4.0 CFE LOADED HYDROGEL SHEET: PREPARATION, OPTIMIZATION AND CHARACTERIZATION

Hydrogel is a three dimensional network of hydrophilic polymers. Hydrogels consist of swellable hydrophilic materials such as starch, cellulose; synthetic polymers like polyvinyl alcohol, polyacrylic acid, polyvinyl pyrrolidone, poly(methacrylates) or other plant- or animal – derived polysaccharides and contain up to 96% water (1). Hydrogels are capable of absorbing large volumes of water because of the presence of hydrophilic chains, which allow them to swell extensively without changing their gelatinous nature (2). They can be used on dry, sloughy, or necrotic wound (3). They are one of the most promising materials for biomedical applications, having several advantages for wound dressing, contact lenses, drug delivery systems, etc (4). These wide applications of hydrogels are due to their biocompatibility with blood, body fluids, and tissue. Ionizing radiation is recognized as a very suitable tool for the formation of hydrogels due to easy process control, possibility of joining hydrogel formation and sterilization in one step and finally, it is not necessary to add any chemical initiators for crosslinking process (5).

CHAPTER 4: CFE-HYDROGEL SHEET

4.1 MATERIALS

Materials used in preparation and characterization of CFE-hydrogel sheet are mentioned in table 4.1

TABLE 4.11: List of materials used in CFE-hydrogel sheet preparation and characterization

Sr. no.	Material name	Source
01	Hydroglycolic extract of *Calendula officinalis* flower	Amine Pvt Ltd, (Gujarat, India)
02	Polyvinyl alcohol (Mw. 1,25,000-1,50,000)	Sigma-Aldrich (Germany)
03	Propyl paraben	Sigma-Aldrich (Germany)
04	Methyl paraben	Sigma-Aldrich (Germany)
05	Sodium carbonate	SD fine chemical (Mumbai, India).
06	Aluminum chloride	SD fine chemical (Mumbai, India).
07	Folin Ciocalteu's phenol reagent	SRL Pvt. Ltd. (Mumbai, India).
08	Gallic acid	SRL Pvt. Ltd. (Mumbai, India).
09	Carrageenan	SRL Pvt. Ltd. (Mumbai, India).
10	Quercetin dehydrate extrapure	SRL Pvt. Ltd. (Mumbai, India).
11	2,2-Diphenyl-1- picrylhydrazyl	SRL Pvt. Ltd. (Mumbai, India).

4.2 PREPARATION AND OPTIMIZATION OF CFE LOADED HYDROGEL SHEET

Hydrogel sheet was prepared by gamma irradiation technique (6) which consisted of two steps which are shown in figure 4.1:

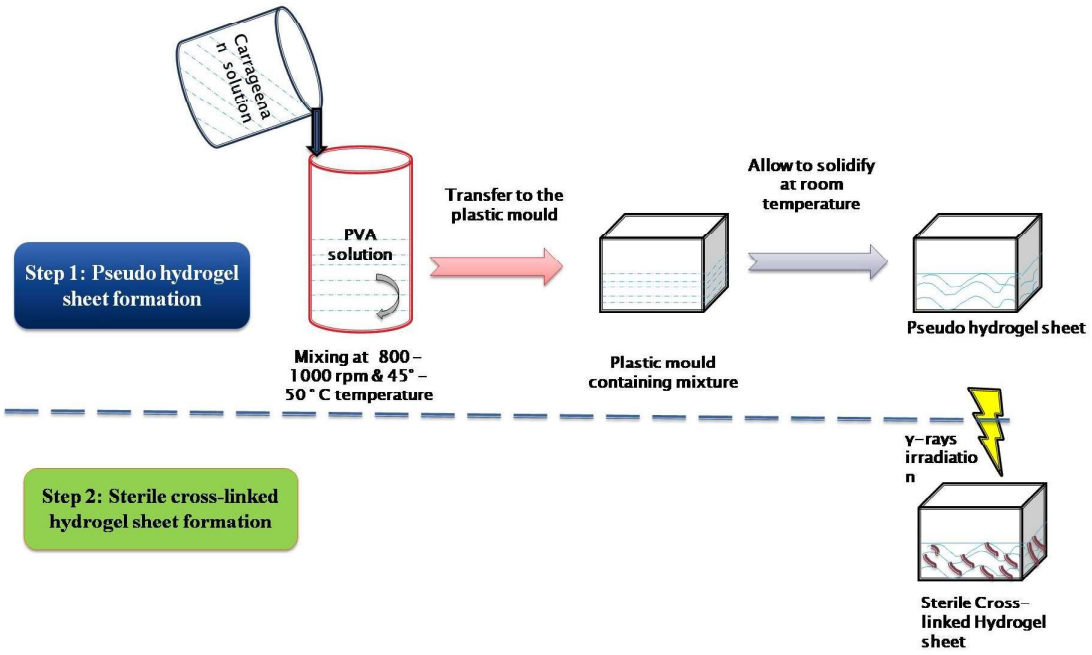

FIGURE 4.1: Preparation of CFE loaded PVA hydrogel sheet by γ-rays irradiation

Step-1: Preparation of pseudo-hydrogel sheet:

1) In one beaker, polyvinyl alcohol (PVA), methyl paraben and propyl paraben were dissolved into hot distilled water (80° to 85° C) with stirring at 800-1000 rpm using overhead mechanical stirrer.
2) In another beaker, aqueous solution of carrageenan and hydroglycolic extract of *Calendula* flower (CFE) were prepared by dissolving into warm distilled water (45° to 50° C) with stirring (800 rpm to 1000 rpm) using overhead mechanical stirrer.
3) Both solutions were mixed at 45° - 50° C with stirring (400 – 500 rpm) for 10 min.

4) After complete homogenous mixing, resulting mixture was poured into anti-adherent moulds which were kept overnight at room temperature for solidification (pseudo-hydrogel sheet formation).

Step-2: Crosslinking and sterilization of pseudogel by irradiation:

5) After 24 hr, the pseudo-hydrogel containing plastic moulds were packed into pouch and sent for γ-rays irradiation which was carried out by γ-rays from a Co-60-source with a selected dose at dose rate of 9kGy/h for crosslinking and sterilization.

Preliminary experiments were carried out to find minimum and maximum concentration of PVA and carrageenan required for hydrogel sheet formation which are represented in table 4.2

TABLE 4.2: Composition of preliminary hydrogel sheet trials for screening of PVA content and carrageenan content to prepare hydrogel sheet

Batch Code	Composition			
	PVA conc. (%w/w)	Carrageenan conc. (%w/w)	CFE conc. (%w/w)	Water (%w/w)
CHS-1	2.0	1.0	2.0	Upto Quantity sufficient
CHS-2	4.0	1.0	2.0	
CHS-3	6.0	1.0	2.0	
CHS-4	8.0	1.0	2.0	
CHS-5	10.0	1.0	2.0	
CHS-6	12.0	1.0	2.0	
CHS-7	8.0	0.5	2.0	
CHS-8	8.0	0.75	2.0	
CHS-9	8.0	1.25	2.0	
CHS-10	8.0	1.5	2.0	

The prepared hydrogels were evaluated for physical parameters before and after gamma irradiation process. Further formulation optimization was carried out by Box-Behnken Design (BBD) to understand the effect of various formulation and process variables on hydrogel sheet properties. From preliminary experiments, the parameters (independent factors) that most significantly affected fluid absorption capacity, hardness and gel fraction of hydrogel sheet (dependent factors) were found to be

- Polyvinyl alcohol (PVA) content (%w/w),
- *Calendula* flower extract (CFE) content (%w/w),
- γ-rays irradiation dose (kGy) and
- Carrageenan content (%w/w)

The formulation was optimized by desirability criterion of good fluid absorptivity capacity and mechanical strength with more than 60% GF. BBD was selected for further optimization using design using Design Expert® 7.0 (Stat-Ease Inc., MN) which gave total 28 runs. The actual values for BBD are shown in Table 4.3.

TABLE 4.3 : Coded and actual levels of independent variables for BBD

Sr. no.	Independent variables	Code	Low value	High value
1	PVA content (%w/w)	A	8	10
2	Carrageenan content (%w/w)	B	0.75	1.25
3	Gamma Radiation dose (kGy)	C	25	45
4	CFE content (%w/w)	D	2	4

4.3 Characterization of CFE loaded PVA hydrogel sheet (CFE-hydrogel sheet)

4.3.1 MECHANICAL PROPERTIES: Hardness of CFE-hydrogel sheet was measured by using CT3 Texture analyzer (Brookfield Ametek, USA) (7). The sample (40 mm x 20mm x 10mm) was taken on sample holder. TA3/100 probe was compressed upto 4 mm distance into the hydrogel sheet sample and redrawn with 0.5 mm/s speed rate (Figure 4.2). Hardness was determined from the resultant force-time plot. The maximum force in the force-time plot represents the hardness of the hydrogel sheet (7).

FIGURE 4.2: Measurement of mechanical properties of CFE-Hydrogel sheet by CT3 Texture analyzer

4.3.2 FLUID ABSORPTION CAPACITY: Fluid absorption capacity of CFE-hydrogel sheet was found with simulated wound fluid (SWF, pH 7.4±0.2) to obtain realistic swelling behavior when it is used on wound site. SWF (7.4±0.2) was prepared by dissolving 0.68 g of NaCl, 0.22 g of KCl, 2.5 g of $NaHCO_3$, and 0.35 g of NaH_2PO_4 in 100 ml of deionized water. Hydrogel sheet sample (40 mm x 20 mm x 10 mm) was immersed into the SWF at room temperature (8).

Periodically, samples were taken out, excess fluid was removed using filter paper and weighed. Sample was kept in SWF until no increase in weight was observed. % Fluid absorption capacity (%A) was calculated by equation (4.1).

$$\% A = \frac{Wt - W0}{Wo} * 100 \quad \text{.....Equation (4.1)}$$

Where, Wt is the final weight of sample and Wo is the initial weight of sample

4.3.3 pH: The pH of CFE-hydrogel sheet was determined using digital pH meter (Lab India, India) at room temperature (9). About 10 g of sample was taken into glass beaker containing 50.0 ml of distilled water and covered with aluminum foil to prevent water loss during experiment. The beaker was kept in a water bath at 37°C for 72 hr. After 72 hr, distilled water containing sample was analyzed for pH (9).

4.3.4 GEL FRACTION: %Gel fraction (%GF) of CFE-hydrogel sheet was find out gravimetrically (10). One sample (10.0 gm) was dried to constant weight at 40° C without removal of water soluble part that was the initial weight of the dried sample (Wo). Second sample (10.0 g) was kept in distilled water at 40^{o} C for 72 hr to remove water soluble part from sample. After 72 hr, samples were dried to constant weight (W_f) at 40° C. The %GF of the samples was measured using equation (4.2)**.**

$$\%Gel\ Fraction = \frac{Wf}{Wo} * 100 \quad \text{.....Equation (4.2)}$$

Where, Wo is the initial weight of the dried hydrogel sheet and Wf is the final weight of dried hydrogel sheet after extraction in distilled water.

