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DESIGN, DEVELOPMENT, CHARACTERIZATION AND SYNERGISTIC ACTIVITY OF LIPOSOME OF CLOVE AND CINNAMON OIL IN ORAL HEALTH CARE

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ABSTRACT

Periodontal diseases are identified as the most important oral health problem globally. Chronic exposure to acidic foods leads to development of dental erosion, abrasion and decay. Essential oils having antibacterial, antifungal and antioxidant properties have recently gained importance as dental pharmaceuticals. But they are chemically unstable in presence of air, light, moisture and high temperature. Hence novel methods like liposomal encapsulation of oils have been introduced to enhance the stability and bioavailability. This research paper focuses on two essential oils, Clove oil obtained from flower buds of *Syzygium aromaticum* Linn (Fam. Myrtaceae) containing eugenol and β caryophyllene and Cinnamon oil from *Cinnamomum cassia* bark (Fam. Lauraceae) containing cinnamaldehyde. The objective was to develop a liposomal gel containing these oils and study their activity against dental pathogens *Lactobacillus acidophillus* and *Streptococcus mutans.* Liposomes were prepared by thin film hydration method using soya lecithin and cholesterol. The optimized liposome was formulated as carbopol gel and evaluated for pH, spreadability, viscosity, drug content and diffusion studies. Liposome showed an entrapment efficiency of 67% for clove oil and 75% for cinnamon oil. They had synergistic activity against dental pathogens. The percent drug content was estimated by RP-HPLC and 94.11 % of eugenol and 91.5%w/w cinnamaldehyde was obtained. The invitro diffusion studies showed a cumulative drug release of 77.23% eugenol and

82.71%cinnamaldehyde at the end of 5hrs. The optimized gel was found to be stable when stored as per ICH guidelines.

Keywords: Anticaries activity, Cinnamaldehyde, Eugenol, Liposomes, Synergistic activity

INTRODUCTION

The development of topical therapy in the past two decades has increased dramatically with more advancements in novel drug delivery systems and their incorporation in dosage forms such as gels, ointments and sprays. It is a better delivery system as it avoids systemic absorption. The oral cavity is lined with mucus membranes with a total surface area of 200cm^2 and is composed of an outermost layer of stratified squamous epithelium, below this lies the basement membrane, a lamina propria followed by the submucosa as the innermost layer. The epithelium of the buccal mucosa is about 40–50 cell layers thick **[1]**. The permeability of buccal mucosa is 4-4000 times greater than that of skin. The periodontal diseases are generally degenerative or neoplastic in nature. The periodontal pocket provides diverse environment for the colonization bacteria like *Streptococcus mutans* **[2]** and *Lactobacillus acidophilus* **[3]**. Essential oils having antibacterial, antifungal and antioxidant properties have recently gained importance as dental pharmaceuticals. However, they are chemically unstable in presence of air, light, moisture and high temperature. They contain unstable oxygen group which act as an oxidizing agent. Additionally, essential oils are poorly soluble in water, hence novel methods like liposomal encapsulation of oils have been introduced to enhance the stability and bioavailability. Liposomes are vesicles having concentric phospholipids bilayer molecules from low molecular weight to high molecular weight. They are biocompatible, completely biodegradable, non-toxic, flexible and nonimmunogenic for systemic and non-systemic administrations. They offer several advantages by enhancing the pharmacokinetics and biodistribution of encapsulated molecules. They also decrease the toxicity and provide target selectivity **[4, 5]**. This research paper focusses on two essential oils Clove oil obtained from flower buds of *Syzygium aromaticum* Linn (Fam. Myrtaceae) containing eugenol and β caryophyllene and Cinnamon oil from *Cinnamomum cassia* bark (Fam. Lauraceae) containing cinnamaldehyde. Clove buds contains 18% of clove oil**;** the major component is eugenol (70-80%) [6]and minor amounts of β-caryophyllene Essential oil extracted from the cinnamon bark contains

70-80% of cinnamaldehyde **[7]**. Literature reports various delivery systems for these constituents. Shende PK *et al* prepared serratiopeptidase and clove oil buccal patch by emulsification method using HPMC and Eudragit L100 **[8]**. The antibacterial activity of liposome-encapsulated clove oil against *S. aureus* in tofu was investigated **[9]**. Literature also reports the study of various essential oils as liposomes for topical purpose **[10, 11]**.

