5.0 MATERIALS & METHODS: CFE LOADED COLLAGEN FILM (CFE-collagen film)

5.1 MATERIALS: Materials used in preparation and characterization of CFE loaded collagen film are mentioned in table 5.1

TABLE 5.1: List of materials used in CFE-collagen film preparation and characterization

Sr. no.	Material name	Source
1.	Bovine collagen	Mil Laboratories Pvt Ltd
2.	Propylene glycol	Merck, India
3.	Sodium carbonate	SD fine chemical (Mumbai, India)
4.	Aluminum chloride	SD fine chemical (Mumbai, India)
5.	Folin Ciocalteu's phenol reagent	SRL Pvt. Ltd. (Mumbai, India)
6.	Gallic acid	SRL Pvt. Ltd. (Mumbai, India)
7.	Carrageenan	SRL Pvt. Ltd. (Mumbai, India)
8.	Quercetin dehydrate extrapure	SRL Pvt. Ltd. (Mumbai, India)
9.	2,2-Diphenyl-1- picrylhydrazyl	SRL Pvt. Ltd. (Mumbai, India)
10.	Disodium EDTA	SRL Pvt. Ltd. (Mumbai, India)
11.	Pepsin 1:3000 ex. Procine (stomach Mucosa extrapure)	SRL Pvt. Ltd (Maharashtra, India)
12.	N,N-Methylene Bisacrylamide 3x cryst.	SRL Pvt. Ltd (Maharashtra, India)
13.	Bis-Acrylamide solution	SRL Pvt. Ltd (Maharashtra, India)
14.	Hi-range 2 protein marker	SRL Pvt. Ltd (Maharashtra, India) Biolit™
15.	Acrylamide extra pure	SRL Pvt. Ltd (Maharashtra, India)
16.	Coomassie Brilliant Blue R-250	Thermo Fisher Scientific, India

5.2 PREPARATION AND OPTIMIZATION OF CFE-COLLAGEN FILMS

CFE-collagen film was prepared by solvent evaporation technique (1) which consisted of two steps (figure 5.1).

Step 1: Preparation of collagen slurry:

- Bovine collagen was taken into the vessel containing distilled water and homogenized using over head homogenizer at 800 - 1000 rpm at 15° - 25° C for 30 min.
- After 10 min homogeneous collagen slurry was form.
- Hydroglycolic extract of *Calendula* flowers (CFE), sodium salt of hyaluronic acid (HA), di-sodium ethylenediamine tetraacetic acid (EDTA), and propylene glycol (PG) were dissolved into distilled water into another vessel with stirring using overhead mechanical stirrer. Resulting solution was added to collagen slurry and mixed for 20 min at 800 -1000 rpm using mechanical stirrer.
- Air bubbles were formed due to aeration which were removed by vacuum.

Step 2: Air drying of collagen slurry:

- Prepared slurry was transfer into the trays and air dried under laminar flow for 24hr at room temperature.
- After 24 hr, films were dried which was carefully removed from the trays.
- The dried films were packed into the pouch and sterilized by γ-rays irradiation at 25kGy radiation dose (Universal Isomed, Gujarat, India).

Various trials were taken with different collagen content (0.25% to 1.25%) and plasticizer (PG) content (0 -1.5% w/w) using One-variable-at-a-time (OVAT) method to optimized formulation. The prepared films were characterized for physicochemical parameters such as appearance, thickness, folding endurance, pepsin digestion time, and fluid absorption capacity. The optimized batch of CFE-collagen film was further evaluated for DSC, FT-IR, AFM and SDS-PAGE.

