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Design and development of antimicrobial wafers for chronic wound healing

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ABSTRACT

The present study aims to develop the antimicrobial wafers for chronic wound healing by lyophilisation technique. Cohesive, non-friable, porous, disc shape wafers were successfully produced with sodium alginate, guar gum (1:1 ratio) and Neomycin as an antimicrobial agent. FT–IR studies confirmed that there was no chemical interaction between the pure drug and used excipients. SEM studies showed that the formulation contains porous network and spongy like structure. Viscosity, tensile strength, drug content, water uptake study, hydration study, antimicrobial study, in vitro study, in vivo study on excision rat model of formulations having various concentrations of polymers was evaluated. Formulation F5 containing neomycin sulphate having viscosity of 12.89±0.93Pa.s, tensile strength 0.25±0.29 N/m2 and drug content 99.62 ±0.006%. Wate r uptake studies showed 715.6±52.37 % for control a nd 509.7±67.23% for drug loaded wafer. Hydration studi es showed for formulation F5 was 97.91±0.02 % and a lso exhibited the most desirable wound dressing characteristics with the highest in vitro drug release. Formulation F5 exhibits greatest inhibition against both gram positive and gram negative bacteria. Formulation F5 showed better wound healing compared to Neomycin Sulphate cream. From the study it was concluded that antimicrobial wafers was successfully prepared for use in chronic wounds infected with bacteria.

Keywords: Antimicrobials; wafers; lyophilized; neomycin

INTRODUCTION

Infectious diseases can be the result of the colonization of the body by various microbes. The principal barrier against microbial invasion is the skin. It constantly interacts with the external environment and is colonized with a diverse population of microbes [1]. Some dressings, such as hydrocolloids can cause infections, and open wounds have a high risk of infection (i.e. up to 6.4%) when it is treated with primary gauze, which lead to an average hospital stay of 10.6 days [2]. Many commercial dressings are available that contain silver, such as Aquacel® hydrofiber, Acticot® absorbent, Urgocell® silver and PolyMem® silver [3].

The use of natural polymers, also known as biopolymers, in conjunction with the application of the freeze drying process in formulation of dressings possessing a high absorbency, led to innovative technologies in the field of wound management [4]. Lyophilized wafers as a topical drug delivery system for the treatment and management of a wide range and types of non-healing exudative wounds have been developed [5]. The philosophy behind the design of wafers relies on the ability of a three-dimensional, porous and regular matrix structure to turn into high viscosity, physical gels [6]. The technology which supports the formulation of such structures is a sophisticated process, in terms of temperature and pressure, known as freeze drying or lyophilisation [7]. In other words, wafers are shape-adaptive or mouldable formulations as they take shape from the containers where the gels are placed prior to lyophilisation [8]. The term ‘wafer’ originated as the initial lyophilized polymers were relatively thin and porous. Lyophilized wafers can be suitable formulations for topical drug delivery on every moist biological membrane as

they can be self-adhesive. Adhesion at the site of delivery can provide targeted delivery of the appropriate therapeutic compounds [9].

Porosity and hydrophilicity have been considered as the most dominant parameters which govern the swelling behaviour of drug-loaded, hydrophilic polymers as the drug is being released as the polymer swells [10]. Lyophilized wafers can be considered as ideal carriers of therapeutic agents, including antimicrobials and therefore are thought to be efficient systems to deliver antimicrobial treatment on a wide range of suppurating chronic wounds [11]. Neomycin sulphate is an aminoglycoside antimicrobial that is commonly used to treat infections of the skin, mucous membranes, wounds and burns [12]. Antimicrobials, such as neomycin which is broad spectrum antimicrobials and commonly used to treat infected wounds [13]. Neomycin sulphate is freely soluble in water and can easily incorporate into water-based polymers to form freeze-dried wafers [14]. Neomycin sulphate has the ability to provide bactericidal effects at minute concentrations and is not toxic toward human cells of gram-positive and gram-negative microorganisms [15].

