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FORMULATION AND DEVELOPMENT OF COMBINATIONAL ANTI-BACTERIAL *IN-SITU* GEL TO TREAT PERIODONTITS

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ABSTRACT

Periodontitis is a disease that begins in the gingival tissue and progresses to the underlying tissue, altering bone homeostasis and resulting in tooth loss if left untreated. The main aim and objective of this study is to design and development of the combinational *in-situ* gel to treat periodontitis. Doxycycline, metronidazole and ciprofloxacin are the most widely used drugs in treating this disease belongs to three different antibiotic classes which acts against the microbes at various stages. The drug-excipient compatibility studies were done using FT-IR and determining the MIC's using broth dilution method helps to select the triple combination as best choice to decrease the recurrence of the disease. *In-situ* gels of various concentrations using carbopol934 and HPMCK100 were formulated and evaluation tests such as physical appearance, pH (6.5±0.56), viscosity (29065.25±1.35 to 29815.70±1.2), gelation time (≤2 min), syringeability(≤1min) were performed for the developed formulations. Application of DoE (3^2 factorial design) helps in the enhancement of research work to determine the optimized formulation. Anti-microbial assay for the optimized formulation was carried out and largest zone of inhibition against the microorganisms was observed. From this research work, it can be assumed that the formulated triple drug anti-microbial *in-situ* gel was active even at lower concentrations along with prolonged release helps to decrease the dosage regimen and also decrease the recurrence of the disease.

Keywords: Periodontitis, MIC, *In-situ* gels, sol-gel theory, pH dependent polymers, 3^2 factorial design & DoE

INTRODUCTION:

Periodontitis is a disease which affects the teeth leading to its destruction and it is one of the most common diseases associated with oral cavity. the term "periodont" means 'structure surrounding the teeth' and "itis" implies 'inflammation' **[1]**. It is linked to a 19% increase in the risk of cardiovascular disease, with a 44% increase in relative risk among those who aged 65 and up. Also related to Type 2 diabetes, parental infection, premature births, low birth weight, and pre-eclampsia **[2]**. Over the last two decades, dentists and microbiologists have embraced periodontal antibiotic therapy as a powerful adjunct for therapeutic management. Antibiotics are defined as naturally occurring or synthetic organic substances that inhibit or kill selective microorganisms in low concentrations. There are numerous antibiotics that could be employed to treat periodontal infections, but it is often unclear which antibiotic would provide the greatest benefit to a patient with a specific periodontal infection, with minimal adverse effect **[3]**. Ciprofloxacin hydrochloride is a fluoroquinolone antibiotic that is commonly used to treat bacterial infections especially caused by gram negative bacteria. The antibacterial action is primarily based on the inhibition of DNA gyrase and topoisomerase IV which are the most unique enzymes for the transcription and replication of DNA in prokaryotic cells. Doxycycline belongs to tetracycline class of antibiotics which is used mostly in the treatment of destruction of biofilm formed on teeth. It acts to stop the bacterial growth (bacteriostatic) by binding to the 30S ribosomal subunit in the bacteria during protein synthesis. Metronidazole is a nitroimidazole derivative which shows bactericidal action by inhibiting DNA synthesis. Targets obligate gram positive and negative anaerobes **[3]**. *In-situ* gelling system is considered as one of the viable local drug-delivery systems because it has the ability to retain high drug levels in the gingival crevicular fluid for prolonged periods in order to achieve the desired clinical benefits **[4]**. These are preparations in which they involve sol-gel theory i.e., when administered at the target site, liquid preparation becomes as a gel due to change in the pH or temperature(external stimuli) at the site and helps to delay the release of drug. As, the phase transformation from sol to gel is critical to the success of *in-situ* gels in periodontitis treatment, numerous *in-situ* gels have shown the capability to achieve gelation in periodontal pockets for adhesion and retention such as thermo-sensitive gels, light-responsive gels, and pH-dependent gels. These systems use various polymers Carbopol, cellulose derivatives (pH dependent) & poloxamer, chitosan xyloglucan (temperature dependent).

