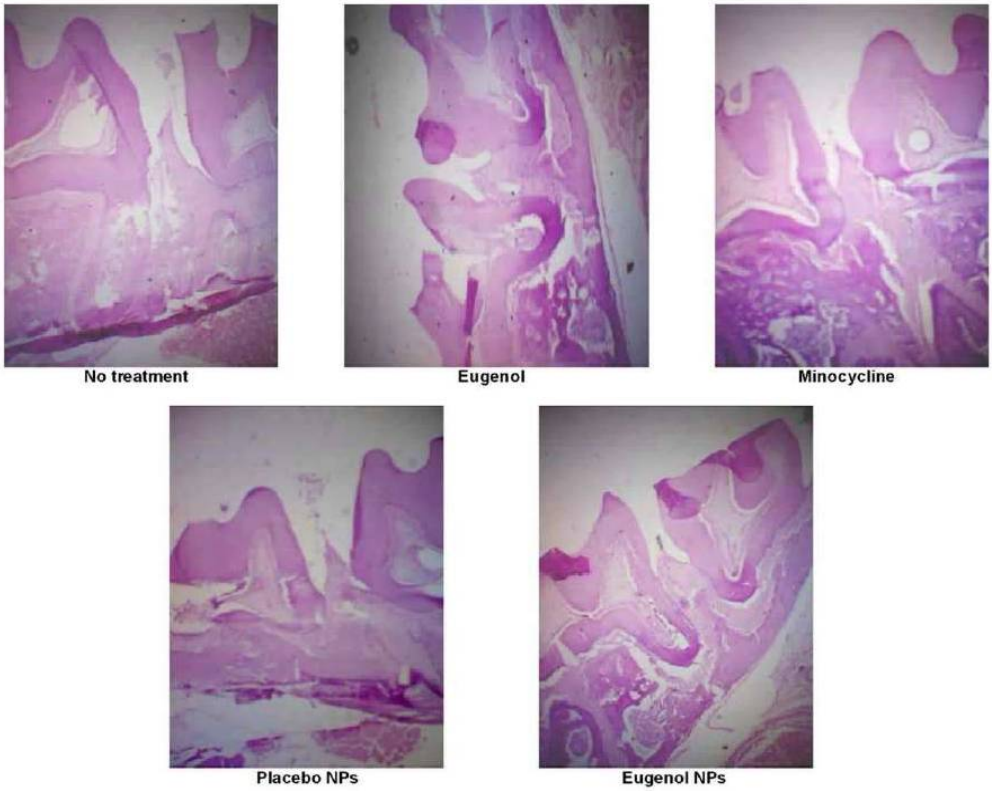

Fig. 8. Histological results of the periodontium of rats in different treatment groups subjected to ligature-induced periodontitis

Fig. 8 shows the histological results of the periodontium of rats in different treatment groups subjected to ligature-induced periodontitis. The cementoenamel junction (CEJ)-bone distance and septal bone resorption could be used to assess the bone loss in periodontitis (Xie et al., 2011). It can be observed from the figures that there is no clear demarcation of cementoenamel junction (CEJ)-bone distance in any of the treatment groups. Thus in terms of the cementoenamel junction (CEJ)-bone distance there was no apparent difference between the treatment groups. But in the case of septal bone resorption, it can be noticed that in the control, pure eugenol and placebo groups there is an apparent destruction of the septal (alveolar) bone. The reduced activity of pure eugenol could be attributed to its rapid washout by gingival crevicular fluid. But in the case of minocycline and eugenol nanoparticles treated groups there is no or minimal destruction of septal bone observed. Thus it could be concluded that the eugenol loaded nanoparticles could prevent septal bone resorption in periodontitis

Accelerated stability studies of nanoparticles

Accelerated stability testing according to ICH guidelines

Controlled temperature and humidity condition

The stability studies were performed for six months as mentioned above. The samples were analyzed at time intervals of 0, 1, 2, 3 and 6 months to determine any change in particle size, polydispersity index (PDI), zeta potential, in vitro drug release and % drug remaining of the optimized eugenol loaded nanoparticles and the results are as shown in Table 12.

Table 12: Particle size, PDI, zeta potential and % drug remaining of eugenol loaded nanoparticles stored at 40±2°C and 75±5% RH

Time (months)	*Mean Particle Size ± SD (n=3)	*Mean PDI ± SD (n=3)	*Mean zeta potential (mV) ± SD (n=3)	Similarityfactor (f_2) for *in vitro* drug release	Percentagedrug remaining (%) ± SD (n=3)
0	212.4±7.9	0.178±0.013	-29.2±3.8	-	100
1	221.3±10.5	0.162±0.013	-30.1±5.3	62.82	99.67±0.24
2	207.8±12.1	0.173±0.010	-27.6±5.2	74.42	99.07±0.16
3	209.9±8.2	0.181±0.009	-28.7±4.9	62.43	98.81±0.23
6	215.9±9.7	0.183±0.008	-31.2±3.9	65.84	97.76±0.32