4.3.5 TOTAL POLYPHENOLIC CONTENT: *C*olorimetric method (11) was used for the determination of total polyphenolic conent of CFE-hydrogel sheet. Aqueous extract of hydrogel sheet were used for analysis. 1.0 ml of sample was taken into 10.0 mL amber colored volumetric flask. 2.0 ml of Folin Ciocalteu's phenol reagent (10% v/v) and 1.0 ml of Na_2CO_3 solution (20%, w/v) were added to flask. The mixture was mixed and

diluted upto 10.0 mL with distilled water and incubated for 30 min at room temperature. After 30 min, the absorbance of mixture was measured using UV-Visible spectrophotometer (Shimadzu, Japan) at 725 nm. The total polyphenolic content was expressed as mg gallic acid equivalent per gm of sample (mg GAE/g), and it was calculated using a calibration curve of a freshly prepared gallic acid solution (2-12 µg/mL) (12).

4.3.6 TOTAL FLAVONOID CONTENT: The flavonoid content of CFE-hydrogel sheet was determined spectrophotometrically (12). Aqueous extract of hydrogel sheet was used for analysis. 1.0 ml of sample was taken into 10.0 mL amber colored volumetric flask. 0.3 mL of Al_2Cl_3 solution (10% w/v), 0.3 mL of sodium nitrite solution (5% w/v) and 2.0 mL of 1.0 M NaOH solution were added to the flask. The mixture was mixed and diluted upto 10.0 mL with distilled water and incubated for 30 min at room temperature (12). Thereafter, solutions were analyzed using UV-Visible spectrophotometer (Shimadzu, Japan). Total flavonoid content was calculated using calibration curve of quercetin solution (2 -20 µg/mL) as a standard. The flavonoid content was expressed in mg of quercetin equivalent per gram of sample (mg QE/g).

4.3.7 ESTIMATION OF ANTIOXIDANT ACTIVITY BY DPPH ASSAY: The radical scavenging capacity of the CFE-hydrogel sheet was determined by the colorimetric assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (13). Aqueous extract of hydrogel sheet were used for analysis. 1.0 ml of sample was taken into 10.0 mL amber colored volumetric flask. 1.0 ml of 1.0 mM DPPH methanolic solution was added into flask. The mixture was mixed and diluted upto 10.0 mL with methanol and incubated for 30 min at room temperature. After 30 min, absorbance was measured at 517 nm by UV-Visible spectrophotometer (Shimadzu, Japan). The radical scavenging activity was calculated using equation 4.3. The results were expressed as quercetin antioxidant activity equivalent (µmol QE/g of sample). The inhibitory percentage of DPPH was calculated according to equation (4.3).

$$\%\ Inhibition = \frac{A\ (blank) - A(sam)}{A\ (blank)} * 100 \quad \text{.....Equation (4.3)}$$

Where, A (blank) = absorbance of blank

A (sam) = absorbance of sample

4.3.8 DSC STUDY: DSC analysis of PVA, carrageenan, CFE, physical mixture and hydrogel sheet were carried out using a Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan). Samples were weighed directly into DSC aluminum pan and scanned in the temperature range of 25–300 °C under an atmosphere of dry nitrogen. Heating rate of 10°C/min was used and thermo-grams obtained were observed.

4.3.9 FT-IR STUDY: FT-IR-spectrum of formulation, PVA, CFE, carrageenan and physical mixture of PVA, CFE and carrageenan were measured by preparing a potassium bromide (KBr) pellet. The pellets were scanned over a wavelength range of 4000-400 cm^{-1} and spectrum was obtained by using a FTIR spectrometer - 430 (Shimadzu 8400S, Shimadzu).

4.3.10 MICROBE PENETRATION TEST: Microbe penetration test was performed to evaluate efficiency of prepared hydrogel dressing to prevent penetration of microorganisms (14). Hydrogel dressings with thickness of around 3 mm and a size of 1x1cm^2 were used for this test. The upper surface of sample was contaminated with 0.1 ml of *S. aureus*, *E.coli* and their mixture (1:1) with a concentration of about 10^5 cells/ml, and then the sample was incubated at 37° C for 24 hrs (15). The lower surface of the hydrogel was visually observed for bacterial growth after 24 hrs incubation.

4.3.11 STERILITY: The formulations were subjected to sterility testing as per procedure described in Indian Pharmacopoeia 2018. Hydrogel sheet was incubated in fluid thioglycolate and soyabean casein digest medium for 14 days and observed for any microbial growth at 1,3,5,7 and 14 days (16).

4.3.12 *IN-VITRO* HAEMOLYSIS STUDY: The haemolytic activity of the hydrogels was determined by direct contact method (17). Red blood cells (RBC) were separated from fresh blood of mice and suspended into saline. 250 µL of RBC suspension was

taken into five tubes. 850 µL saline solution (positive control), 100 µL of 1% Triton solution (negative control), 250 ppm solution CFE (sample1), 500 ppm CFE solution (sample 2) and CFE loaded hydrogel sheet extract equivalent to 500 ppm CFE (sample 3) were added to each tubes. Volume of each tube was adjusted upto 1.0 mL by adding sterile saline solution and incubated for 1 hr at room temperature. After 1 hr incubation, all tubes were centrifuged at 10,000 rpm for 5 min at 4° C and supernant was analyzed using UV visible spectrophotometer (Shimadzu, Japan) at 541 nm (18). The % haemolysis was calculated by using equation. 4.4.

$$\%\ \boldsymbol{Haemolysis} = \frac{\boldsymbol{As-An}}{\boldsymbol{Ap-An}} * \mathbf{100} \quad \text{..... Equation. (4.4)}$$

Where, As is the absorbance of sample, An is the absorbance of negative control, Ap is the absorbance of positive control

4.4 RESULTS AND DISCUSSION

In general, hydrogel sheets are prepared by a crosslinking process of polymer which can be done by many methods e.g. chemical reaction, high energy irradiation, enzymatic reaction and physical reaction (e.g. ionic interaction, crystallization of the polymeric chain, hydrogen bond between chains, protein interaction, or design of graft copolymers (19). In recent decades, the utilization of irradiation technique to produce hydrogel sheets has been preferred over the chemical crosslinking method because of the toxic nature of chemical agents (19). Radiation reactions utilize electron beams, gamma ray, x-rays or ultraviolet light to excite a polymer and produce a crosslinked structure (19). Ionizing radiation is recognized as a very suitable tool for the formation of hydrogels due to easy process control, possibility of joining hydrogel formation and sterilization in one step (5). The radiation crosslinking can be easily adjusted by controlling the radiation dose and it is reproducible. Commercialized hydrogel dressings like Vigilon, Ivalon, and Aqua-gel and Kik gel are prepared by this technique. Many other researchers also reported this method (20-23). This technique is simple and acceptable by industry. Hence in our study, we used gamma irradiation to prepare hydrogel sheet.

4.4.1 HYDROGEL SHEET PREPARATION AND OPTIMIZATION:

- **Preliminary Screening**: PVA was selected for the preparation of hydrogel sheet because it is non toxic, non-carcinogenic, biocompatible, non-expensive, highly crystalline, water soluble polymer with good film forming and high hydrophilic properties (24, 25). Other advantages of PVA hydrogel are permeability to small molecules, barrier for bacteria, transparency and soft-consistency (26). But one limitation of PVA hydrogel sheet is its weak mechanical strength which can be overcome by blending with other polymers (27, 28). Carrageenan improves mechanical strength and water absorption capacity of PVA hydrogel sheet (29). Other function of carrageenan in the mixture is to solidify the mixture and form thermoreversible gel for easy handling of the material before γ-rays irradiation. κ-Carrageenan is most widely used among the different types due to its structure resemblance with natural glycosaminoglycans (30). κ-Carrageenan forms

thermotropic hydrogel upon cooling by coil to helix transition which lead to helical aggregation and form pseudo hydrogel sheet which is brittle in nature and poor mechanical strength so that crosslinking is necessary (31). Upon irradiation, thermoreversible hydrogel sheet which is produced by carrageenan is destroyed and simultaneously crosslinked hydrogel sheet formed. Trials were carried out for preliminary screening of optimum concentration of PVA and carrageenan for the preparation of the hydrogel sheet which is shown in table 4.4.

TABLE 4.4: Preliminary trials for the preparation of hydrogel sheet

Batch	γ-rays radiation dose (kGy)	Observation (before crosslinking by γ-rays radiation)	Observation (after crosslinking by γ-rays radiation)
CHS-1	Not Performed	Solidification of solution not occurred, Gel sheet not formed	NA
CHS-2	Not Performed	Solidification of solution not occurred	NA
CHS-3	Not Performed	Solidification of solution not occurred	NA
CHS-4	25	Solidification of solution occurred and converted into transparent, brittle sheet	Flexible, transparent gel sheet formed
CHS-5	25	Solidification of solution occurred and converted into transparent, brittle sheet	Flexible, transparent gel sheet formed
CHS-6	25	Solution was very viscous, difficult to pour into the mould	Rigid, transparent gel sheet formed
CHS-7	25	Solidification of solution not occurred	Gel sheet not formed
CHS-8	25	Solidification of solution occurred and converted into transparent, brittle sheet	Flexible, transparent gel sheet formed
CHS-9	25	Solidification of solution occurred and converted into transparent, brittle sheet	Flexible, transparent gel sheet formed
CHS-10	25	Solution was very viscous, Solidification of solution occurred and converted into very brittle sheet	Very brittle, transparent gel sheet formed

Each formulation was observed before radiation process for its solidification properties, homogeneity and physical stability by keeping it at room temperature. There was no solidification seen in formula CHS-1, CHS-2 and CHS-3 as PVA amount was not sufficient to form hydrogel sheet. Below 0.75% w/w carrageenan, there was not proper solidification observed, while above 1.25% w/w carrageenan, very brittle hydrogel sheet form which was difficult to handle. From preliminary trials, it was found that PVA conc. (8%, 10% and 12%) with carrageenan (0.75% to 1.25%) gave stable and homogenous gels. 12%w/w PVA solution was highly viscous which led to high aeration during mixing and hence was omitted for further optimization.

❖ **Optimization by BBD**: Further optimization was carried out by BBD to understand the effect of variables on hydrogel properties and result are shown in table 4.5.