Carbopol and HPMC comes under both pH and temperature dependent polymers **[4, 5]**. In the present research , the study involves the development of *in-situ* gel by using three different categories of antibacterial agents in combination to enhance the pharmacological action for the treatment periodontitis by determining their MIC's (individual & combinations) along with the application of DOE for optimization of polymer concentrations used in the preparation.

MATERIALS AND METHODS

Materials:

Ciprofloxacin(C), Doxycycline(D), Metronidazole(M), Carbopol934 from Loba Chemie Pvt.Ltd Mumbai, HPMC K100(Hydroxy Propyl Methyl Cellulose) from Merck, Sodium hydroxide from Fischer scientific Mumbai, Potassium dihydrogen phosphate and Hydrochloric acid from Thermo Fischer Scientific Mumbai, Sodium chloride from Fischer scientific Mumbai, Distilled water and all other excipients used were of analytical grade**.**

Methods:

Organoleptic studies: These studies include the identification of colour, odour, physical appearance of APIs and excipients.

Compatibility Studies:

These studies were performed to check the compatibility among the API's and major excipients used to develop the formulation. Mainly FTIR was used in this research to determine the compatibility.

FTIR: In this, an IR spectrum was obtained by plotting the Wave Number on x-axis and Transmittance on the y-axis. APIs and excipients were mixed in the1:1 ratio. The physical mixtures were further mixed with KBr of IR grade in the ratio of 1:2 and compressed into pellets using pellet presser. Those pellets were then scanned using FTIR spectrophotometer (Bruker, Alpha II) in the range of 4000cm^{-1} to 500 cm^{-1}. The FTIR spectra of physical mixtures obtained were compared with that of FTIR spectra of the standard **[6]**.

Determination of MIC:

Minimum inhibitory concentration (MIC) was defined as the lowest antimicrobial concentration that inhibits the visible growth of a microorganism after an overnight incubation (expressed in mg/L or µg/mL). This can be determined by dilution methods or by gradient methods. This research involves one of the dilution methods **[7]**.

Broth Dilution method: MIC of individual drugs and their combinations (A, B, C, D) were determined by using Broth Dilution technique. In this method, the nutrient broth was prepared first and then transferred equal volumes of broth into each sterile test tube(15 to 20) and the 1st test tube was added with drug solution in the concentration of 1mg/ml followed by serial dilutions then inoculated with selected microorganism

(Pseudomonas) in the aseptic room under laminar air flow system and were kept in the incubator (Kemi) for incubation up to 24hrs at 37^{o}C and observed for the visual turbidity which indicates the growth and clear solution indicates inhibition of microorganisms **[8, 9]**.

Preparation of *In-situ* gel: *In-situ* gel was prepared by using pH triggering systems using Dispersion method having pH responsive polymer Carbopol-934 and the viscosity enhancing polymer HPMCK100. These polymers and other excipients (preservatives, pH adjusters) were added to the suitable solvent with continuous stirring under mechanical stirrer and kept aside for overnight. Selection of solvent/diluent was based on various trials using different buffers such as pH6.8 phosphate buffer, 0.01NHCl, DMSO, normal saline, 0.1NHCl, distilled water: DMSO etc. The drug solution was then dissolved in the selected solvent and added to the polymer solution under continuous stirring till uniform solution was obtained.

Evaluation of formulations:

Physical appearance: The prepared formulations were examined for their appearance and clarity by visually.

pH: The pH of the formulations was measured by using digital pH meter (Lab India). The pH range of the oral cavity lies between 6.2-7.6.

Gelation time: The time taken for the prepared formulation to transfer into the gel from its solution form was evaluated. The formulation (pH≤ 7) was added to the pH7.4 buffer and examined the time for gelling visually.

Viscosity: Viscosity is an important parameter which gives the flow properties for the prepared formulations. It was measured by using Brookfield viscometer of appropriate spindle and rpm. It was expressed in cps.

S**yringeability:** All the developed formulations were tested for syringeability by using 21-gauge needle and the time was noted after the emptying of contents from the syringe by continuous application of constant force.