MATERIALS AND METHODS

2.1. Materials

Neomycin sulphate was obtained as gift sample from B. M Pharma and chemicals, Hyderabad, India. Sodium alginate and guar gum was purchased from Loba chemicals Mumbai, India. All other chemicals used were of analytical grade and obtained commercially.

2.2. Preparation of Antimicrobial wafers

The polymers were taken separately in the ratio 1:1 and then with required quantity of water it was converted into gel using water to dissolve the polymers and this mixture was kept aside for few minutes to eliminate the air bubbles. To this, Neomycin sulphate was added and mixed thoroughly, poured in the petri plates and kept for primary freeze drying for -75 °C and then subjected to secondary freeze drying where the product is kept under vacuum for about -55°C for 25 h .The obtained product was dried and placed in a tightly closed container. The various compositions of antimicrobials were tabulated in **Table 1.**

2.3. Characterization of Antimicrobial Wafers

2.3.1. Fourier Transform Infrared spectroscopy

The FT-IR spectral measurements were taken at ambient temperature using a Shimadzu, Model 8033(USA), about 2g of the pure drug and prepared wafers were selected separately .Pure drug and the prepared crystals were dispersed in KBr powder and the pallets were made by applying 6 tons pressure .FT-IR spectra were obtained by powder by powder diffuse reflectance on FT-IR spectrophotometer.

2.3.2. Differential scanning calorimetry (DSC)

DSC measurements were performed by DSC 60, Shimadzu, differential scanning calorimeter with a thermal analyzer .Accurately weighed samples (about 1mg of Neomycin sulphate) were crimped using crimper in aluminium pans and heated under nitrogen flow (20ml/min) at a scanning rate of 10°C min^{-1} from 25°C to 250°C. An empty aluminium pan was used as reference.

2.3.3. Scanning electron microscopy

The Scanning electron microscopic (Joel-LV-5600, USA with magnification of 250X.Photographs were obtained to identify and confirm nature and morphological characteristics of the wafers.

2.4. Evaluation of antimicrobial wafers

2.4.1. Viscosity

The viscosity of the wafers was determined at 25°C using a viscometer (Brookfield viscometer) and viscosity was measured in Pa.s. The measurement of each formulation was done in triplicate and average values were calculated.

2.4.2. Tensile strength

Tensile strength of the films was determined with Universal strength testing machine. The sensitivity of the machine is 1 gm. It consists of two load cell grips; the lower one is fixed and the upper one is movable. The test film of specific size (4 x 1 cm^2) was fixed between these cell grips and force was gradually applied till the film breaks. The tensile strength of the film was taken directly from the dial reading in kilograms.

Tensile strength (N/cm^2) = Breaking force/ Area of cross section (1)

2.4.3. Drug content
Wafers (2 mg) was taken in a 100 ml volumetric flask, methanol was added to make up the volume. Drug concentration was determined by measuring the absorbance of solution at 315 nm using UV-Vis spectrophotometer (Shimadzu 1800, Japan).

2.4.4. Water uptake studies
The water uptake capacity of different lyophilized controls and drug loaded wafers when placed on top of a constantly hydrated cellulose membrane for 24 hours. Wafer weights were recorded prior to being placed on the membrane (W_0) and after 24 hour of water uptake (Wt), using an analytical balance, Water uptake (W_U) was calculated in percentage terms (%) using the simple formula:

$$W_U = (Wt/ W0) \times 100 \quad (2)$$

Where, Wt = weight of swollen wafer after 24 hours of hydration and W_0 = weight of lyophilized wafer at time zero.