Not significant (p > 0.05)

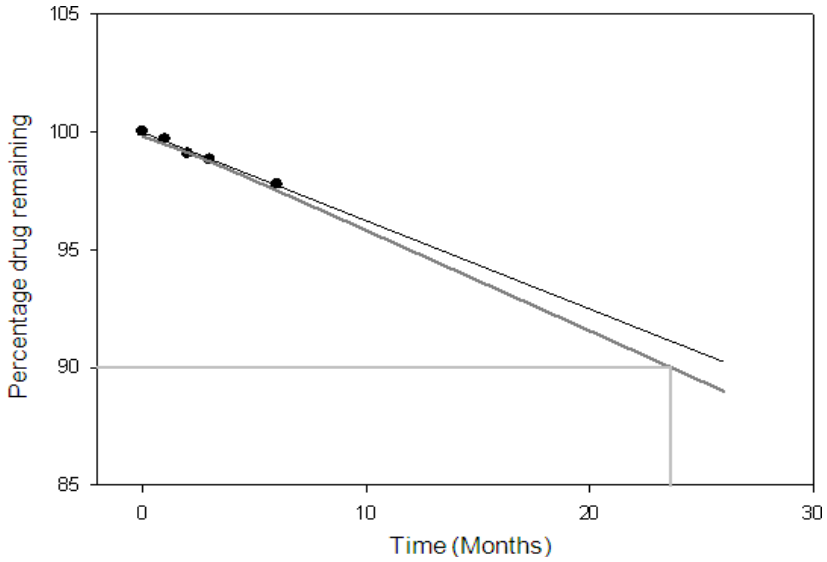

Fig 9. Shelf life determination of eugenol loaded nanoparticles

The plot obtained using SigmaplotTM 12 software (Cranes Software International, Bangalore, India) for the determination of shelf life is shown in Fig. 9.

The changes in the observed parameters were found to be statistically not significant (p >0.05) which indicated that the optimized formulations were stable. Similarity factor values greater than 50 ensured that there is no difference between the in vitro drug release profile of the samples on storage at accelerated conditions of temperature and humidity. Stability studies as per ICH guidelines at 40 ± 2°C and 75 ± 5% RH predicted a eugenol degradation of 2.24 %, in the nanoparticles at the end of 6 months. From the plot obtained using the software, the shelf life of eugenol loaded nanoparticles was found to be 23.60 months.

Photostability testing

The accelerated photostability study was carried out according to International Conference on Harmonisation (ICH) Q1B guidelines (ICH, 1996). The percentage drug remaining after the photostability testing was found to be 99.54±0.36% (n=3). The result indicated that the packed eugenol loaded nanoparticles are resistant against photodegradation.

Accelerated stability study by conventional method using Arrhenius equation

The percentage drug remaining in the optimized nanoparticles when stored for 3 months at elevated temperatures of 30 ± 2 ºC; 40 ± 2 ºC and 50 ± 2 ºC at 55 ± 5% RH were determined and the results are as shown in Table 13.

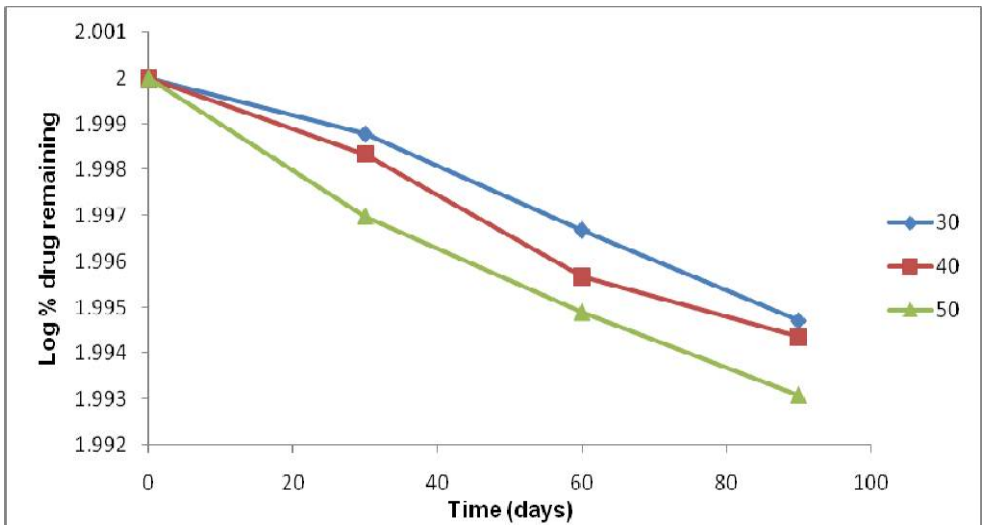

Fig 10. Log percent drug remaining versus time plot for eugenol loaded nanoparticles

Table 13: Percent drug remaining in eugenol loaded nanoparticles stored at elevated temperatures (30 ± 2 ºC; 40 ± 2 ºC and 50 ± 2 ºC)