TABLE 4.5: BBD for optimization of calendula flower extract loaded hydrogel sheet

Std	Run	Factor 1 A PVA conc. (% w/w)	Factor 2 B Carragee-nan conc. (% w/w)	Factor 3 C Radiation dose (kGy)	Factor 4 D CFE Conc. (%w/w)	Response 1 % Fluid absorptivity	Response 2 %GF	Response 3 Hardness (g)
22	1	10	1.25	25	4	78.09 ± 1.8	64.99 ± 2.0	8 ± 1
2	2	9	0.50	35	3	62.54 ± 2.4	73.21 ± 3.2	6 ± 1
17	3	9	1.00	35	3	84.14 ± 3.1	59.55 ± 1.4	16 ± 1
14	4	11	1.00	35	3	54.11 ± 2.9	70.38 ± 1.1	22 ± 1
7	5	8	1.25	45	2	46.72 ± 4.1	70.90 ± 2.5	48 ± 3
6	6	9	1.00	35	3	81.42 ± 3.7	60.45 ± 1.4	15 ± 1
9	7	8	0.75	25	2	56.07 ± 1.8	56.79 ± 1.8	19 ± 1
24	8	9	1.00	35	3	80.10 ± 2.7	65.69 ± 2.0	15 ± 1
20	9	8	0.75	45	4	75.00 ± 3.0	35.22 ± 1.2	23 ± 2
5	10	9	1.00	35	5	62.78 ± 1.4	55.55 ± 2.8	9 ± 1
11	11	9	1.00	55	3	46.51 ± 2.0	67.85 ± 1.5	38 ± 3
18	12	10	0.7	45	4	39.12 ± 3.2	70.77 ± 3.1	22 ± 1
8	13	10	0.75	25	2	37.07 ± 1.4	58.12 ± 5.1	7 ± 1
19	14	10	0.75	25	4	35.41 ± 2.0	66.20 ± 2.1	9 ± 1
16	15	9	1.00	15	3	50.31 ± 2.6	61.29 ± 2.7	13 ± 1
21	16	9	1.00	35	3	82.50 ± 3.5	58.90 ± 1.6	17 ± 2
13	17	9	1.00	35	3	81.21 ± 2.2	59.20 ± 1.7	16 ±1
1	18	9	1.50	35	3	67.70 ± 1.5	74.15 ± 2.5	10 ± 1
15	19	8	0.75	25	4	78.09 ± 1.1	66.75 ± 2.8	6 ± 1
12	20	8	1.25	25	4	79.85 ± 1.8	46.71 ± 1.6	6 ± 1
27	21	7	1.00	35	3	92.00 ± 3.5	77.22 ± 3.2	31 ± 2
4	22	8	1.25	25	2	60.63 ± 1.4	69.83 ± 2.6	34 ± 3
3	23	10	1.25	25	2	64.45 ± 1.5	70.83 ± 2.1	27 ± 2
26	24	10	1.25	45	2	59.07 ± 2.3	73.60 ± 3.6	35 ± 2
25	25	9	1.00	35	3	82.10 ± 3.7	59.55 ± 1.4	16 ± 1
10	26	8	1.25	45	4	62.10 ± 2.6	59.65 ± 1.7	15 ± 1
28	27	9	1.00	35	1	62.54 ± 1.9	75.48 ± 2.9	39 ± 1
23	28	10	1.25	45	4	41.12 ± 2.5	66.25 ± 3.2	18 ± 1

The physical properties of the hydrogel sheet depend on composition and γ-rays irradiation dose. An ideal wound dressing must be able to absorb wound exudates and provide a wet environment for the wound, so that fluid absorption ability is important for hydrogel wound dressing. Wound dressing should withstand some frictional stresses during day to day activities when applied on the wound. So that if there are any accidental frictional stresses, the membrane will absorb the energy without breaking and will thus provide it's protective effect over the wound. Hydrogel sheet should be able to resist the physiological stress caused by the movement of body and at the same time provide close and prolonged contact between gel and skin area. Gel hardness, which expresses the applicability of the gels to the skin, is directly correlated to the polymer concentration. Hydrogel sheet dressing is more patient friendly as it did not break into pieces during application and removal on wound site.

4.4.1.1 Influence on % fluid absorptivity (%A)

i. **Statistical analysis for % fluid absorptivity:** Based on the F value for model selection (table 4.6), quadratic model was selected for the response 1 (% fluid absorptivity) and from p value (<0.0001), it was confirmed that the model was significant.

TABLE 4.6: Statistical analysis for % fluid absorptivity

Sequential Model Sum of Squares [Type I]						
Source	Squares	Df	Square	Value	Prob > F	
Mean vs Total	96831.75	1	96831.75			Suggested
Linear vs Mean	865.8056	4	216.4514	0.851491	0.5095	
2FI vs. Linear	2874.456	6	479.076	3.035417	0.0407	
Quadratic vs. 2FI	**2166.919**	**4**	**541.7297**	**126.9182**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	33.30598	5	6.661195	3.551728	0.0952	Aliased
Residual	9.3774	5	1.87548			
Total	102781.6	25	4111.264			

ii. To identify the significant parameters and their interaction, analysis of variance (ANOVA) was performed for each parameter (Table 4.7) which showed that the effects of factors were significant and hence the model was significant for water absorptivity (%A). From the F value we can say that PVA content was most affecting % water absorptivity and other variables were the least affecting variables.

iii. **Mathematical equation for % fluid absorptivity:** In terms of actual components, the inter-relation between independent and dependent variables was derived. The equation (4.5) indicated the complexity between the input and output variables. The interrelation between the dependent and independent variables is shown in 3D response surface curve (figure 4.3).

$$\%\textbf{Fluid absorptivity} = 81.91 - (8.95715625 * A) + (1.443822917 * B) - (1.132989583 * C) + (1.13665625 * D) + (5.43346875 * A * B) + (1.19778125 * A * C) - (6.96828125 * A * D) - (10.65021875 * B * C) + (4.73471875 * B * D) - (7.60096875 * C * D) - (2.313453125 * A^2) - (4.118515625 * B^2) - (8.296015625 * C^2) - (4.733515625 * D^2) \quad \text{……Equation (4.5)}$$

TABLE 4.7: Effect of various variables on % fluid absorptivity

Response	**1**	**% Fluid absorptivity**			
ANOVA for Response Surface Quadratic Model					
Analysis of variance table [Partial sum of squares - Type III]					
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	**5907.18**	**14**	**421.9415**	**98.85382**	**< 0.0001**	**Significant**
A-PVA content	749.6002	1	749.6002	175.6188	< 0.0001	
B-Carrageenan content	31.89227	1	31.89227	7.471824	0.0211	
C-radiation dose	19.63855	1	19.63855	4.600982	0.0575	
D-CFE Content	22.65403	1	22.65403	5.307459	0.0440	
AB	174.1424	1	174.1424	40.79865	< 0.0001	
AC	8.462628	1	8.462628	1.982652	0.1894	
AD	428.6406	1	428.6406	100.4233	< 0.0001	
BC	752.263	1	752.263	176.2426	< 0.0001	
BD	132.2326	1	132.2326	30.97989	0.0002	
CD	340.791	1	340.791	79.84163	< 0.0001	
A^2	63.67756	1	63.67756	14.91858	0.0031	
B^2	437.8806	1	437.8806	102.5881	< 0.0001	
C^2	1776.697	1	1776.697	416.2504	< 0.0001	
D^2	578.4181	1	578.4181	135.5137	< 0.0001	
Residual	42.68338	10	4.268338			
Lack of Fit	**33.30598**	**5**	**6.661195**	**3.551728**	**0.0952**	**not significant**
Pure Error	9.3774	5	1.87548			
Cor Total	5949.864	24				

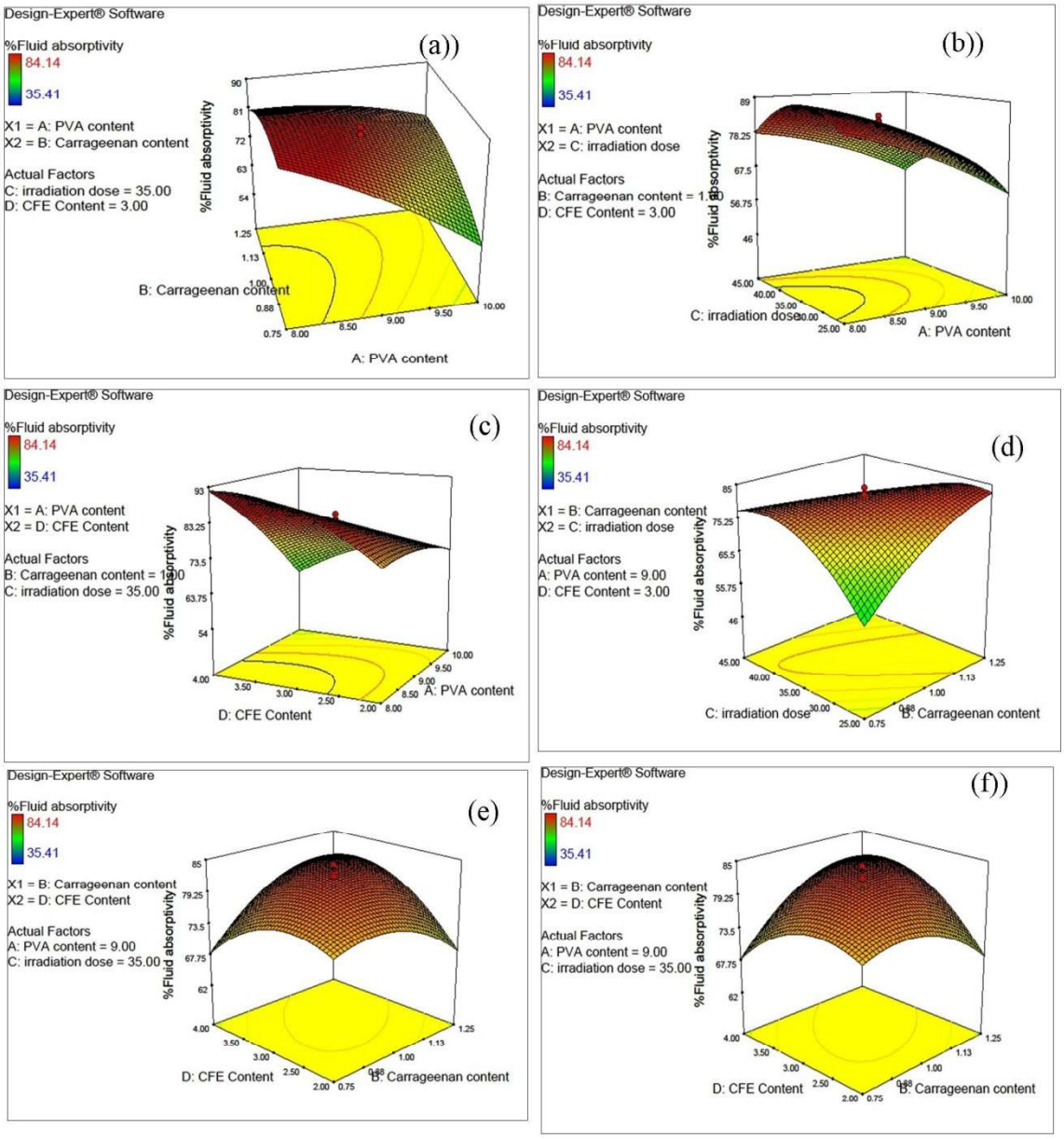

FIGURE 4.3 : Response surface (3D) plots showing combined effect of (a) PVA content and carrageenan content, (b) PVA content and radiation dose, (c) CFE content and PVA content (d) radiation dose and carrageenan content, (e) CFE content and carrageenan content and (f) CFE content and radiation dose on % fluid absorptivity of hydrogel sheet

All hydrogels had minimum 40-92% fluid absorptivity which is sufficient for the application as wound dressing (32). Hydrogel sheet swells by absorbing water but does not dissolve due to crosslinking of polymer chain. It has the ability to hold water within its polymeric network. Swelling of hydrogel is induced by the electrostatic repulsion of

the ionic charges of its network, which blocks the aggregation of the polymer chains and acts to expand the hydrogel (33). Fluid absorption capacity of PVA hydrogel decreased with an increase in radiation dose and PVA content. The reduction of fluid absorption capacity with increased radiation dose may be due to increased cross-linking by the radiation (21, 33). Degree of swelling is inversely proportional to cross linking density. Increased cross-links reduce the available scope of free spaces for water in the polymer network. This can be explained by the phenomenon that higher number of cross-links diminishes voids spaces and reduces water absorption capacity of polymer (32). The fluid absorption capacity of hydrogel sheet was increased with increase in carrageenan and CFE content. Carrageenan is an anionic sulfated polysaccharide polymer composed of D-galactose and 3,6-anhydrogalactose units. Increased carrageenan content increases number of hydrophilic groups (-OSO3) in the hydrogel networks which ionize in swelling medium, creating an electrostatic repulsion which increase water absorption capacity (32, 34). Another reason might be that the fragment of degraded carrageenan by γ-rays coming out from hydrogel sheet during swelling and make gel sheet more porous which infusing more fluid into hydrogel sheet (29). The absorption capacity decreased with increase in %GF because increased in crosslinking of polymer. Higher irradiation dose increases the crosslinking of polymer which reduces absorption capacity of hydrogel (21, 33).