2.4.5. Hydration studies
The hydration study was undertaken with the drug-free wafers only. Gelatin agar was prepared by dissolving 12 g of gelatin agar powder in 300 mL of distilled water (4% w/v of gelatin) at 60 °C, and the solution was stirred until it was completely dissolved. The hot gelatin solution was poured into individual petri dishes (diameter 100 mm) and allowed to cool at room temperature (25 ± 2 °C) overnight. The swelling properties and hydration testing of the wafers were carried out by placing the drug-free wafer with a known diameter at the centre of the petri dish on top of a gelatin agar surface, and the dish was covered with a lid. The wafer should absorb water from the agar and expand and increase in diameter. The wafer was left at room temperature (25 ± 2 °C) for 24 h. Grid paper was placed beneath the petri dish to accurately measure the expansion of wafer diameter. Three replicates were performed for every formulation to obtain the expansion ratio of each formulation. The expansion ratio was calculated using:

$$\text{Expansion ratio} = D_t/D_o \quad (3)$$

Where Do is the initial diameter of the wafer and D_t is the expansion diameter of the wafer 24 h after being placing on the gelatin agar Surface.

2.5. *In vitro* drug diffusion studies
The wafers were prepared and (approximately 25 mg) were immersed in a 50 mL of acetate buffer (pH 5.5). The samples were incubated at 37°C and stirred at 100 rpm. Sample (1 mL) were taken from the release medium at 60 minutes intervals and diluted to 5 mL with fresh buffer solution to assess the quantity of drug released at various time interval for 6 hrs. The samples were analyzed by UV- Spectroscopy at 315 nm.

2.6. Antimicrobial tests
Antimicrobial efficacy was measured using the disc diffusion method. *E. coli* (Gram negative) and *S. aureus* (Gram positive) bacteria were chosen for the study. Nutrient agar was used as the culture media, while Gelatin agar was used for inoculation and antimicrobial efficacy tests. The wafer containing Antimicrobials was punched into 6 mm diameter discs to resemble commercial antimicrobial discs. The positive control was prepared as a 6 mm filter paper disc impregnated with 0.5mg of antimicrobials, and a drug-free wafer was punched into 6 mm diameter disc as negative control. All the procedures followed aseptic techniques and were carried out in a Grade A clean room in a laminar flow cabinet. After 30 minutes the fresh overnight cultures of inoculums (100 µl) of culture were spread on to solidified nutrient agar plates. One standard antibiotic containing disc was placed in each plate. The cultured agar plates were incubated at 37 $^{\circ}$C for 24 h. The zone of inhibition was investigated after incubation period of 24 hours.

2.7. *In vivo* studies
Healthy wistar rats weighing between 170-200 g was used for the study. The animals were anesthetized. After anaesthesia, the dorsal hair was carefully shaved using an electric clipper. The wound site will have marked with a sterile circular (2.2 cm diameter) disc, stain with methylene blue. A full-skin thickness wound of 6 mm diameter will be created at the marked site by excising the skin flap with sterile scissors and forceps, then the wafers with neomycin sulphate should be given in different concentration. The wound healing activity will be observed at 0,7th and 14th day of the study. The wound healing rate was calculated as a percentage of the size of the unhealed wound area compared with the size of the initial dorsal wound area.

2.8. Stability studies

Optimized formulations of freeze dried wafers was selected for stability studies .Short term stability studies were carried out for a period of three months as per ICH guidelines.

Table 1. Composition of antimicrobial wafer formulation

Sl. no	Ingredients (gm)	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	Neomycin sulphate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2	Sodium alginate	0.1	0.5	1	1.5	2	2.5	3	3.5	4
3	Guar gum	0.1	0.5	1	1.5	2	2.5	3	3.5	4
4	Glycerin	2	2	2	2	2	2	2	2	2
5	Methyl Paraben	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
6	Propyl Paraben	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
7	Water q.s	100	100	100	100	100	100	100	100	100

Table 2. Viscosities of various formulations

Batch no	Viscosity (Pa. S) (Mean ± SD)*
F1 (0.1%)	3.79 ± 0.30
F2 (0.5%)	4.09 ± 0.34
F3 (1.0%)	6.48 ± 0.33
F4 (1.5)	7.40 ± 0.55
F5 (2%)	12.89±0.93
F6 (2.5%)	13.17±0.44
F7 (3%)	13.86±0.13
F8 (3.5%)	14.08±0.22
F9 (4%)	14.83±0.33