Time (Days)	Storage conditions	% Drug remaining	Log % drug remaining
0	30 ± 2 ºC and 55 ± 5% RH	100.00	2.0000
30		99.72	1.9988
60		99.24	1.9967
90		98.79	1.9948
0	40 ± 2 ºC and 55 ± 5% RH	100.00	2.0000
30		99.62	1.9983
60		99.01	1.9957
90		98.71	1.9944
0	50 ± 2 ºC and 55 ± 5% RH	100.00	2.0000
30		99.31	1.9970
60		98.83	1.9949
90		98.42	1.9931

Table 14: Degradation rate data for eugenol in optimized nanoparticles

Temperature (ºC)	TemperatureT (ºK)	(1/ T) × 10^{-3} ($ºK^{-1}$)	Slope × 10^{-5}	k × 10^{-4}	Log k
30	303	3.3003	-5.9855	1.3785	-3.8606
40	313	3.1949	-6.5280	1.5034	-3.8229
50	323	3.0960	-7.6180	1.7544	-3.7559
25	298	3.3557	-	1.2759	-3.8942

The order of degradation of eugenol in the nanoparticles was determined as given in Table 14 and was found to follow first order kinetics (r2 values of 0.988, 0.984 and 0.985 at 30, 40 and 50°C respectively) as shown in Fig. 5.6.16. The reaction rate constant 'k' for the degradation was measured. Plot of the logarithm of k values at each elevated temperature against the reciprocal of absolute temperature was drawn (Arrhenius plot) and is shown in Fig. 11 . From the plot, k value at 25ºC (k25) was determined and used to calculate shelf life

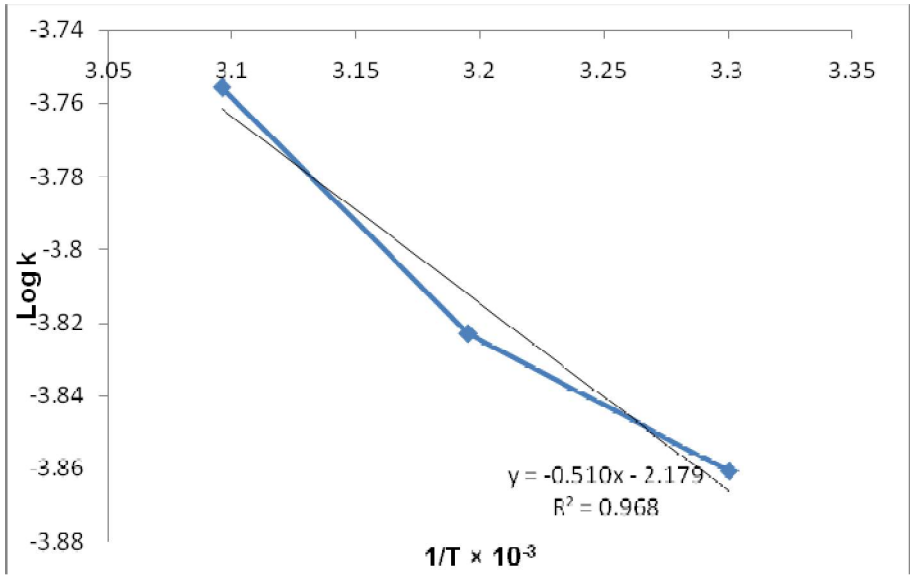

Fig. 11. Arrhenius plot for optimized eugenol loaded nanoparticles

Shelf life = t0.9 = 0.1052 / k25

Shelf life of eugenol loaded nanoparticles = 0.1052 / 1.2759× 10-4 = 824.5 days = 27.11 months

The shelf life of the optimized eugenol loaded polycaprolactone nanoparticles at 25ºC was determined to be 27.11 months

CONCLUSION

Nanoemulsion gels offer several advantages for the treatment of periodontitis, including improved penetration, sustained release, stability, reduced risk of systemic side effects, and improved patient compliance. Several studies have demonstrated the potential of nanoemulsion gels containing antimicrobial agents for the treatment of periodontitis. From the animal study, It can be observed from the figures that there is no clear demarcation of cementoenamel junction -bone distance in any of the treatment groups. Thus in terms of the cementoenamel junction-bone distance there was no apparent difference between the treatment groups. But in the case of septal bone resorption, it can be noticed that in the control, pure eugenol and placebo groups there is an apparent destruction of the septal (alveolar) bone. The reduced activity of pure eugenol could be attributed to its rapid washout by gingival crevicular fluid. But in the case of minocycline and eugenol nanoparticles treated groups there is no or minimal destruction of septal bone observed. The results of the study to evaluate the tissue disruption in the interdental papilla is given in Table 11. However, further studies are needed to optimize the formulation and evaluate the efficacy of these formulations in clinical settings. Overall, the development of nanoemulsion gels for periodontitis represents an exciting area of research that has the potential to improve the management of this inflammatory condition.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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