Dental caries remains the most prevalent and costly oral infectious disease worldwide. Dental disorders require a prolonged contact of the active agent at the site of action which can be achieved by the controlled drug delivery systems as the treatment is based on the removal of the pathogenic bacteria from the periodontal pocket The objective was to develop and evaluate a liposome encapsulating both these oils and then evaluate its synergistic activity against dental pathogens.

MATERIAL AND METHODS

Standards Eugenol and Cinnamaldehyde was procured from Loba chemicals Pvt. Ltd. having 99% purity. Buds *of Syzygium aromaticum and* bark of C*innamomum cassia* were procured from local market and authenticated by comparing the morphological features with the sample present in the Pharmacognosy laboratory of the institute. HPLC grade acetonitrile and methanol was purchased from Thomas Baker and Water HPLC grade from Anant Pharmaceuticals Pvt. Ltd.

Extraction of essential oil

Extraction of oils was done using Clevenger's apparatus. 50 gm of cinnamon bark powder and 50 gm of clove buds was extracted in mixture of glycerin and distilled water (20:80) using Clevenger's apparatus for 3-4 hours. Extraction of both the oils was done separately. The volume of essential oils was determined using a calibrated trap.

Characterization of the oil

A stock solution of eugenol and cinnamaldehyde (1000 µg/ml) was prepared. It was diluted to obtain a working standard of 100 µg /ml. The calibration curve was prepared for both eugenol and cinnamaldehyde standard using methanol as solvent. Linearity was established for eugenol in the concentration range 5ppm to 25ppm at λmax 282nm and for cinnamaldehyde in the concentration 1ppm to 5pmm at λmax 286nm. A stock solution of extracted clove oil (100µg/ml) and cinnamon oil (100µg/ml) was prepared in methanol. It was suitably diluted to obtain a 20ppm solution. Absorbance of this solution was taken at the stated λmax values and the concentration of eugenol in clove oil and cinnamaldehyde in cinnamon oil

was estimated using the standard equation of calibration curve.

Preparation of liposomes

The thin film hydration method was used for the preparation of liposomes **[5, 12]**. Soya lecithin and cholesterol were used as lipids. For the preparation of liposomes, 20mg of either clove oil or cinnamon oil was dissolved in chloroform and methanol mixture. The organic solvent was then added to an accurately weighed amount of soya lecithin and cholesterol as indicated in Table 1. This mixture was then sonicated for 10 min. The resultant mixture was then transferred to a rotary evaporator and the organic solvent was evaporated under reduced pressure and above lipid transition temperature so that a clear thin film of uniform thickness could be formed. Traces of solvent was removed by maintaining the lipid film in vacuum oven overnight. The film on complete drying was hydrated with phosphate buffer (pH 5.5). After hydration the liposomes were separated from the unentrapped drug by centrifugation at 10000 rpm and 4^0C temperature using cooling centrifuge. Formulation LF1 to LF6 was prepared with clove oil varying the proportion of soya lecithin and organic solvent and formulations LF7 TO LF12 was prepared with cinnamon oil **(Table 1)**.

Table 1: Preparation of Liposomes

Ingredients	LF 1	LF 2	LF 3	LF 4	LF 5	LF 6	LF 7	LF 8	LF 9	LF 10	LF 11	LF 12
Soya lecithin (mg)	45	50	55	45	50	55	45	50	55	45	50	55
Cholesterol (mg)	15	15	15	15	15	15	15	15	15	15	15	15
Chloroform:Methanol	2:1	2:1	2:1	3:1	3:1	3:1	2:1	2:1		3:1	3:1	3:1
Clove oil (mg)	20	20	20	20	20	20	-	-	-	-	-	-
Cinnamon oil (mg)	-	-	-	-	-	-	20	20	20	2:1	20	20
Entrapment Efficiency (%)	24	28	35	46	57	54	26	34	42	68	75	71

Evaluation of liposomes [13]

The evaluation was done on the basis of vesicular size and entrapment efficiency

i. **Vesicular size determination:** The drop of liposome suspension was taken on a glass slide with a drop of glycerine. For visualization rhodamine red was added and the liposomes were observed under Digital Motic Optical Microscope. Scanning electron microscopy was carried out at the National Centre for Nanosciences and Nanotechnology, University of Mumbai.

ii. Entrapment efficiency: Aliquots of liposomal suspension (10 ml) was subjected to centrifugation using cooling centrifuge at 10,000 rpm for 90 minutes. Clear supernatant was separated from liposome pellet. The pellet was dried in vacuum oven. Accurately weighed 25 mg of pellet was dissolved in 10ml of methanol and sonicated for 20-30 min to lyse the liposomes. Concentration of

eugenol and cinnamaldehyde was determined spectrophotometrically at λmax 282 nm for clove oil liposomes and λmax 286 nm for cinnamon oil liposome respectively, using, empty lysed liposomes as blank. Concentration of eugenol and cinnamaldehyde in supernatant and sediment gave a total amount of drug in the dispersion. The percent entrapment was calculated using the formula, % entrapment= amount of active constituent in sediment/amount of active constituent added ×100.