Design of experiment (DoE): Full Factorial design $\mathbf{3^2}$ was applied for the prepared formulations by taking the gelation time and syringeability as responses (dependent variables) 1&2 using Design Expert software. 3^2 indicates that, the concentrations were optimized at 3 levels i.e., low, medium & high whereas power 2 implies the two independent variables (Carbopol 934 & HPMCK100). By using this experimental design, the interaction between the dependent and independent variables can be established.

Anti-microbial assay: Antimicrobial activity was determined by agar diffusion test employing cup plate technique for the

optimized formulation. Prepared and sterilized agar medium was poured into petri dishes which were previously seeded with selected microorganisms Pseudomonas aeruginosa (MTCC code:1035) and Staphylococcus aureus (MTCC code:737) under aseptic conditions. The solutions of minimum inhibitory concentration of standard/control and optimized formulation containing selected combination of drugs (test) were prepared (Mandal S *et al*., 2012) **[10]** and observed for the zone of inhibition after 24hrs of incubation at 37^0C in the incubator.

RESULTS & DISCUSSION

The following graphs and figures were the results obtained after performing the mentioned compatibility and evaluation methods. The results shown in the above graphs **(Figure 1 & 2)** reveals the compatibility of the API's and the excipients. FT-IR spectrum of triple drug combination shows the principal peaks of absorption at 3530 which gives the O-H stretch(alcohol), 3379 indicates N-H stretch (aliphatic amine), 3220 & 3099 implies O-H stretch (alcohol, carboxylic acid), 2925 & 2845 shows C-H stretch(alkane), 1100 - 1000 indicates C-F (halo compound), 817&744 gives C-Cl stretch (halo compound), 2145 CΞN stretch (cyano group present in metronidazole),1900-1850 C-H bending (aromatic compound). The drug combination when added with the excipients namely Carbopol 934 and HPMC K100 did not show the deviation from the observed peaks. So, it can be said that the drugs and excipients were compatible with each other without having interactions.

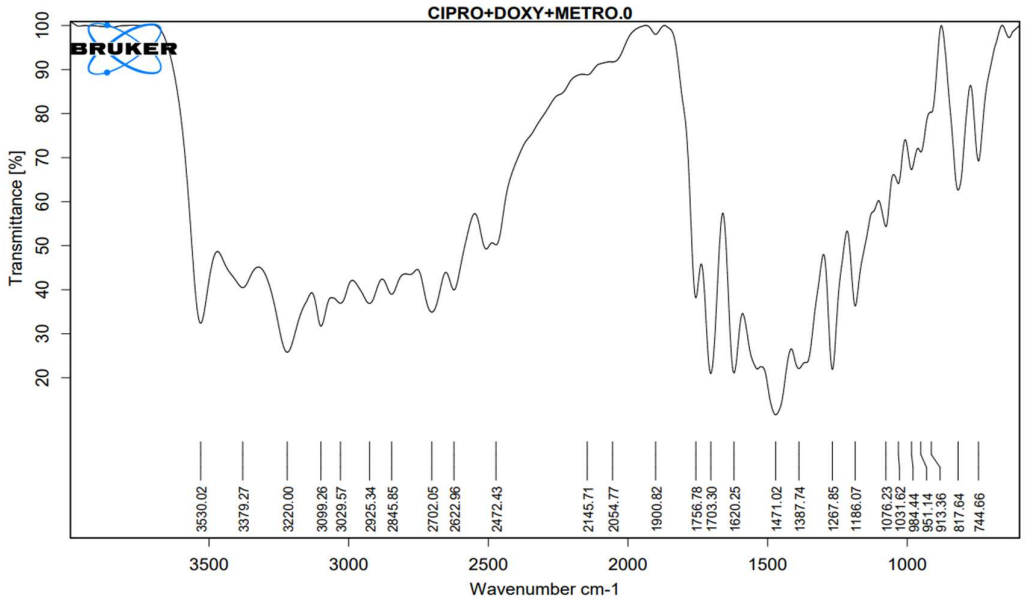

Figure 1: FT-IR graph of drug combination(Ciprofloxacin, Doxycycline, Metronidazole)