**Standard deviation n = 3*

Table 3. Tensile strength of various formulations

Batch no	Observed value (N/m^2) (Mean ± SD)*
F1	0.04±0.16
F2	0.05±0.03
F3	0.07±0.03
F4	0.12±0.03
F5	0.25±0.29
F6	0.81±0.03
F7	1.39±0.36
F8	1.54±0.04
F9	1.98±0.04

**Standard deviation n = 3*

Table 4. Drug content of wafer formulation

Batch no	%Drug content (Mean ± SD)*
F1	98.88±0.0059
F2	98.50±0.0052
F3	97.38±0.0058
F4	99.12±0.0153
F5	99.62±0.0068
F6	97.34±0.066
F7	99.25±0.125
F8	98.13±0.0058
F9	97.28±0.0062

**Standard deviation n = 3*

Table 5. Water Uptake capacity of Control and Drug loaded wafers

Sl. No.	Batch No.	Control		Drug loaded Wafers	
		W1	W2	W1	W2
1	F1	2.894	7.786	3.036	6.268
2	F2	2.866	8.704	3.008	6.873
3	F3	2.873	9.092	3.015	7.147
4	F4	2.812	9.785	2.954	7.893
5	F5	2.804	9.96	2.946	8.043
6	F6	2.842	9.78	2.984	7.982
7	F7	2.881	9.08	3.023	7.447
8	F8	2.856	8.28	2.998	6.886
9	F9	2.891	8.05	3.033	6.028

Table 6. Hydration studies of various formulation

Batch no	Percentage Hydration (Mean ± SD)*
F1	92.14±0.053
F2	92.89±0.032
F3	93.47±0.014
F4	95.68±0.052
F5	97.91±0.024
F6	93.48±0.068
F7	92.72±0.042
F8	91.83±0.044
F9	90.08±0.016

Table 7. Zone of inhibition data

Codes of wafers	Diameter of zone of inhibition (cm) (Mean ± SD)*	
	E. coli	***S. Aureus***
Neomycin sulphate cream	2.20±0.08	2.22±0.060
F5 (Blank)	1.5±0.072	1.52±0.040
F5 (drug loaded)	2.12±0.08	2.02±0.055

Table 8. Stability data of prepared antimicrobial wafers

Batch No.	Storage condition	Drug Content				Physical appearance
		0 month	1st month	2nd month	3rd month	
F5	30± 2°C /RH 65± 5%	99.57±0.11	98.27±0.28	97.18±0.16	98.23±0.16	**No change**
	40± 2°C /RH 75± 5%	99.41±0.67	98.96±1.1	98.76±0.74	98.47±0.21	**No change**