Preparation of topical gels:

For the preparation of topical gels carbopol 976P NF (0.5-2%) was soaked in water for a period of 2 hours. The mixture was stirred for 1 hour with the help of magnetic stirrer. To this propylene glycol 1% and glycerine 1% was added. The formulation was neutralized by drop-wise addition of 50% (w/w) triethanolamine until transparent gel appeared **[14, 15]**. The liposome LF5 and LF11 was selected for incorporation into the gel. Liposomal pellets of clove and cinnamon (1% each) was mixed with vehicle (gel) to produce the final concentration of both the drugs in the gel as 2.0 % (w/w). Formulation was allowed to equilibrate for 24 hours. Four formulations (F1 to F4) were prepared using varying concentration of carbopol 976P from 0.5% to 2%.

Evaluation of liposomal gel:

The liposomal gel was evaluated for colour, appearance, pH, homogeneity, viscosity by using Brookfield viscometer with spindle no 7 at 100 RPM at room temperature. The drug content in the gel was carried out by RP-HPLC. For this 1gm of liposomal gel was dissolved in 10ml methanol. After filtration 1ml of solution was further diluted till 10ml and analysed by HPLC. The method was developed using Jasco HPLC with UV detector equipped with Chromnav 2.0 software for recording and interpretation of data. Column used was Athena C_{18} (5µm C_{18}100 Å, LC Column 250 x 4.6 mm). The mobile phase that gave the best resolution consisted of HPLC grade methanol and water, MeOH : H_2O (70:30) It was delivered as an isocratic system at a flow rate of 1 ml/ min and detection done at 284nm for simultaneous determination of eugenol and cinnamaldehyde. The injection volume was 20µl. The developed method was validated as per ICH Q2A (R1) guidelines.

Anticaries Activity

Clove oil and cinnamon oil are known to possess anticaries activity. Hence liposomal formulation which showed the best entrapment efficiency was taken up further for this study. It was carried out by agar well plate method **[16]**. Microbial cultures *Lactobacillus*

acidophillus (MTCC No. 10307, MRS broth) and *Streptococcus mutans* (MTCC No .890, Brain Heart Infusion agar) were obtained from IMTECH Chandigarh. All glass wares were sterilised in hot air oven at 160^0C for 2 hrs. Medium was sterilized in autoclave at 121^0C and 15 psig pressure for 15 minutes. Standard inoculums of microbial culture was prepared by comparing it with McFarland No.1 turbidity standard solution and then 1:500 dilution was made with sterile water. Activity against dental pathogen was tested for liposome LF5 and LF11 which had maximum entrapment efficiency. The test samples included 5mg of liposomes LF5 dissolved in 1ml of dimethyl formamide (sample 1) and 5mg of liposomes LF11 dissolved in 1ml of dimethyl formamide (sample 2). Sample 3 included 2.5 mg of clove liposome LF5 and 2.5 mg of cinnamon oil liposomes LF11 together dissolved in 1ml of DMF. Sample 4 included the gel formulation, F2. For the study of zone of inhibition in agar well plate, 0.3 ml of constant solution of test samples were added and incubated at 37 ± 0.5 ^{0}C.

RESULTS AND DISCUSSION:

Clove buds and Cinnamon bark were identified by comparing its morphological features with those given in the literature and with the authenticated samples of the institute. Clove oil and cinnamon oil were individually extracted using Clevenger's apparatus. The yield of oil obtained from cinnamon bark was 3%(w/w) and clove buds was 2.5%(w/w). Standard eugenol had a λ max of 282nm. It was found to be linear in the range 5ppm to 25ppm, having a standard equation of $y=0.028x + 0.1937$ with $R^2 = 0.993$. Cinnamaldehyde had a λ max of 286nm. It was linear in the range 1ppm to 5pmm, having standard equation $0.1031x+0.013$ with $R^2 = 0.999$. The concentration of eugenol in clove oil was found to be 62.25%(w/w) and concentration of cinnamaldehyde in cinnamon oil was found to be 7.2%(w/w) using the standard equation of calibration curve