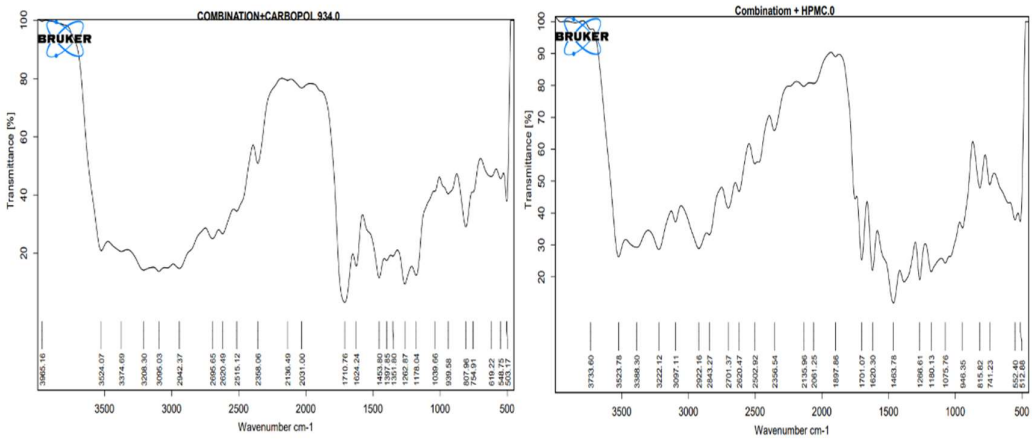

Figure 2: FT-IR graphs of physical mixtures

The MIC of the individual drugs ciprofloxacin, doxycycline, metronidazole was determined by observing for the turbidity (growth). Their MICs were observed as 15.625mg/L, 32mg/L, 62.5mg/L respectively. For the combinations A,B,C,D obtained MICs were 3.9mg/L, 62.5mg/L, 7.81mg/L, 1.95mg/L respectively. From the obtained results, combination D showed best MIC compared to other combinations i.e., the microbial growth inhibits at the lowest concentration. By this, the main point drawn was, combination of three drugs can be selected for the formulation of *in-situ* gel which shows synergistic effect over the periodontal pathogens. Visual examination of prepared formulations confirmed that they were free from particulate matter and appeared as clear pale yellow-coloured solutions. Prepared formulations were within the acceptable pH range (6.5-7.0) thus reduces the oral discomfort and when administered into the periodontal pockets (7.4) transformed into gelling systems. The gelation time of the formulations was found to be less than ≤2 minutes which indicates the optimum time for the conversion of solution to its gel form when administered into the periodontal pocket and avoids leakage from the site. Viscosity of the formulations were reported such that before gelation the viscosity is less and after gelation the viscosity increases gradually. The viscosity of the formulations was in the range of 29065.25±1.35 to 29815.70±1.2 after gelling. The *in-situ* gels using carbopol forms the low viscosity gels compared to other polymers such as sodium alginate etc. This effects the drug release time from the gelling systems. Hence optimization of the concentrations and the medium for the dispersion of polymers needs to be done carefully. The formulations were shown in the **Table 2** implies that the syringe ability was within the range up to 1 minute so that it can easily be injected into the periodontal pockets directly and reduces the patient's incompliance towards the treatment.