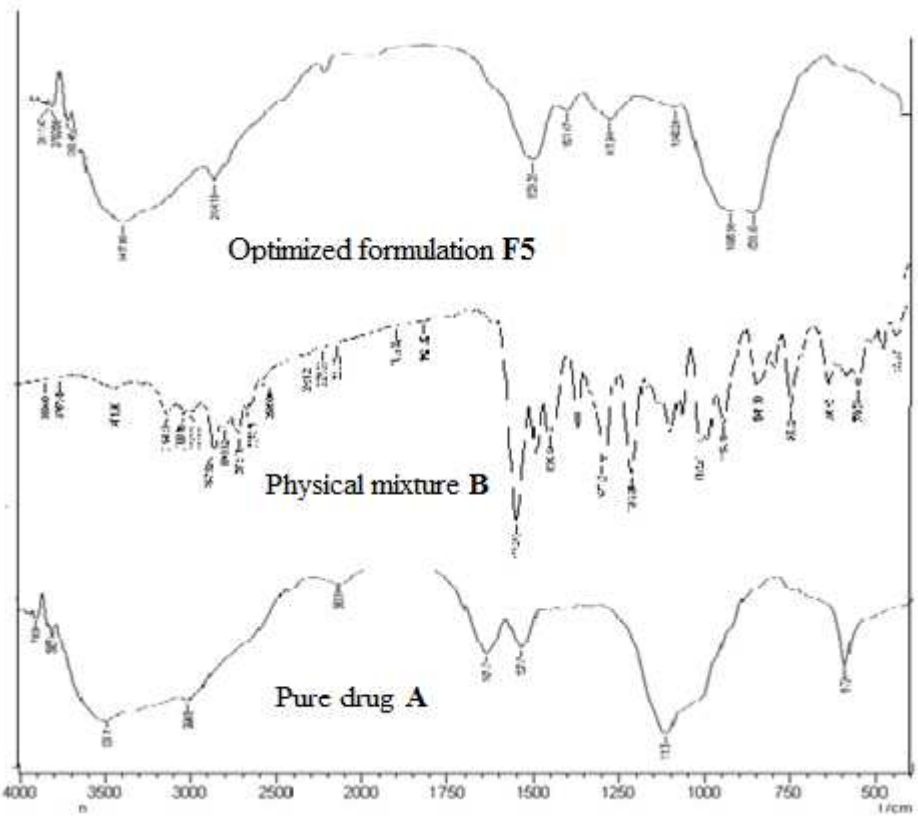

Figure 1: FT-IR spectra of Pure Drug (A), Physical mixture (B) and optimized formulation (F5)