Colloidal suspensions of liposomes containing 20mg of clove oil or 20mg of cinnamon oil was prepared by film rehydration method. Variation in different formulation were brought about by changing the concentration of phospholipid from 0.5% to 2% and varying the solvent proportion (chloroform: methanol). Twelve different liposomal vesicles were prepared (six containing clove oil and six containing cinnamon oil) and they were visualized using a digital motic microscope and their entrapment efficiency was calculated. It was observed that liposomes were round shaped with particle size ranging from 10 μm to 50 μm **(Figure 1)**.

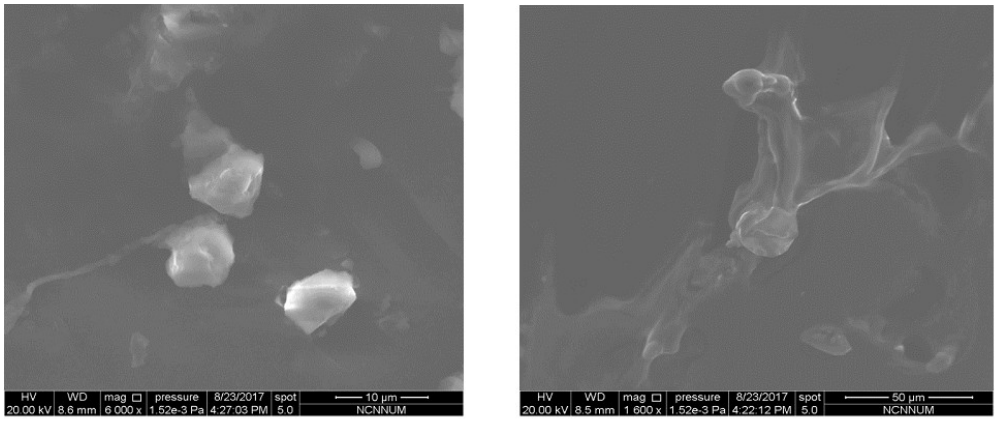

Figure 1: SEM analysis of Clove oil liposomes and Cinnamon oil liposomes

In order to determine the drug holding capacity of the prepared liposomes, the entrapment efficiency was evaluated **(Table 1)**. The formulation LF5 containing clove oil was found to have an entrapment efficiency of 57%. Similarly the formulation LF11, containing cinnamon oil was found to have an entrapment efficiency of 75%. Both, the amount of lecithin and solvent proportion, influenced the entrapment of the herbal extract inside lipid vesicles in a positive way. However, increase in phospholipid concentration to 55mg, decreases the entrapment efficiency. 50mg phospholipid concentration showed maximum entrapment efficiency. The ratio of organic solvents also played an important role in drug entrapment. Maximum entrapment efficiency was observed in chloroform: methanol (3:1). Anticaries activity was studied on the optimized liposomes LF5, LF11 and their equal combination.

The liposomal vesicle LF5 and LF11 was then taken up for further studies. A topical gel of 1 % w/w concentration was prepared using carbopol 974P. The concentration of carbopol 974P was varied in the range of 0.5, 1, 1.5 and 2%w/w. The prepared formulations were evaluated for physical characteristics like homogeneity, spreadability, pH and drug content. It was observed that increasing the concentration of polymer increased the viscosity. Higher viscosity leads to low spread ability values. The result showed that the developed herbal gel was yellowish in colour, translucent in appearance and showed good homogeneity with absence of lumps and skin irritation. Formulation F2 was the ideal one containing 1.0% carbopol 974P with a spread ability of 35±0.43, pH 6.9 and viscosity of 9600cps. The % drug content was established using RP-HPLC. Simultaneous estimation of eugenol and cinnamaldehyde in liposomal gel could not be determined by UV

spectrophotometer. So HPLC method with Methanol:Water (70:30) was developed. An isocratic system at a flow rate of 1 ml/ min and detection done at 284nm was used for the simultaneous method development. Drug content was found to be 91.5% for eugenol and 94.11 % for cinnamaldehyde **(Figure 2, Table 2)**.

The method was validated as per ICH guidelines Q2A (R1). The results of the validation studies is given in **Table 4**. The anticaries activity was carried on the gel formulation F2 by the agar cup method with the test organism. A synergistic activity was observed for the gel which contained eugenol and cinnamaldehyde **(Table 4, Figure 3)**. An increase in the zone of inhibition showed a synergistic activity and it also indicated good diffusion of the drug from the gel.