CHAPTER 5: CFE-COLLAGEN FILM

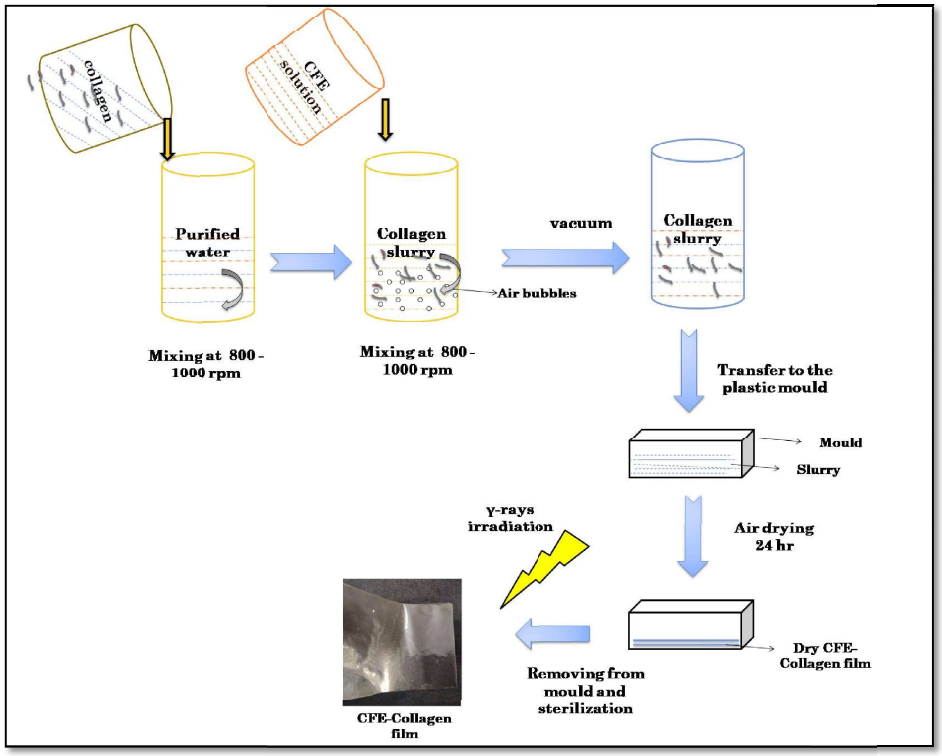
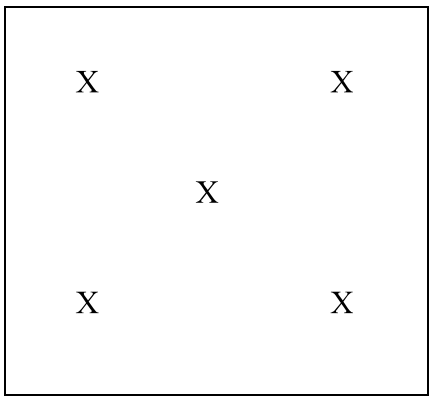


FIGURE 5.1: Flow diagram for the preparation of CFE-Collagen film

5.3 CHARACTERIZATION OF CFE-COLLAGEN FILMS

5.3.1 PHYSICAL APPEARANCE: The prepared films were visually observed for surface smoothness, transparency, stickiness and other physical appearance.

5.3.2 THICKNESS: At different 5 positions, CFE-collagen film was analyzed for thickness by using digital micrometer. A 2.5 cm x 2.5 cm film sample was used for study.



5.3.3 pH: pH of CFE-collagen film was measured using digital pH meter. For that, about 0.5 g sample was taken into 50.0 ml of distilled water. Sample was stirred for 30 min at 100-200 rpm using magnetic stirrer. Then after pH of water was checked using digital pH meter.

5.3.4 FOLDING ENDURANCE: It was determined manually by repeatedly folding the film at same place till it broke. The no. of times the film could be folded at the same place without breaking gave the value of folding endurance.

5.3.5 FLUID ABSORPTION CAPACITY (G/G): Pre-weighed film was placed in 15 ml of simulated wound fluid and the weight of the film was noted periodically. Every time after noting the weight. Fluid absorption capacity of the film was determined in triplicate and calculated using equation 5.1.

$$\text{Fluid absorption capacity (g/g)} = \frac{Final\ weight\ (g) - Initial\ weight(g)}{Initial\ weight\ (g)}$$

….Equation (5.1)

5.3.6 MOISTURE CONTENT: %Moisture content of collagen film was determined using loss on drying (LOD) method. About 0.250 g of film was taken into clean, dry LOD bottle and kept in hot air oven at 115° C till constant weight *(final weight)*. % Moisture was calculated using equation 5.2.

$$\%Moisture\ content = \frac{Initial\ weight\ of\ sample\ (g) - Final\ weight\ of\ sample\ (g)}{Initial\ weight\ of\ sample\ (g)} * 100$$

…Equation (5.2)

5.3.7 PEPSIN DIGESTION TIME: Collagen film was placed into test tube containing 15 ml of 1% w/v pepsin solution prepared in 0.1N HCL at 37° C. Time required for complete digestion of sample was noted.

5.3.8 DIFFERENTIAL SCANNING CALORIMETRY (DSC) STUDY: DSC analysis was 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 obtained thermo-grams were observed.

5.3.9 FOURIER-TRANSFORM INFRARED (FT-IR) SPECTROSCOPY: FT-IR-spectrum of formulation, collagen, CFE and physical mixture 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).