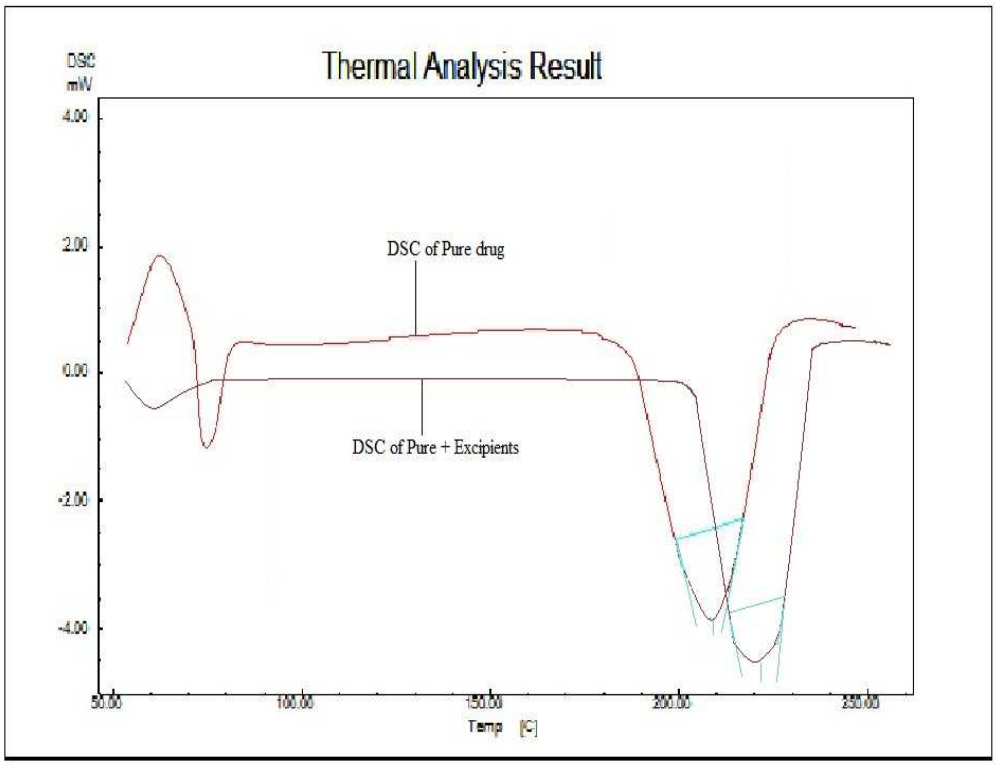

Figure 2: DSC Thermogram of Pure drug and drug with excipients

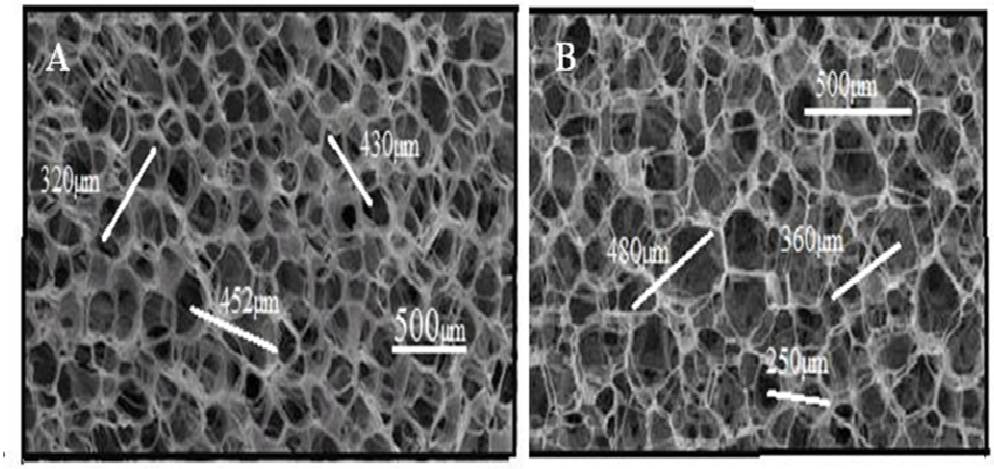

Figure 3: SEM photographs of drug free wafers (A) and drug loaded wafers (B)

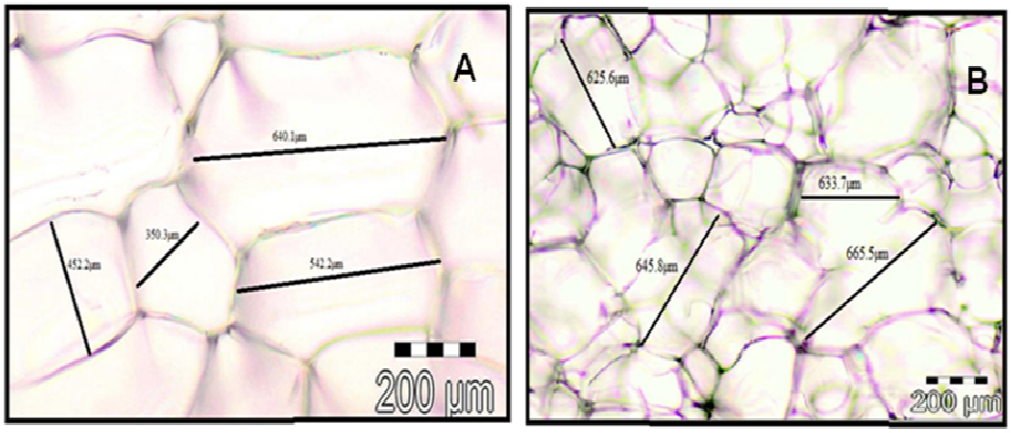

Figure 4: Microscopic images of drug free wafers (A) and drug loaded wafers (B)