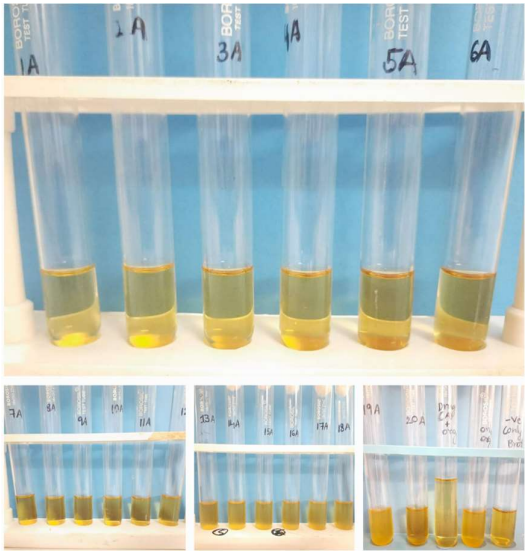

Figure 3: MIC of combination A(C+D)

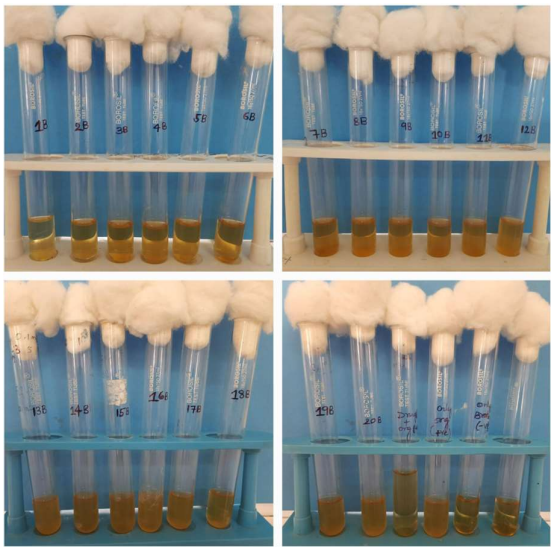

Figure 4: MIC of combination B(C+M)

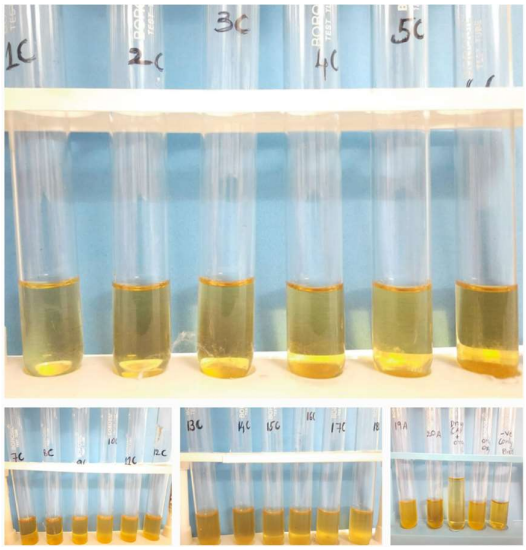

Figure 5: MIC of combination C(D+M)

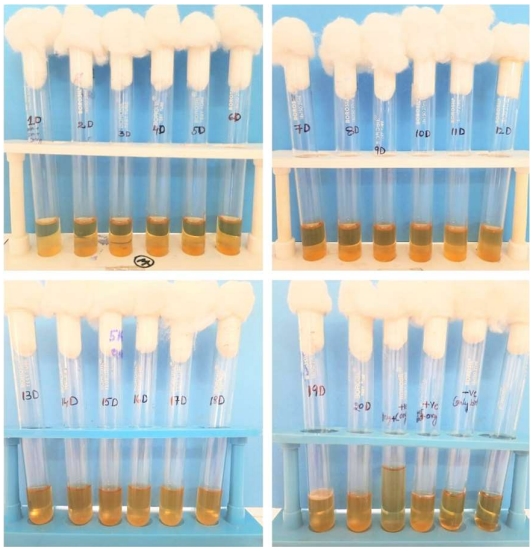

Figure 6: MIC of combination D(C+D+M)

Table 1: Evaluation of *In-situ* gel; CPS means centipoise

Formulation code	Physical appearance	Range of pH at 25^{0}C	Viscosity (CPS)
F1-F9	Clear, pale yellowish	6.5±0.56	29065.25±1.35 to 29815.70±1.2