5.3.10 TOTAL POLYPHENOLIC CONTENT: Colorimetric method based on Folin Ciocalteu's phenol reagent was used for the determination of total phenolic content of CFE-collagen film *(2).* Sample was mixed with 2 ml of Folin Ciocalteu's phenol reagent (10% v/v) and 1 ml of aqueous sodium carbonate

(20%, w/v). The mixture was vortexed and diluted with water to a final volume of 10 mL and incubated for 30 min at room temperature. The absorbance was measured at 765 nm using UV-Visible spectrophotometer (Shimadzu, Japan). The total phenols were expressed as mg of gallic acid equivalents per g of sample (mg GAE/g) and it was calculated using a calibration curve of a freshly prepared gallic acid solution (2-10 ppm) (3).

5.3.11 TOTAL FLAVONOID CONTENT: The flavonoid content was determined spectrophotometrically based on the formation of complex flavonoid aluminum, having the maximum absorption at 310 nm (3). Sample was mixed with a 10% solution of aluminum chloride, 5% sodium nitrite solution and 1 M NaOH solution (3). After 30 min, samples were analyzed using and total flavonoid content was calculated using quercetin calibration curve. The flavonoid content was expressed in mg of quercetin equivalent per gram of sample (mg QE/g).

5.3.12 ESTIMATION OF ANTIOXIDANT ACTIVITY BY DPPH ASSAY: Colorimetric assay based on 2,2-diphenyl-1-picryllhydrazyl (DPPH) was used to estimate the radical scavenging capacity of the CFE-collagen film (4). The samples were treated with 1.0 ml of 1.0 mM DPPH methanol solution for 30 min and absorbance was measured at 517 nm by UV-Visible spectrophotometer (Shimadzu, Japan). The radical scavenging activity that is % inhibition of DPH free radical was calculated using the blank sample and the formulation samples (equation 5.3). The results were expressed as quercetin antioxidant activity equivalent per g of sample (µmol QE/g of sample).

$$\%\ Inhibition = \frac{A\ (blank) - A\ (sample)}{A\ (blank)} * 100 \quad \text{..... Equation (5.3)}$$

5.3.13 *IN-VITRO* HAEMOLYSIS STUDY: 3 ml of blood from rat was drawn directly into 0.1% K_2-EDTA containing centrifugation tubes to prevent coagulation which was centrifuged at 3000 rpm for 10 min at 4° C for separation of hematocrit (red, lower layer) and plasma (yellowish, upper layer) content in blood. From that,

plasma was aspirated using micropipette and equal volume was replaced with sterile saline solution. After gentle mixing, centrifugation was repeated. Plasma was replaced with sterile saline solution and gentle mixing was carried out. From that, 150 µl was taken into five tubes and labeled as following (a) positive control (850 µl saline solution), (b) negative control (100µl of 1% Triton solution), (c) test sample 1(500 µg CFE) and (d) test sample 2 (CFE-collagen film equivalent to 500 µg CFE). Volume of each tube was adjusted upto 1.0 ml by adding sterile saline solution and incubated for 1 hr at room temperature. After incubation period, all tubes were centrifuged at 10,000 rpm for 5 min at 4° C and supernant was analyzed using UV spectrophotometer at 541 nm wavelength. % Haemolysis was calculated using equation 5.4.

$$\%\ Haemolysis = \frac{A\ (positive\ control) - A\ (sample)}{A\ (negative\ control) - A(positivve\ control)} * 100$$

……. Equation (5.4)

5.3.14 ATOMIC FORCE MICROSCOPY (AFM): The microstructure of the film was observed by AFM (SHIMADZU SPM 9600, Japna) in dynamic mode at room temperature (~20 degree C). Each sample was scanned with a scanning rate of 1 Hz and AFM images were obtained.

5.3.15 SODIUM-DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE): The gel separation (SDS-PAGE) was performed following the protocol described by Sambrook and Russel (5). The bands were observed visually and the molecular weight was determined by comparing with the protein marker (Hi-range 2 protein marker, SRL, India).

Procedure :

i. Preparation of 8% separating gel: An 8% acrylamide solution was prepared and poured into the plates for casting. The solution was allowed to stand for 45 min to completely polymerize the gel (figure 5.2).

ii. Preparation of 5% staking gel over the separating gel: after 45 min, 5% stacking gel solution was poured over the separating gel. A comb was inserted and kept for 45 min to completely polymerize the gel.

iii. Comb was removed from the gel and gel sheet was washed with the distilled water.

iv. Tank buffer was added to the top reservoir and bottom tank in gel electrophoresis instrument.

v. The samples were dissolved in SDS sample buffer solution (1% SDS, 1% mercaptoethanol, 20% glycerol). The sample (40 microliter) was subjected to gel electrophoresis (Vertical midi gel system-05-03, GeNei^{TM} , Merck specialities pvt ltd, Mumbai, India) at a constant current of 5 V/cm. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 and the gel images were captured.