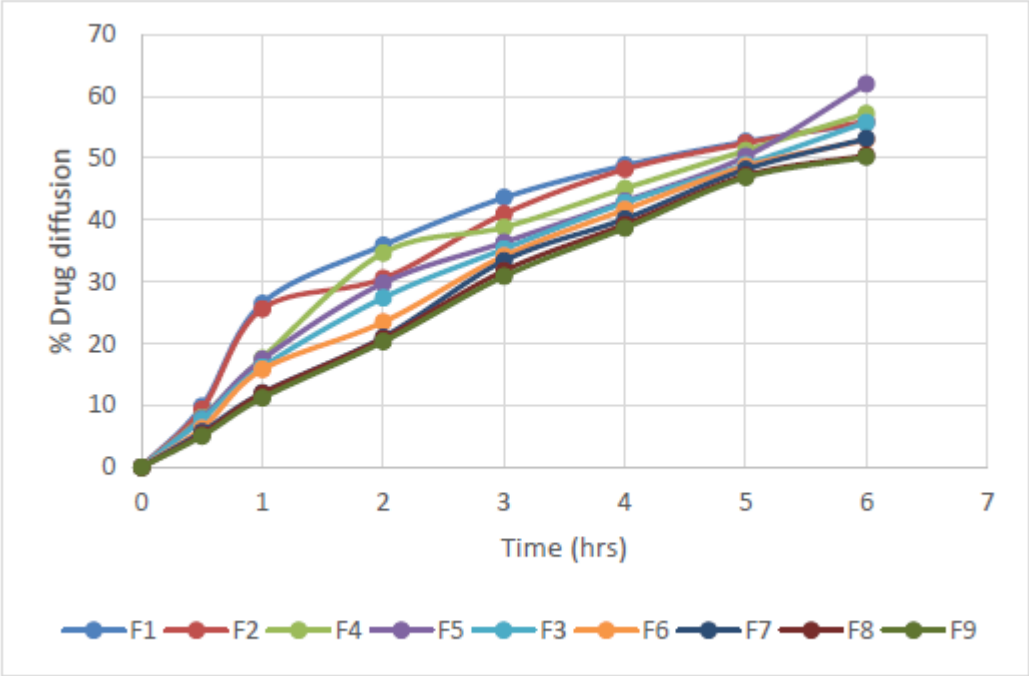

Figure 5: Percentage diffusion studies of the batch F1-F9

Figure 6. Decrease in the size of the wound area

RESULTS AND DISCUSSION

3.1. Preparation of Antimicrobial wafers

Weighed polymer powders were added gradually into distilled water and mixed for at least 3 h using a magnetic stirrer. For sodium alginate, stirring was carried out at room temperature (25 ± 1 ºC). Guar gum gels were heated up to 45 ºC and 60 ºC, respectively. It was important that all powder was dissolved and a homogenous gel was formed before the mixture was removed from stirring. The hydrogels were left to stand for another 6 h to eliminate bubbles. Then, 7 g of the hydrogel was poured into a 60 ml jar with a 40 mm diameter. The hydrogel was allowed to cool to room temperature in the jar. Then, the jars were placed in a - 80 ºC freezer for the next 24 h. For the preparation of gels containing antimicrobials, the antimicrobials were dissolved in distilled water before the polymer powders were added. Each wafer formulation was coded based on the wafer polymer and the antimicrobials contained. Wafer liophillization involved cooling the gel to -80 ºC and then heating them in a series of thermal ramps to room temperature (20 ºC) under reduced pressure from 1 atm to 0.001 mBar. The entire freeze-drying process took place over 26 h.

3.2. FT-IR Spectroscopic Analysis

The characteristic peaks of pure drug were compared with the peaks of physical mixture and freeze dried product. There were no chemical interactions of the drug with guar gum and alginate. It can be concluded that the characterization peaks of pure drug were unaltered by the excipients and the method used to prepare wafers **(Figure 1)**.

3.3. DSC studies

DSC thermo grams showed a melting endothermic peak of Neomycin at 209°C. The DSC curve of mixture of drug and excipients showed a melting point at 225.68°C. The thermo gram of the drug and the freeze dried wafer are shown in **Figure 2.**

3.4. Scanning Electron Microscopy (SEM)

The scanning electron microscopy (SEM) studies were carried out to investigate the morphology of the drug free wafers and the drug loaded wafers **(Figure 3)**. The morphology of the wafer was observed using a microscope (Olympus BX41) and was captured using a camera. The wafer was cut into thin layers using a razor blade and placed onto the glass slide and carefully covered with a cover slip. The porous structures of wafers were observed

under 10x magnification. SEM images showing the Porous structure of the wafer formulation .The average diameter of the wafer was found to be 430µm. Microscopic images of drug loaded wafers display an interconnecting, porous network and a spongy-like structure **(Figure 4)**.