Table 2: Application of DOE

Formulation code (F)	Carbopol934 concentration (%w/v)	HPMCK100 concentration (%w/v)	Response1 (sec)	Response2 (sec)
F1	-1	-1	68	9.92
F2	+1	0	53	20.52
F3	0	-1	64	11.7
F4	-1	+1	57	17.13
F5	+1	-1	60	16.48
F6	-1	0	62	13.52
F7	0	+1	50	21.49
F8	0	0	58	18.37
F9	+1	+1	46	24.62

R1=Gelation time, R2=syringeability

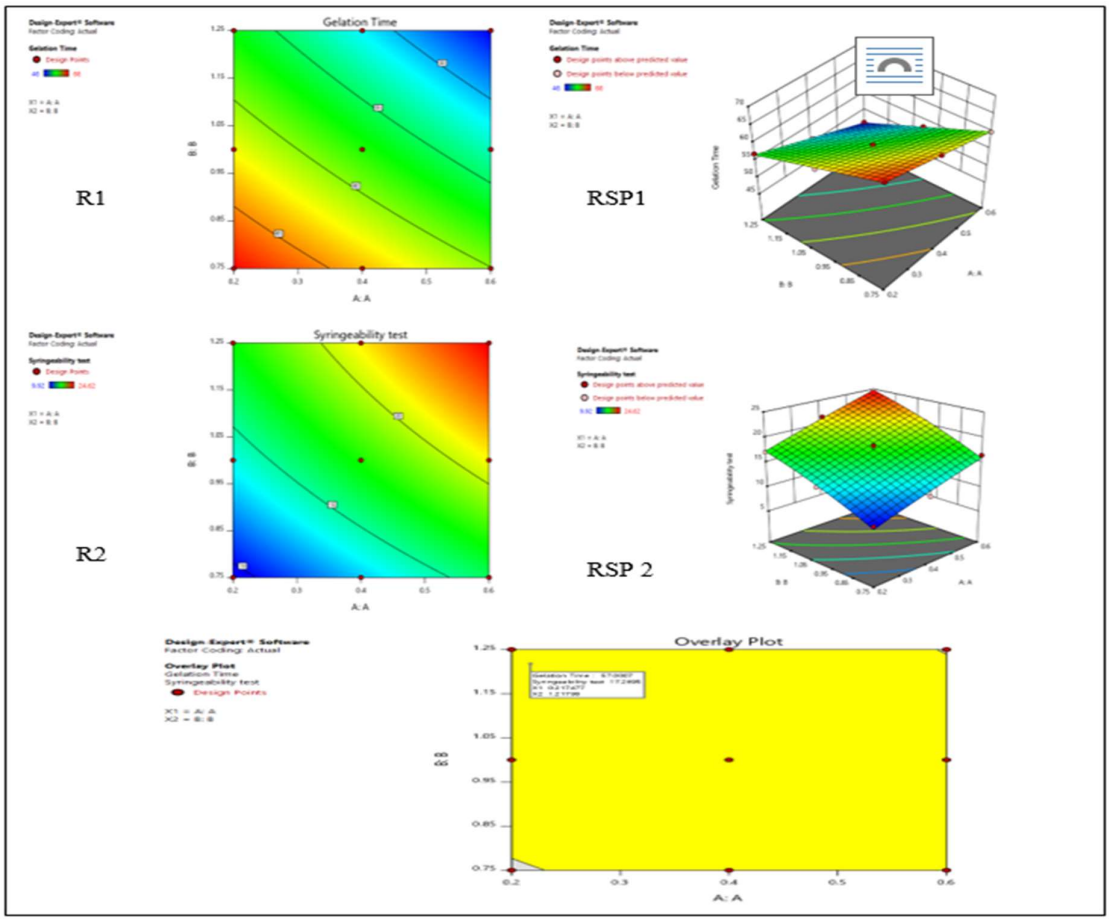

Figure 7: DoE graphs
R1=Contour plot of gelation time; RSP1=Response surface plot of gelation time
R2=Contour plot of syringeability; RSP2=Response surface of plot of syringeability