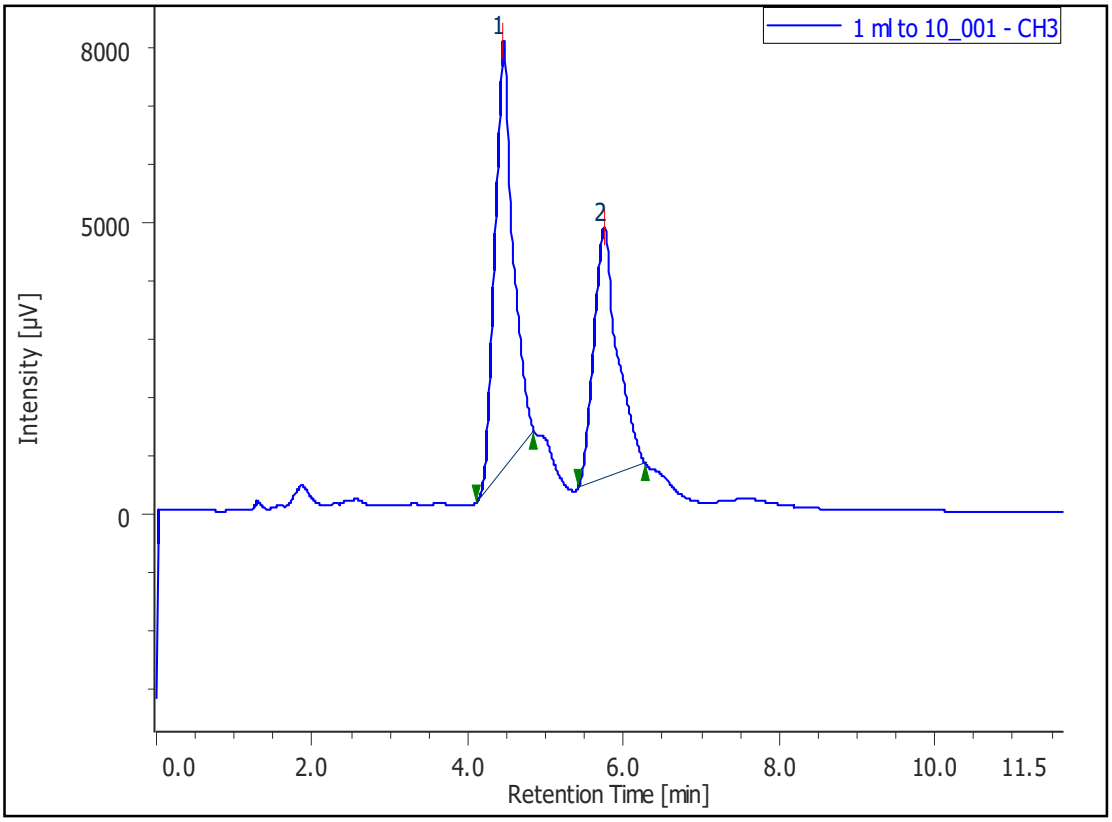

Figure 2: HPLC chromatograph of liposomal gel, Formulation F2

Table 2: Drug content for liposomal gel F2

Sr no.	Peak name	Tr	Area	NTP	Resolution	Symmetry Factor	% drug content
1	Cinnamaldehyde	4.5	107043	2041	2.88	1.166	94.11
2	Eugenol	5.7	97043	2075	N/A	1.302	91.5

Table 3: Validation studies for simultaneous analysis of Eugenol and Cinnamaldehyde

Parameter	Eugenol	Cinnamaldehyde
Linearity	y = 8624.6x + 5383.2	y = 47363x + 10421
R^2	0.997	0.9888
Range	5-9 µg/ml	1-5 ug / ml
Specificity	Specific	Specific
Accuracy – 80%	95.28	96.07
100%	94.91	95.29
120%	93.43	95.73
System Precision (%RSD)	1.22	0.57
Method Precision (Intra day)	1.22	0.60
Method Precision (Inter day)	1.01	1.02
Robustness	Robust	Robust
LOD	0.24	0.028
LOQ	0.73	0.086

Table 4: Anti caries Activity of optimized gel

	Zone of inhibition (mm)	
Test Pathogen	*S.mutans*	*L.acidophilus*
Cinnamon oil liposomes: Sample 1	1.7	1.5
Clove oil liposomes: Sample 2	1.8	1.7
Mixture of clove and cinnamon oil liposomes: Sample 3	2.0	1.9
F2 formulation: Sample 4	1.9	1.8