3.5. Viscosity

The viscosity of the formulations wafers was determined at 25°C by using Brookfield viscometer with spindle no. S-96 at 1 rpm and viscosity was measured in Pa.s. The measurement of each formulation was done in triplicate and average values are calculated. Viscosity of the formulations mainly influenced by concentration of the polymer. The concentration of the polymer varied from 0.1% to 4%. From the obtained data it was found that the viscosity of the formulations increases as the polymer concentration increases. The viscosity of the formulations was found to be in the range of 3.79 Pa.s to 14.08 Pa.s **(Table 2)**.

3.6. Tensile strength

Tensile strength test was carried out for the wafers of various concentration. The tensile strength of F5 formulation was found to have optimum tensile strength (**Table 3)**. From the obtained data it can be concluded that as the concentration of guar gum increases the tensile strength also increases up to 2% (0.25 N/m^2) which complies with the control wafer (0.26 N/m^2). Tensile strength more then 2% makes the wafer hard and brittle.

3.7. Drug Content

Drug contents in the prepared wafers were ranging from 97.28 to 99.12 % and lying within Pharmacopeial limit (**Table 4)**.

3.8. Water uptake test

Water uptake capacity (WUC) of lyophilized wafers, varied from 323.2±59.47 to 509.7±67.23% these result suggests that lyophilized wafers can absorb and retain a substantial amount of water **(Table 5)**. The ability to absorb water will depend on the higher inherent hygroscopicity of polymer. But as the concentration increases behind certain level the water uptake capacity was decreased. The decreased capacity to retain water is due to altered properties of the gels mediated from the drug-polymer interactions.

3.9. Hydration studies

The wafer discs were hydrated slowly into a gel form and expanded on a gelatin agar surface. F9 showed least hydration 90.08±0.016%, where F5 showed highest 97.91±0.024 % of Hydration. Initially it was found that the hydration value increases along with concentration of the polymer, then it was start decreasing **(Table 6)**. The pore size of wafer was decreases at higher concentration of polymer and hence wafer was less hydrated at higher concentration.

3.10. Diffusion Studies

Diffusion studies was carried out for various formulation of wafers. From the In-vitro drug diffusion data it was found that, the drug diffusion gradually decreased from F1 to F9 due to increase in the concentration of polymer. The sodium alginate and Guar gum retards the release of the drug from the wafer **(Figure 5)**.

3.11. Antimicrobial test

Antimicrobial test was carried out on gram negative and gram positive organisms *E.coli* and *S. aureus* by diffusion method and for the Neomycin sulphate cream using well plate method **(Table 7)**.

3.12. *In vivo* studies

In vivo studies carried out using wistar rat for 0, 7 and 14 day showed highest decrease in the diameter of the wound treated with drug loaded wafers compared with Neomycin cream **(Figure 6)**.

3.13. Stability studies

The aim of stability studies is to predict the shelf life of a product by accelerating the rate of decomposition, preferably by increasing the temperature and RH. The wafer F5 was selected for stability study. The drug contents observations of all formulations after 3 months were not significantly affected by accelerated stability conditions; indicating chemical stability over a period of 3 months **(Table 8)**.

CONCLUSION

Drug loaded antimicrobial wafers were successfully prepared using lyophilisation technique. FT-IR spectra of antimicrobial wafers exhibit no significant shift in the peaks, which indicate the no interactions between the drug and excipients. DSC thermograms showed chemical compatibility between drug and excipients. SEM images

showed that the wafers prepared were porous in nature. It was found that as the concentration of the polymers increases the viscosity of the formulations increased. The Tensile strength of the optimized formulation was found to be 0.25±0(N/m^2). Drug content of all the formulations was were ranging from 97.28 to 99.12 % and lying within Pharmacopeial limit and ensures dose uniformity. It was observed that F5 released maximum amount of drug over a period of 6 hours compared to other formulations. Antimicrobial studies showed the zone of inhibition of F5 formulation was almost close compared to Neomycin sulphate cream. Based on the results obtained it was observed that drug loaded wafers showed faster wound healing compared to Neomycin sulphate cream.

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