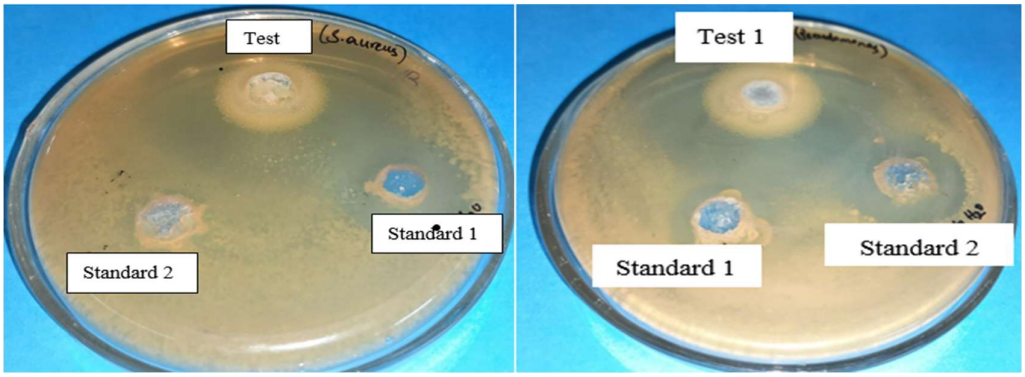

Figure 8: Anti-microbial activity against S. aureus and Pseudomonas species

From **Figure 7** the syringeability time recorded for the gel formulations were found to be within the range of 9.92± 0.57 to 24.62 ± 0.51 Sec. The equation derived for R^2 by the best fit mathematical model was R2 = -14.24333+15.99167A+33.46000B+4.65000 A*B-3.87500A^2-9.28000B^2. The predicted R^2 of 0.8426 was in reasonable agreement with the adjusted R^2 of 0.9575. The equation was found to be significant, as the model F value was found to be 37.02 and the model P value was <0.0001. The response surface

plot and contour plot for R2 which shows a vital linear increasing trend in syringeability time with an increase in factors A and B. This could be accredited to the fact that an increase in the viscosity of the system results in enhanced resistance to flow. The formulations were subjected to their respective gelation times, which resulted in quick gelation in less than 68 ± 1 sec. The equation derived by the best fit mathematical model for response R1 was R1 = +85.4444-15.0000A-14.66667B-15.00000A*B+8.33333A^2-2.66667B^2. The equation shows vital decrease in gelation time with an increase in factors A and B. Decrease in gelation time with increase in factors A and B could be due to the increased viscosity of the system with increasing levels of factors. All the formulations showed gelation time in the range of 46± 2 to 68± 1 sec. ANOVA of the equation suggested the model F value 537.96 and P value < 0.0001, indicating that the model is significant. The predicted R-squared of 0.9605 was also in reasonable concurrence with the adjusted R-squared of 0.9895.

Check point analysis and optimization of design:

A numerical optimization technique using the desirability function and a graphical optimization technique by overlay plot was employed for the optimization of all the responses with respect to individual constraint. The optimized formulation (F10) was acquired by applying constraints of R1=57 sec, R2 = 17 sec on the responses. These constraints were common for all the formulations. Recommended concentrations of the factors were calculated by DoE from the overlay plot which has the highest desirability, near to 1.0. The optimum values of the selected variables obtained using DoE were 0.2 and 1.2 for factor A and B respectively. The results in the **Figure 8** shown that the optimized formulation was tested for its antimicrobial activity and the results showed the largest zone of inhibition compared to the standard ones. Thus, the formulation prepared was suitable to avoid the formation of biofilm on teeth.

CONCLUSION:

This research work helps to bring out the compatibility between the combination of drugs and with the excipients. The MIC reveals the best combination is D which inhibits microorganisms at very lower concentrations. All the formulations prepared were shown good syringeability and gelation time along with viscosity which are the most important parameters for *in-situ* gels. With the application of DoE, the formulation F10 was suggested as a new optimized formula. The antimicrobial study of the optimized formulation (F10) shows its strength against microbial attack. Furthermore, the method development should be done to know the drug release

kinetics followed by clinical trials with the developed formulation which are the main criteria for the future prospective.

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