4.4.1.2 Influence on %gel fraction

Based on the F value for model selection (table 4.8), Quadratic model was selected for the response 2 (%gel fraction). From p value (<0.0128), it was confirmed that the model is significant for the % gel fraction.

TABLE 4.8: Statistical analysis for gel fraction

Response	**2**	**gel fraction**	**Transform:**	**None**		
Sequential Model Sum of Squares [Type I]						
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	**105047.8**	**1**	**105047.8**			**Suggested**
Linear vs. Mean	230.4708	4	57.6177	1.873427	0.1567	
2FI vs. Linear	90.276	6	15.046	0.395888	0.8687	
Quadratic vs. 2FI	**358.2317**	**4**	**89.55793**	**5.93353**	**0.0128**	**Suggested**
Cubic vs. Quadratic	102.8121	4	25.70302	3.890892	0.0843	Aliased
Residual	33.02972	5	6.605944			
Total	105862.6	24	4410.942			

Analysis of variance was performed for each parameter (table 4.9) which showed that the effects of factors were significant and hence the model was significant for %GF. From the F value we can say that CFE content and γ radiation dose were affecting highest to the %GF and carrageenan content was the least affecting variable to the %GF.

TABLE 4.9: Effect of various variables on gel fraction

Response	**2**	gel fraction			
ANOVA for Response Surface 2FI Model					
Analysis of variance table [Partial sum of squares - Type III]					
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	**678.9785**	**14**	**48.49847**	**3.213195**	**0.0419**	**Significant**
A-PVA content	19.82918	1	19.82918	1.313754	0.2813	
B- Carrageenan content	3.594145	1	3.594145	0.238125	0.6372	
C-radiation dose	35.33636	1	35.33636	2.341159	0.1604	
D-CFE Content	105.8819	1	105.8819	7.015054	0.0265	
AB	30.07951	1	30.07951	1.992874	0.1917	
AC	2.679788	1	2.679788	0.177545	0.6834	
AD	40.81114	1	40.81114	2.703883	0.1345	
BC	9.396013	1	9.396013	0.622519	0.4504	
BD	0.040217	1	0.040217	0.002664	0.9600	
CD	12.22195	1	12.22195	0.809745	0.3916	
A^2	47.19895	1	47.19895	3.127095	0.1108	
B^2	306.1604	1	306.1604	20.28421	0.0015	
C^2	36.47251	1	36.47251	2.416433	0.1545	
D^2	52.24154	1	52.24154	3.461187	0.0957	
Residual	135.8418	9	15.09353			
Lack of Fit	**102.8121**	**4**	**25.70302**	**3.890892**	**0.0843**	**not significant**
Pure Error	33.02972	5	6.605944			
Cor Total	814.8203	23				

Mathematical equation for Gel fraction: In terms of actual components, the inter-relation between independent and dependent variables was derived. The Equation (4.6) indicated the complexity between the input and output variables. The interrelation between the dependent and independent variables is shown in 3D response surface curve (figure 4.4).

$$\%\textbf{Gel fraction} = 60.55716667 + (1.682036184 * A) + (0.490805921 * B) + (1.648351974 * C) - (2.663930921 * D) - (2.657490132 * A * B) + (0.676226974 * A * C) - (2.399134868 * A * D) - (1.247792763 * B * C) - (0.090838816 * B * D) - (1.444148026 * C * D) + (2.009568531 * A^2) + (3.477897478 * B^2) + (1.200397478 * C^2) + (1.436647478 * D^2) \quad \ldots\ldots\ldots \quad \text{Equation (4.6)}$$

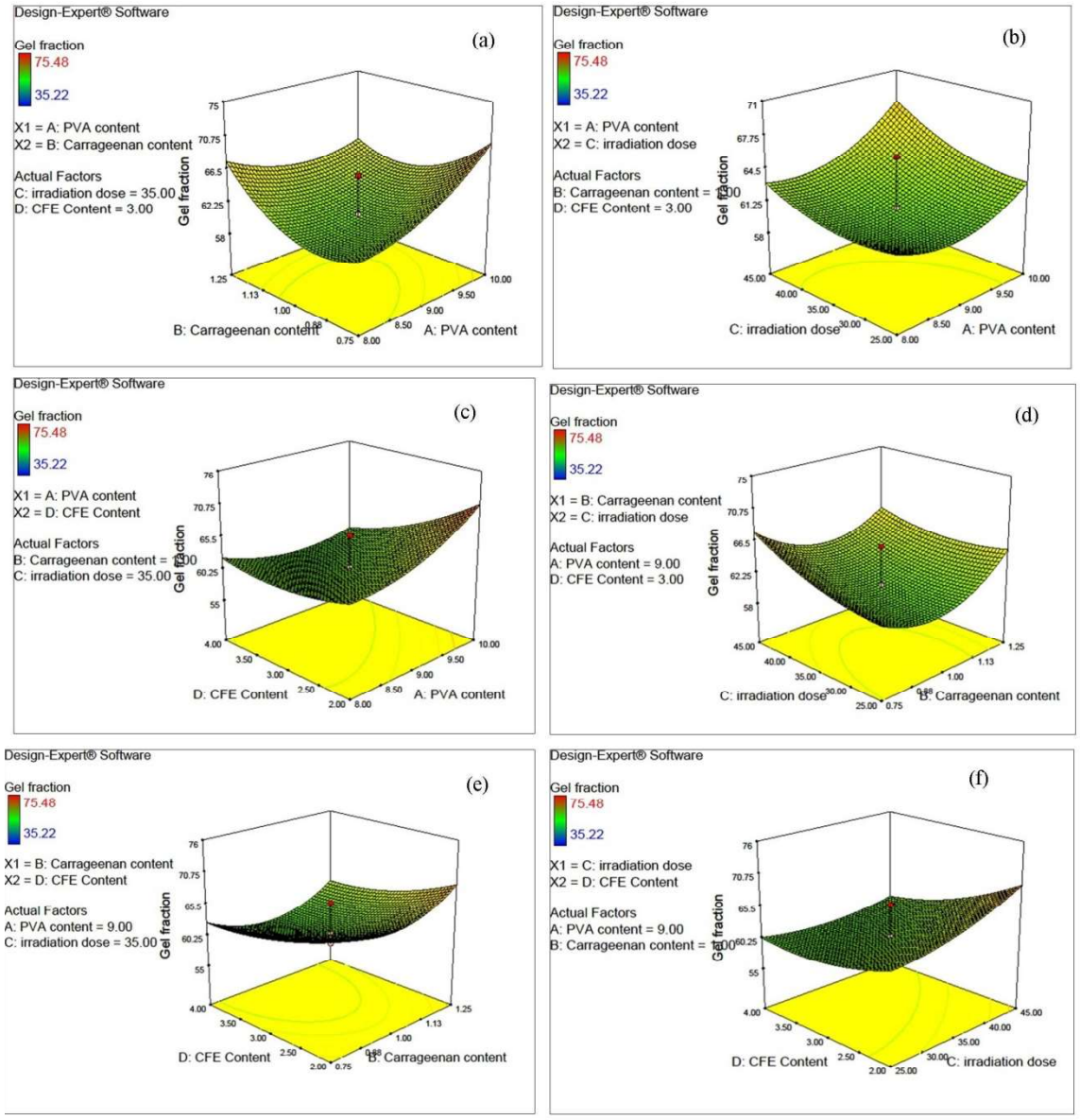

FIGURE 4.4 : Response surface (3D) plot showing combined effect of (a) PVA content and carrageenan content; (b) PVA content and radiation dose; (c) CFE content and PVA content; (d) Radiation dose and carrageenan content; (e) CFE content and carrageenan content; and (f) CFE content and radiation dose on gel fraction of hydrogel sheet

Cross-linking does not occur entirely in hydrogel sheet after gamma irradiation process. Certain amounts of polymer macromolecules with other non crosslinked ingredient such as CFE remain in the network uncross-linked. To evaluate the degree of crosslinking, gel fraction of hydrogel sheet was calculated. Higher the gel fraction means higher crosslinking of polymer. %GF of prepared hydrogel sheets were in the range of 6 – 48 g. Hardness was reduced significantly with incorporation of CFE, decreased slightly

with the increase of carrageenan content, increased with PVA content and γ-rays irradiation dose. %gel fraction and hardness are both mechanical properties of hydrogel sheet and mainly depends on crosslinking of polymer. As explain earlier, radiation dose and PVA content increase the crosslinking of polymeric network which increase the hardness of hydrogel sheet. CFE acts as a plasticizer as well as reduces the crosslinking of polymer due to glycol content which reduces the hardness of hydrogel sheet.

4.4.1.3 Influence on hardness

Statistical analysis for hardness: Based on the F value for model selection as shown in table 4.10, quadratic model was selected for the response 3 (hardness). From p value (<0.0001), it was confirmed that the model is significant for the hardness.

TABLE 4.10: Statistical analysis for hardness

Response	**3**	**Hardness**	**Transform:**	**None**		
Sequential Model Sum of Squares [Type I]						
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Mean vs. Total	8320.19	1	8320.19			
Linear vs. Mean	**1840.573**	**4**	**460.1432**	**14.34482**	**< 0.0001**	**Suggested**
2FI vs. Linear	306.704 5	6	51.11741	2.475032	0.0986	
Quadratic vs. 2FI	**203.0323**	**4**	**50.75808**	**87.01385**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	0.666667	1	0.666667	1.176471	0.3276	Aliased
Residual	2.833333	5	0.566667			
Total	10674	21	508.2857			

The ANOVA for the hardness showed that the effects of factors were significant and hence the model was significant (table 4.11). From the F value we can say that variable C (γ-Radiation dose) was affecting highest to the hardness and variable A (PVA content)

was the least affecting variable. In terms of actual components, the inter-relation between independent and dependent variables was derived.