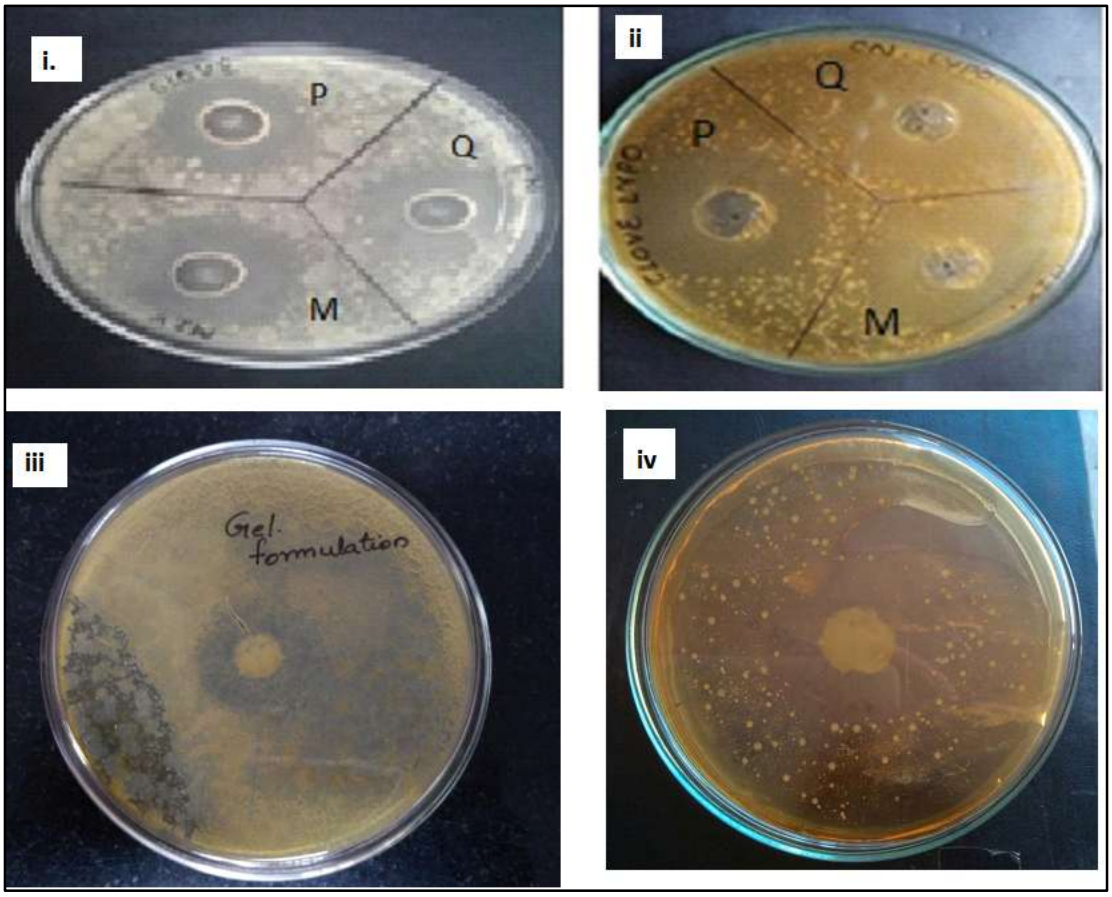

Figure 3: Anticaries activity – i. Liposomes on *L. acidophillus*, ii. Liposomes on *S. mutans* iii. Gel formulation on *L. acidophillus*, ii. Gel formulation on *S. mutans*
(P – Clove oil liposomes, Q – Cinnamon oil liposomes, M- mixture containing both the liposomes.)

CONCLUSION:

Liposome of clove oil and cinnamon oil were successfully formulated by using varying concentration of soya lecithin and chloroform: methanol. The formulation has shown its effectiveness such a synergistic increase in anticaries activity. A simple, precise, accurate HPLC method was developed for simultaneous analysis of eugenol and cinnamaldehyde in the gel formulations. Several pharmaceutical products are being used and developed for the oro- dental delivery, but still, many are in pipeline. Recent advance in the controlled and targeted drug delivery systems offer a large scope and potential for alleviating dental disorders by use of novel techniques

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CONFLICT OF INTEREST: None

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