TABLE 4.11: Effect of various variables on hardness

ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	**2350.31**	**14**	**167.8793**	**287.793**	**< 0.0001**	**Significant**
A-PVA content	26.13333	1	26.13333	44.8	0.0005	
B-Carrageenan content	40.83333	1	40.83333	70	0.0002	
C- γ Radiation dose	494.0833	1	494.0833	847	< 0.0001	
D-CFE Content	225	1	225	385.7143	< 0.0001	
AB	3.846154	1	3.846154	6.593407	0.0425	
AC	8.533333	1	8.533333	14.62857	0.0087	
AD	64.02899	1	64.02899	109.764	< 0.0001	
BC	1.633333	1	1.633333	2.8	0.1453	
BD	59.92908	1	59.92908	102.7356	< 0.0001	
CD	1	1	1	1.714286	0.2383	
A^2	58.68056	1	58.68056	100.5952	< 0.0001	
B^2	16.75362	1	16.75362	28.7205	0.0017	
C^2	140.1667	1	140.1667	240.2857	< 0.0001	
D^2	30.78205	1	30.78205	52.76923	0.0003	
Residual	3.5	6	0.583333			
Lack of Fit	**0.666667**	**1**	**0.666667**	**1.176471**	**0.3276**	**not significant**
Pure Error	2.833333	5	0.566667			
Cor Total	2353.81	20				

The equation (4.7) indicated the complexity between the input and output variables. The interrelation between the dependent and independent variables is shown in 3D response surface curve (figure 4.5).

$$\textbf{Hardness (g)} = 15.83333333 - (2.333333333 * A) + (2.916666667 * B) + (6.416666667 * C) - (7.5 * D) + (1.25 * A * B) - (1.333333333 * A * C) + (3.916666667 * A * D) - (0.583333333 * B * C) - (5.416666667 * B * D) + (0.5 * C * D) + (2.708333333 * A\text{^}2) - (2.833333333 * B\text{^}2) + (2.416666667 * C\text{^}) + (2.041666667 * D\text{^}2) \quad \ldots \textbf{Equation (4.7)}$$

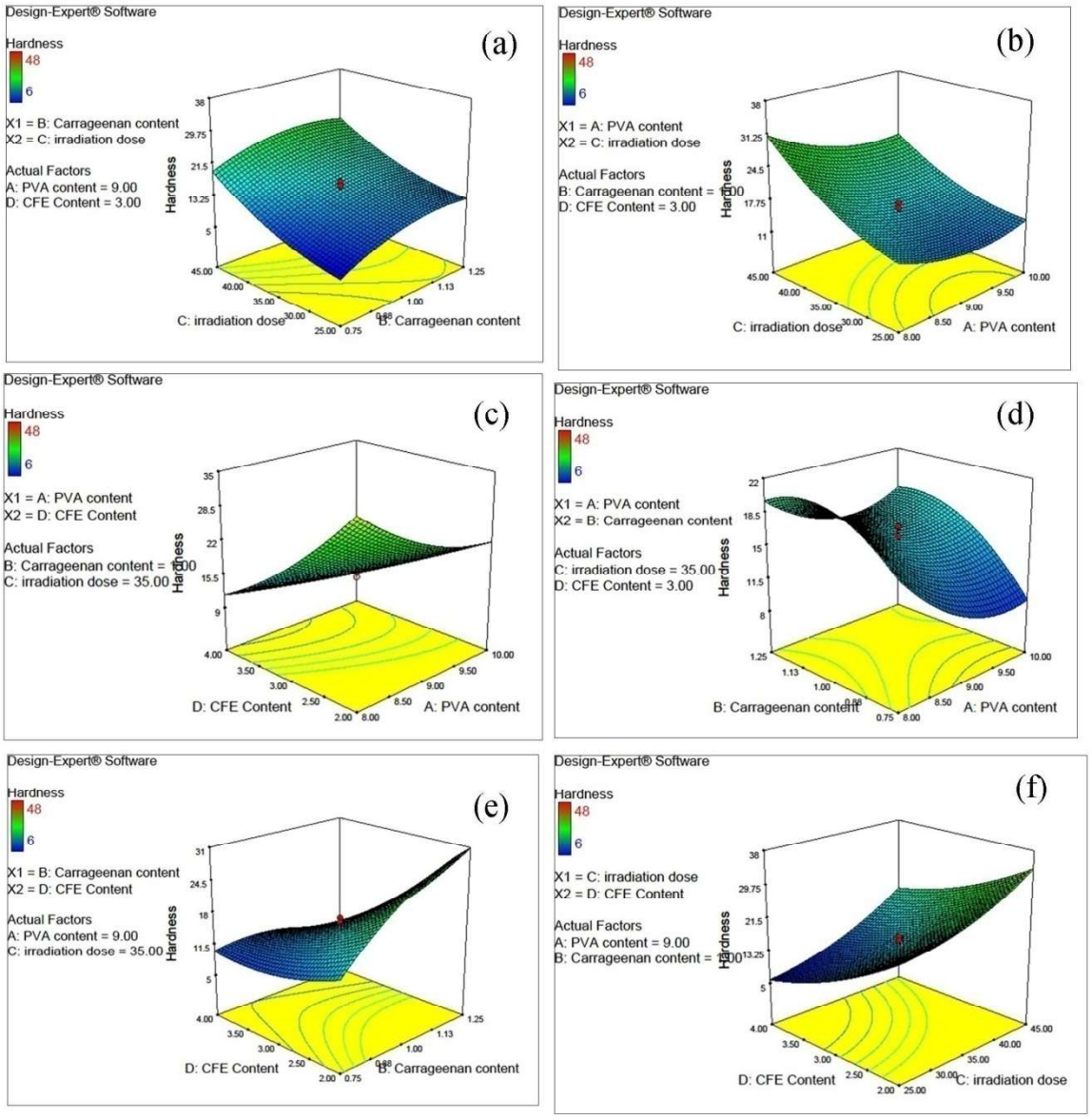

FIGURE 4.5 : Response surface (3D) plot showing combined effect of (a) PVA content and carrageenan content; (b) PVA content and radiation dose; (c) CFE content and PVA content; (d) radiation dose and carrageenan content; (e) CFE content and carrageenan content; and (f) CFE content and radiation dose on hardness of hydrogel sheet

Wound dressing should withstand some frictional stresses during day to day activities when applied on the wound. If there are any accidental frictional stresses the membrane should absorb the energy without breaking and provide it's protective effect over the wound. Hardness of hydrogel sheets were in the range of 23 – 69 %. Gel fraction were reduced significantly with incorporation of CFE, decreased slightly with the

increase of carrageenan content, increased with PVA content and γ-rays irradiation dose. The formation of gel explains the fact that crosslinking network between mono-polymer by gamma rays is formed. Crosslinking transforms a linear polymer into a three dimensional molecule, resulting in a significant increase in molecular mass, lower solubility and improved mechanical properties (35). This crosslinking process results from the coupling of the polymer radicals that were directly and indirectly produced from PVA by gamma rays (36), so that increasing PVA content leads to increase in gel fraction which is due to the crosslinking of PVA (15, 37, 38). Carrageenan degrades to a low molecular weight compound upon γ-rays irradiation which prevent PVA molecule from crosslinking. Carrageenan is relatively acquiescent to radical reaction during irradiation which results in less radical available to PVA chains for cross linking (29). So that as carrageenan content increased, gel content of hydrogel sheet was decreased. The %GF of hydrogel sheet increase with an increased in radiation dose due to increased cross-linked by the action of radiation (32). As hydroglycolic extract of calendula flower contains mixture of water and propylene glycol, as CFE act as plasticizer as well modify the gel properties. %Gel fraction and hardness decrease with increasing in CFE extract due to glycol part of CFE decrease the crosslinking reaction by scavenging the free radical (14, 39).

Establishment of design space: Establishment of design space was done to assess the sensitivity of optimized batch. An overlaid response plot was obtained keeping all four independent variables. Design space was created in such a way that the response would be between a particular limit. Overlay plot was obtained by superimposing contour plots of responses Y1, Y2 and Y3 which displays the area of feasible response values in the factor space. Regions that fit the optimization criteria are colored yellow. Flags were placed in overlay plot that shows predicted values of desired response with optimized values of variables. The software generated an overlaid plot of design space as shown in figure 4.6

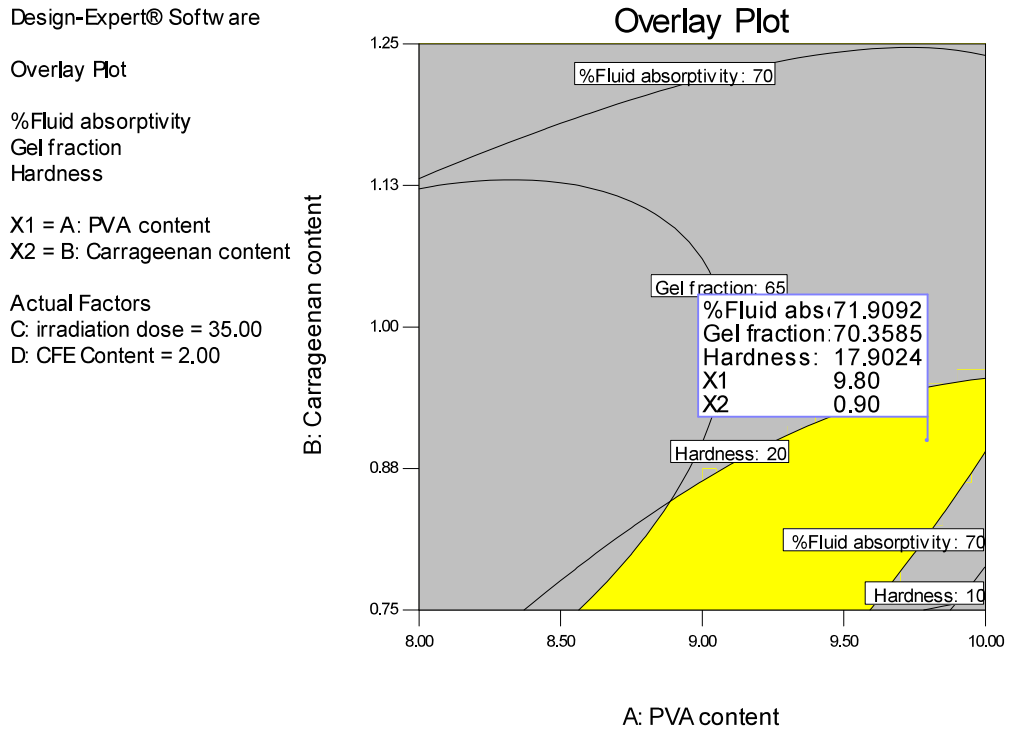

FIGURE 4.6: Software generated overlaid plot of design space

- **Analysis of Design space:** Analysis of design space was performed using desirability curve. From obtained results (Table 4.12), it was revealed that observed responses were found to be closer to predicted value of responses suggested by software DesignExpert 7.0. This proves the robustness of the established design space as predicted by software.

TABLE 4.12: Predicted value and Observed value of check point batch

Response	Predicted	Observed
Y1(% Fluid absorptivity)	71.9092%	73.8±2.1%
Y2 (% Gel Fraction)	70.3585%	68.4±3.8%
Y3 (% Hardness)	17.9024 g	15 g ± 1 g

4.4.2 PHYSICOCHEMICAL CHARACTERIZATION: The optimized formulations were evaluated for its physicochemical parameters such as physical appearance, pH of aqueous extract, microbe penetration test, total flavonoid content, total phenolic content, antioxidant activity and sterility test.

4.4.2.1 Physical appearance: The prepared calendula flower extract loaded hydrogel sheet (fig 4.7) was transparent, non sticky, flexible and easy to remove. It was a slightly yellowish in colour due to CFE.

FIGURE 4.7: Physical appearance of calendula flower extract loaded hydrogel sheet

4.4.2.2 pH: Human skin has an acidic nature (pH 4 to 6.8). Wound healing is promoted when the pH of skin and wound are maintained at a slightly acidic pH (40). Therefore the pH of hydrogels should be in this region. pH value of aqueous extract of developed formulation was 6.5 ± 0.5 which is acceptable for wound dressing.

4.4.2.3 Mechanical properties: Result of mechanical properties study carried out by texture analyzer shown in figure 4.8. The load versus distance plot was generated by the texture analyzer shown in figure 4.9. The maximum load in the plot that is 12 g represents the hardness of the CFE-hydrogel sheet. Hydrogel sheet dressing is more patient friendly as it did not break into pieces during application and removal on wound site.

TexturePro CT V1.8 Build 29 — Brookfield Engineering Labs. Inc.

DATA REPORT

Sample Description

Product Name:	GEL	Note:	
Batch Name:	CCD- 011		
Sample:	1		
Dimensions:			
Shape:	Block		
Length:	0.00 mm		
Width:	0.00 mm		
Depth:	0.00 mm		

Test Method

Test Date:	13-11-2017	Test Time:	16:35:28	
Test Type:	Compression	Recovery Time:	15	s
Target:	4.0 mm	Same Trigger:	False	
Hold Time:	0 s	Pretest Speed:	2.00	mm/s
Trigger Load:	0 g	Data Rate:	10.00	points/sec
Test Speed:	0.50 mm/s	Probe:	TA3/100	
Return Speed:	0.5 mm/s	Fixture:	TA-RT-KIT	
# of Cycles:	3.0	Load Cell:	10000g	
Target Type:	Distance			

Results

Hardness Cycle 1:	12.00	g
Deformation at Hardness:	3.99	mm
Hardness Work Cycle 1:	0.20	mJ
Recoverable Deformation Cycle 1:	2.42	mm
Recoverable Work Cycle 1:	0.10	mJ
Total Work Cycle 1:	0.30	mJ
Rigidity 1:	0.00	g
	at 4.0 mm	
Load at Target:	12.00	g
Deformation at Target:	3.99	mm
Adhesive Force:	4.00	g
Adhesiveness:	0.00	mJ
Resilience:	0.73	
Stringiness Length:	1.57	mm
Stringiness Work Done:	0.00	mJ

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FIGURE 4.8: Result of hardness study of CFE loaded hydrogel sheet by Texture analyzer

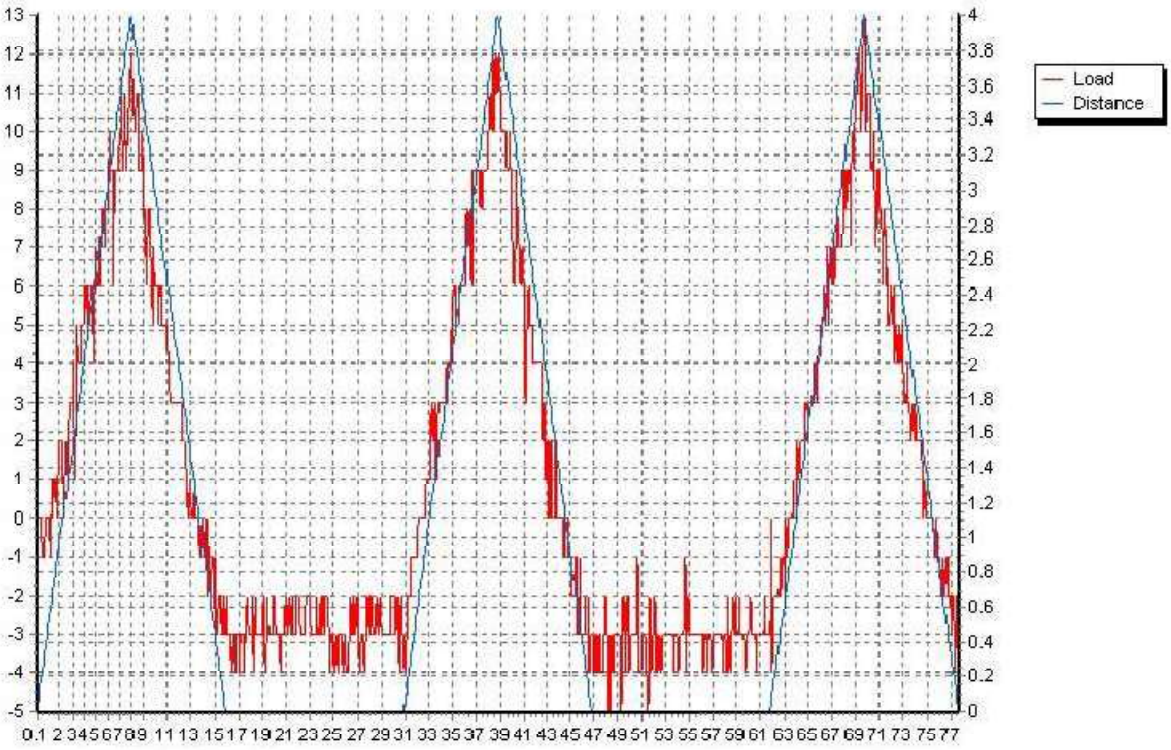

FIGURE 4.9: Load vs. distance plot generated by texture analyzer

4.4.2.4 Total polyphenolic, flavonoid content and DPPH assay: The results of total amounts of polyphenolic, flavonoid determination and DPPH assay are shown in table 4.13 Phenolic compounds play an important role in the capture and neutralization of free radicals due to their chemical structures (41). Free radical scavenging abilities of CFE loaded hydrogel sheet using the DPPH assays were investigated. DPPH is red color free radical which is converted into yellow colour if free radicals have been scavenged. Based on this property, DPPH is used to find out antioxidant activity of substance by its ability to scavenge free radical. Free radical scavenging activity of hydrogel sheet loaded with CFE was observed. Calendula flower extract contain polyphenols, carotenoids and flavonoids which scavenge free radicals (42, 43). Reactive oxygen species are formed during inflammation which damage and retards the normal wound healing process.

Antioxidant activity of formulation reduces the oxidative damage to tissue and helps in wound healing process.

TABLE 4.13: Result of total polyphenolic and flavonoid content determination

	Amount* (per 100 ml extract of 25 g hydrogel sheet)	**Analytical curve (Straight line equation) (raw data are not shown)**
Total polyphenolic content	4.1430 ± 0.634 mg GAE/100 ml extract	Y = 0.092x + 0.055 (R^2 = 0.999)
Total flavonoid content	4.218 ± 0.110 mg QUE/100 ml extract	Y = 0.092x + 0.251 (R^2 = 0.996)
DPHH assay	0.6987 ± 0.146 µmol QUE/100 ml extract	Y = -0.149x + 1.065 (R^2 = 0.989)

GAE (Gallic acid equivalent), QUE (Quercetin equivalent)*±SD, n=3

4.4.2.5 Microbe penetration test: No bacteria were found on the TSA medium. This property is very important for a dressing because it protects the wound from further infection.

4.4.2.6 FT-IR study: FTIR spectra of CFE, PVA, carrageenan, physical mixture and hydrogel sheet are given in figure 4.10 to figure 4.15 respectively. FT-IR analysis of CFE proved presence of alkyl halides (517.53 cm^{-1}, 565.33 cm^{-1}), alcohols phenols (3318.25 cm^{-1}), saturated amine and aliphatic amine groups (1078.27 cm^{-1}, 1105.02 cm^{-1}), alkanes (1438.05 cm^{-1}) and ketones group (1785.63 cm^{-1}) which were also reported by Al-Mussawi et. al., 2019 (44). The FTIR spectrum of hydrogel sheet consisted of all functional groups present in PVA, Carrageenan and CFE. FT-IR spectra of PVA and formulation showed O-H stretching at 3404 to 3416 cm-1 indicating presence of intermolecular hydrogen bonded hydroxyl groups having polymeric association (26). Broadening of O-H stretching vibration appeared between 3,500 due to interaction between O-H groups of polymer by crosslinking in hydrogel sheet. The spectra also

contain characteristic bands of C-O-C and stretching vibrations in between 1078 cm^{-1} to 1106 cm^{-1} due to cross-linking of the polymeric chains (26).

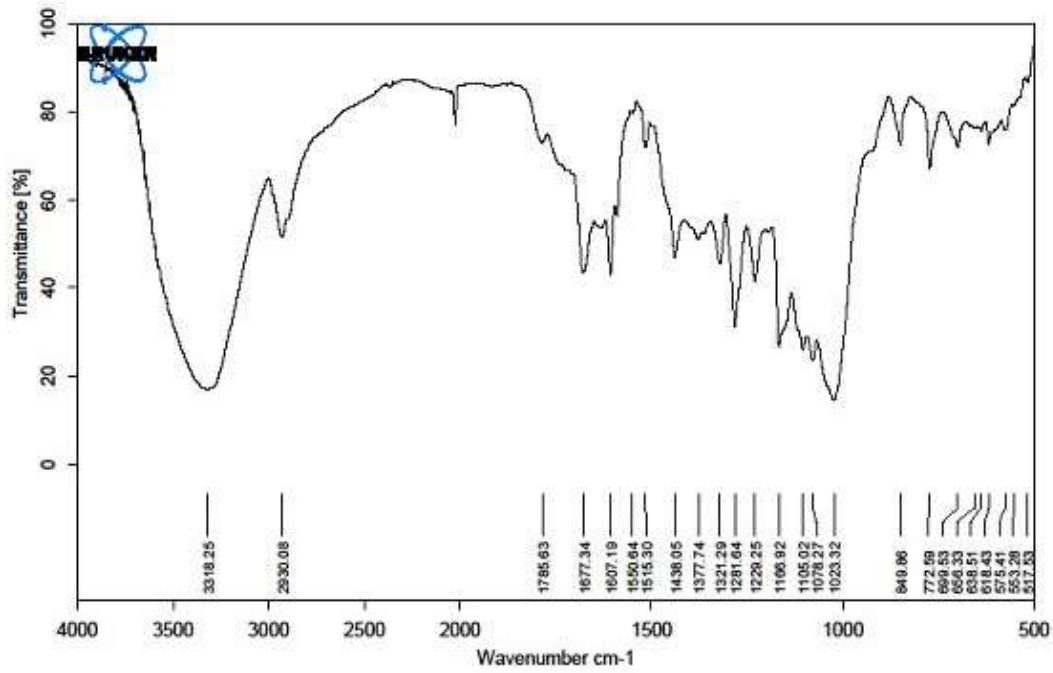

FIGURE 4.10: FT IR spectra of *Calendula* flower extract

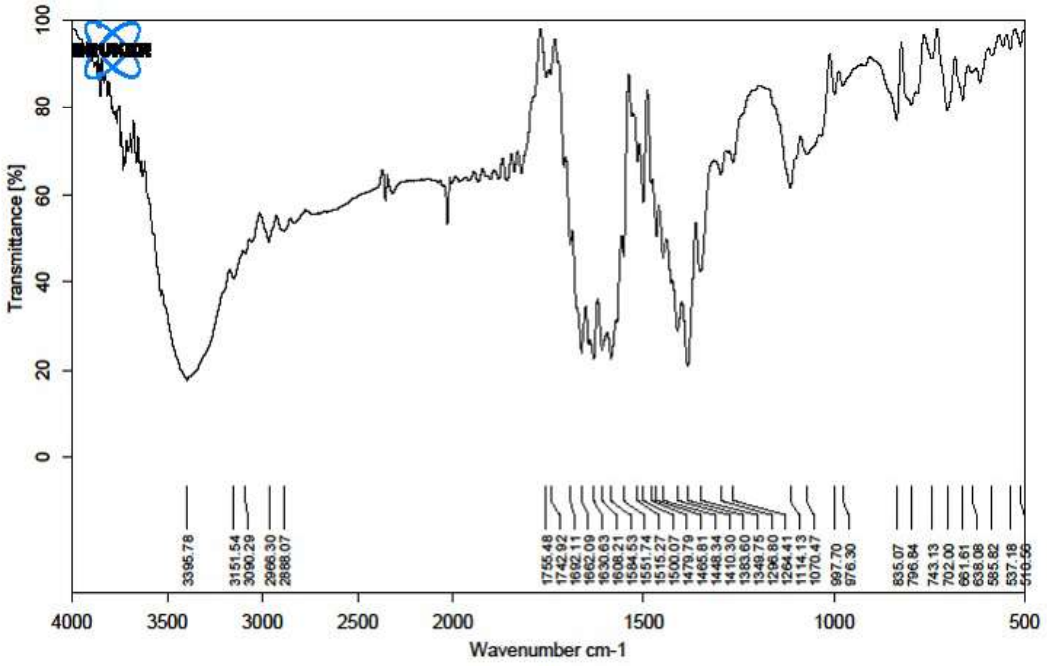

FIGURE 4.11: FT IR spectra of polyvinyl alcohol

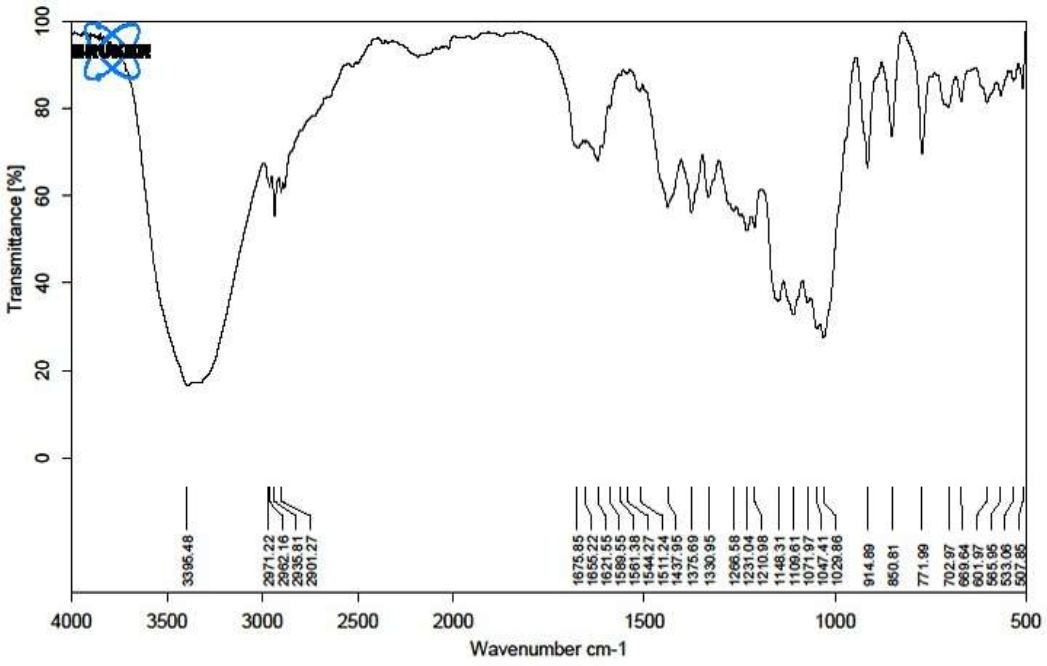

Figure 4.12: FT IR spectra of carrageenan

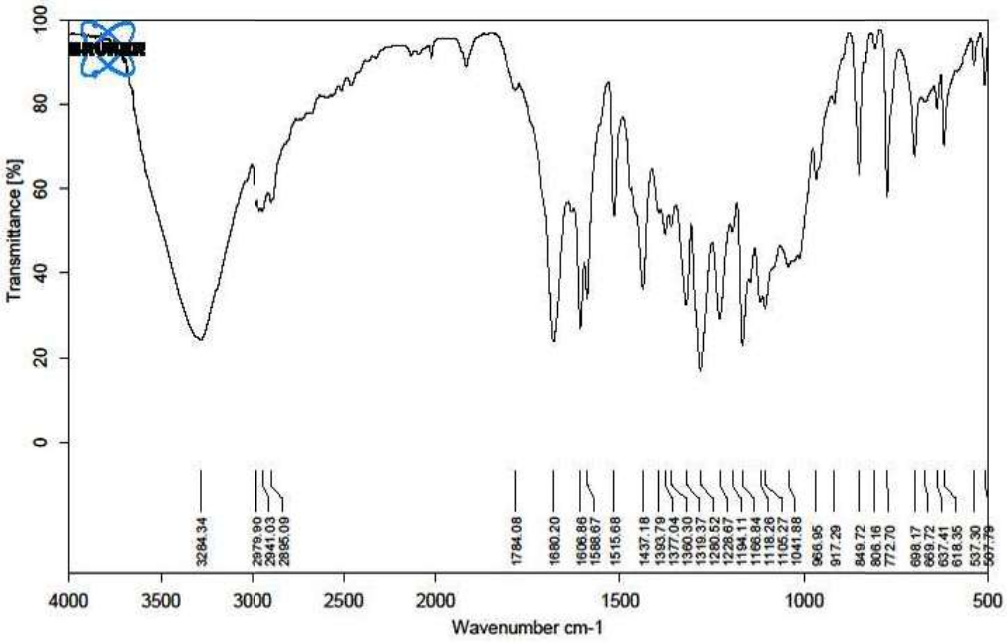

FIGURE 4.13: FT IR spectra of physical mixture of PVA, carrageenan, CFE

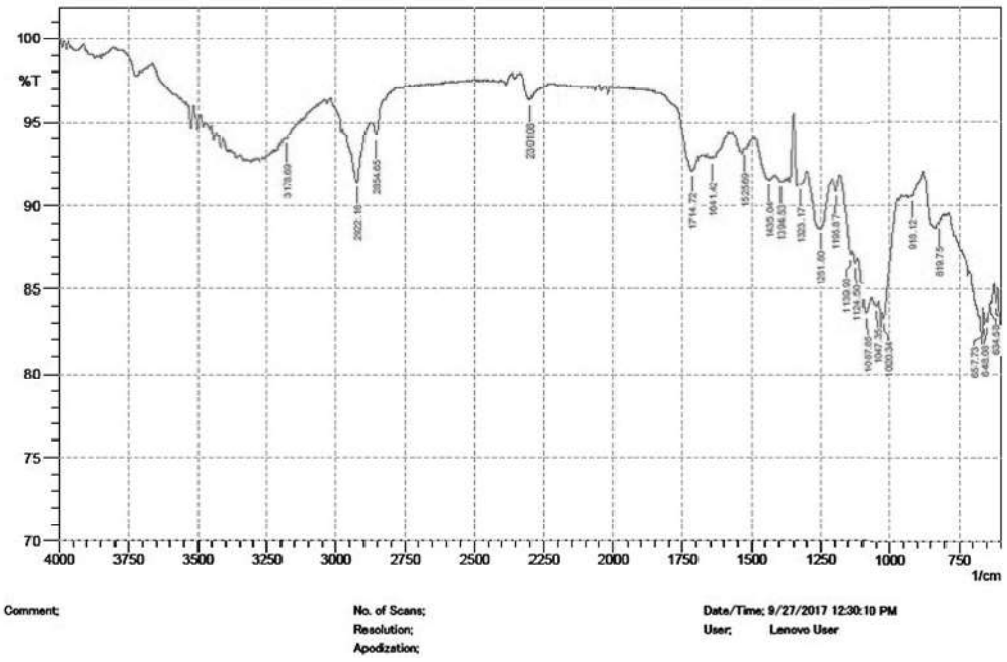

FIGURE 4.14: FT IR spectra of CFE loaded hydrogel sheet

4.4.2.7 DSC study: DSC thermograms of CFE, PVA, carrageenan, physical mixture of CFE, carrageenan, PVA and formulation are presented in figure 4.15 to 4.19. There was broad endothermic peak in CFE's thermogram which indicates its amorphous nature. PVA thermogram has one sharp endothermic peak around 185.62° C which represent its melting point. The physical mixture of all ingredients did not show exothermic peak which indicated that there was no chemical interaction between excipients and CFE. There was broad endothermic peak around 100° - 120° C in CFE loaded hydrogel sheet's thermogram because of loss of water molecules from hydrogels, while other two peaks at 227° and 247° C were due to decomposition of polymer.

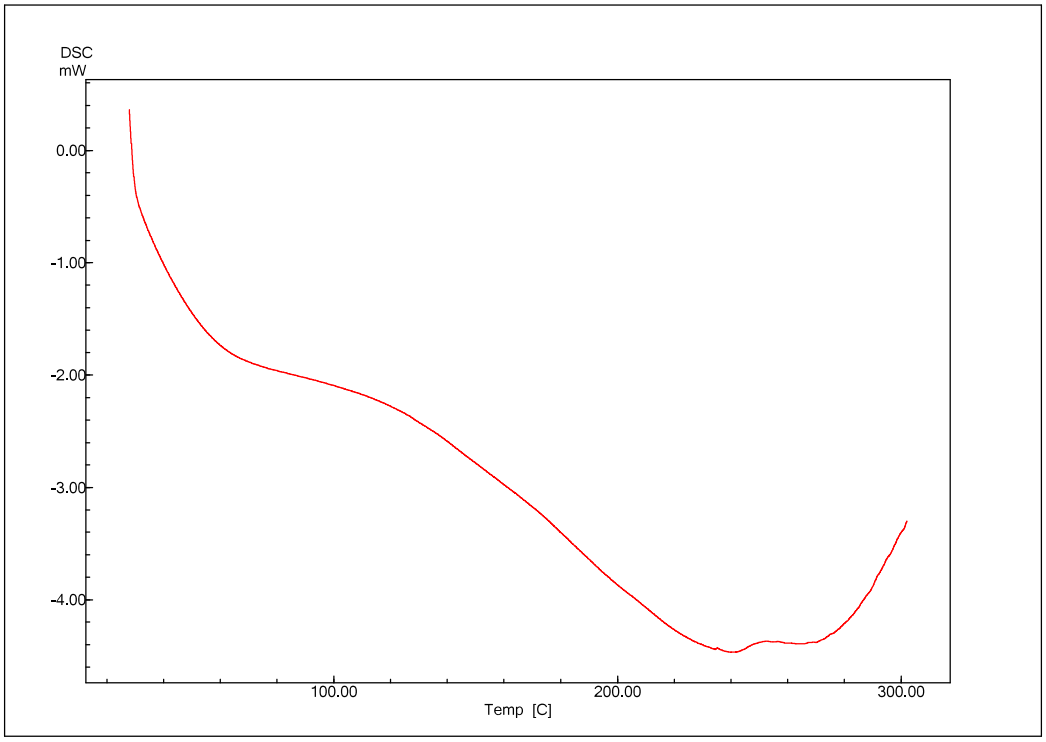

FIGURE 4.15: DSC thermogram of CFE

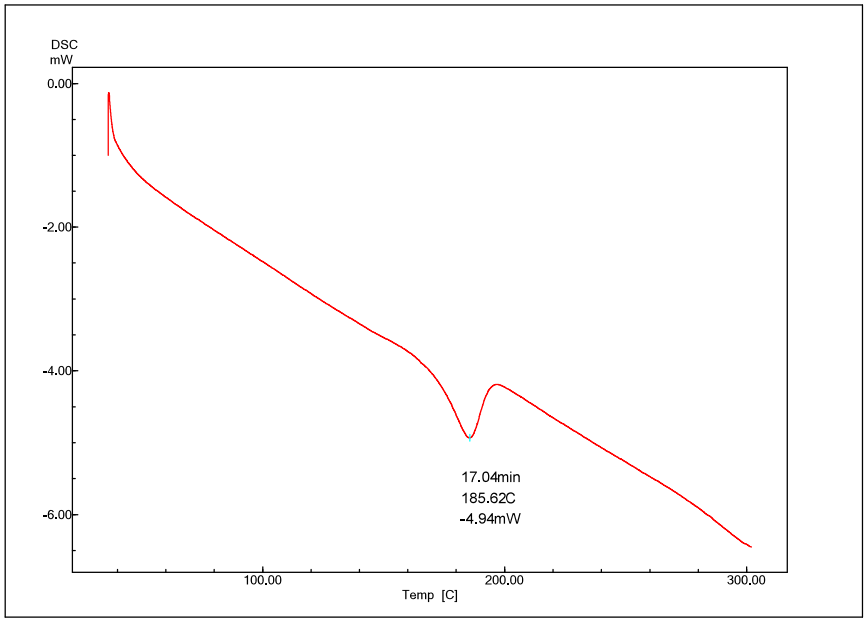

FIGURE 4.16: DSC thermogram PVA

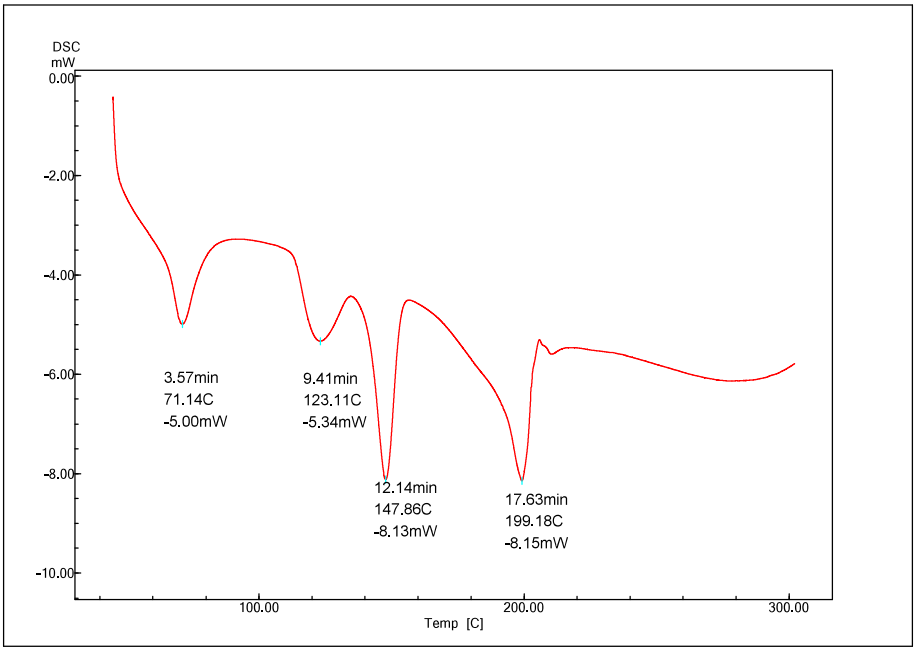

FIGURE 4.17: DSC thermogram of Carrageenan

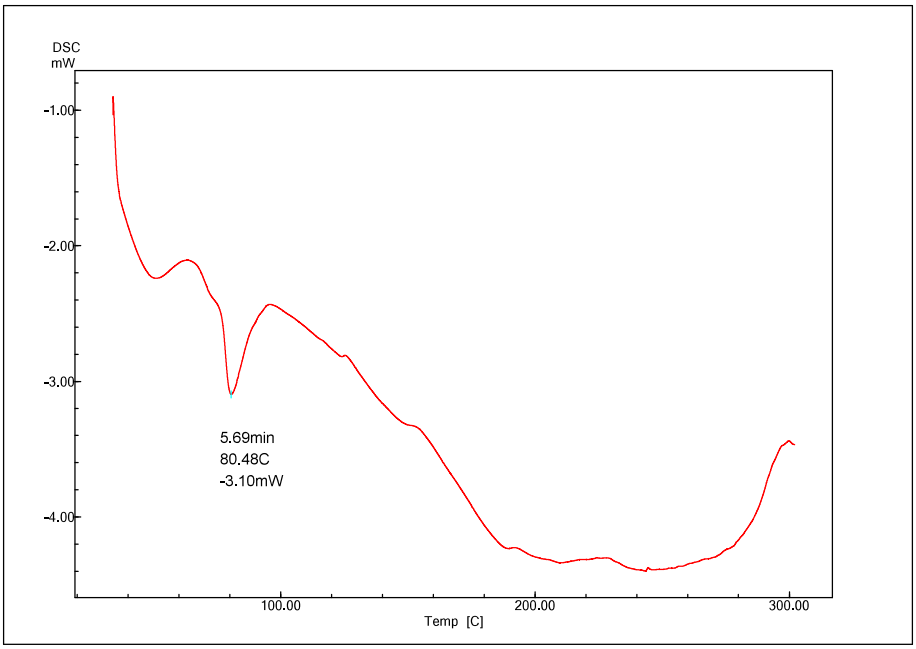

FIGURE 4.18: DSC thermogram of physical mixture of CFE, PVA, carrageenan

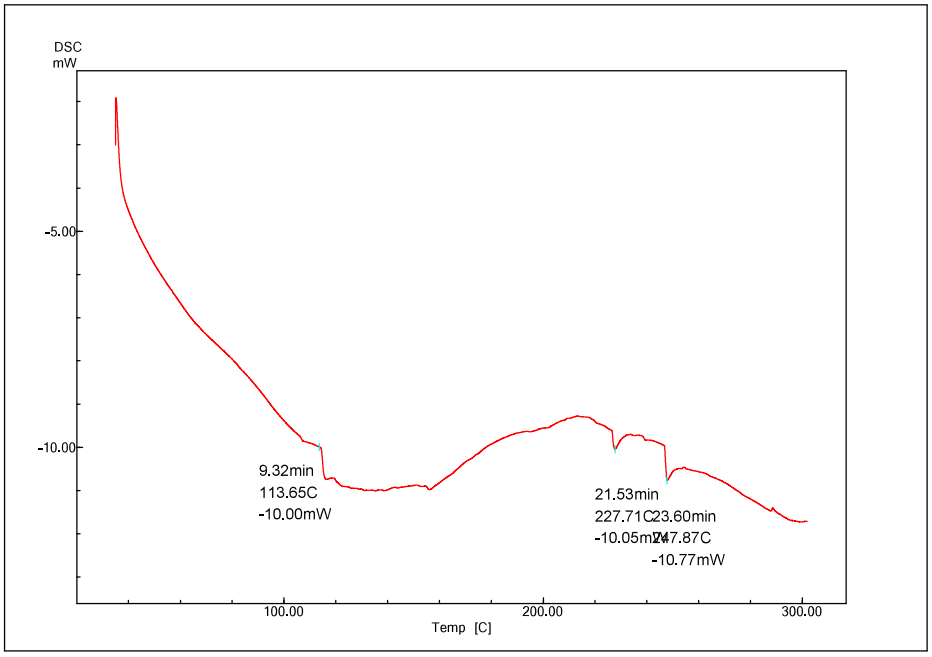

FIGURE 4.19: DSC thermogram of CFE loaded hydrogel sheet

4.4.2.8 ***In-vitro*** **haemolysis study:** Haemolysis can lead to jaundice, anemia and other pathological conditions. Hence product which is directly in contact with blood must be evaluated for its safety in terms of haemolysis potential. In many wounds, there is loss of partial or full skin. So during treatment, there is chance of direct contact of blood with the dressing. Result of *in-vitro* haemolysis study is shown in table 4.14. Less than 1.0% haemolysis was observed with CFE (upto 500 ppm) and CFE loaded hydrogel sheet (equivalent to 500 ppm CFE) which indicated the safety of CFE and developed CFE loaded hydrogel sheet.

TABLE 4.14: Result of % haemolysis study carried out for CFE loaded hydrogel sheet

Sr. No.	Sample Name	UV absorbance *	% Haemolysis (± sd, n=3)
1	Netative control (150 µl erythrocyte + 850 µl sterile saline solution)	0.421 ± 0.004	0
2	Positive control (150 µl erythrocyte + 100 µl of 1% Triton solution + 850 µl sterile saline solution)	0.832 ± 0.023	100 ±0.95012 %
3	Sample 1 (150 µl erythrocyte + 400 µl of CFE-Hydrogel sheet extract + 450 µl sterile saline solution	0.420 ± 0.028	0.243 ± 0.016 %
4	Sample 2 (150 µl erythrocyte + 600 µl of CFE-Hydrogel sheet extract + 350 µl sterile saline solution)	0.419 ± 0.021	0.486 ± 0.004 %
5	Sample 2 (150 µl erythrocyte + 800 µl of CFE-Hydrogel sheet extract + 50 µl sterile saline solution)	0.418 ± 0.026	0.729 ± 0.006%

4.5 REFERENCES

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