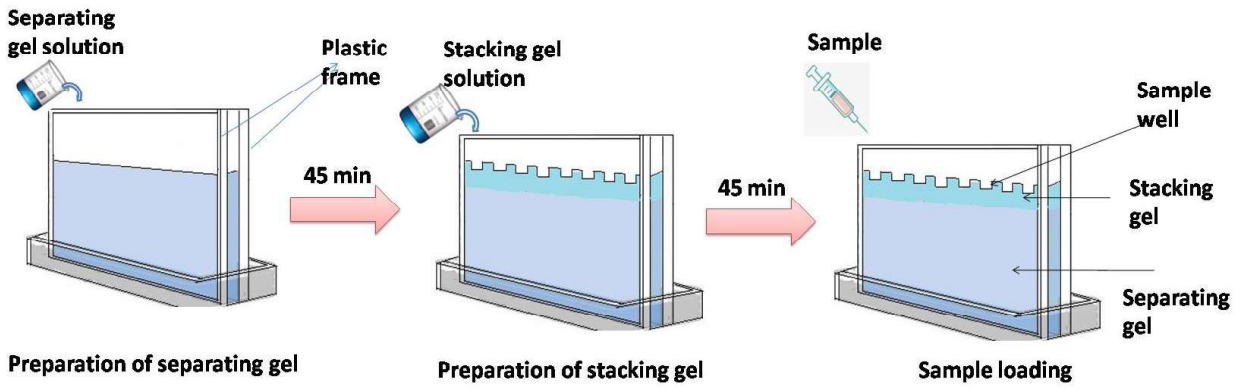


FIGURE 5.2: Gel Electrophoresis: preparation of separating gel and stacking gel

5.4 RESULTS AND DISCUSSION:

5.4.1 PREPARATION OF CFE-COLLAGEN FILM:

- All films were slightly yellowish, transparent and flexible with a smooth surface (figure 5.3).

FIGURE 5.3: CFE-collagen film

- Collagen is a thermo labile protein so that all mixing process and drying process were carried at room temperature.
- Trials were carried out for optimization of collagen content shown in table 5.2.

TABLE 5.2: Effect of collagen content on physicochemical parameters of film

Code	Collagen content (%w/w)	Thickness (mm)*	Folding endurance	Fluid absorption capacity (g/g) *	%LOD*	Pepsin digestion time (min)*	pH*
F1	0.25%	0.023 ± 0.007	50	3.14± 0.44	7.5± 1.4	90	3.7 ± 0.1
F2	0.5%	0.053 ± 0.002	50	5.2 ± 0.174	11.1 ± 0.9	240	3.75 ± 0.1
F3	0.75%	0.076 ± 0.004	80	9.89 ± 0.70	10.4 ± 0.5	240	3.46 ± 0.1
F4	1.0%	0.086 ± 0.004	>100	14.89 ± 1.43	10.5 ± 0.8	270	2.93 ± 0.2
F5	1.25%	0.098 ± 0.012	>150	16.24± 0.78	9.8 ± 0.7	315	2.78 ± 0.2

*±SD, n=3

- The transparency and thickness of the film varied with the collagen content. The film became more thick and opaque with increasing collagen content from 0.25% to 1.25%w/w. Thickness of films incorporated with CFE ranged from 0.01 to 1.1 mm. If collagen film is very thick then it will take more time for bio-degradation so that more time will be required to absorb the collagen into the body. Films prepared with 0.25%, 0.5% and 0.75% collagen were very thin and brittle having less pepsin digestion time due to less collagen content. Films prepared with 1.25% collagen were opaque and thick. Film prepared with 1.0% w/w collagen was thinner than film prepared by 1.25% so seem to more transparent.
- pH of all films were in acidic range (2.7 to 3.8) as collagen used here was acid swollen.
- Folding endurance was determined to find the flexibility of film which is needed to handle the film easily and for comfortable and easy application on wound. Folding endurance time of film was increased from 50 to 150 with increased in collagen content from 0.25% to 1.25%. Films prepared with less than 1.0% collagen were very thin and brittle having poor mechanical strength, less folding endurance time as compare to film prepared with more than 1.0% collagen.
- During drying process, films were shrunk due to interaction between nonpolar and polar amino acid of collagen making them brittle and stiff in nature and also

difficult to remove from mould after drying. To make film more flexible, propylene glycol as a plasticizer was added (27, 28). Results for the optimization of propylene glycol (PG) content are shown in table 5.3.

- Prepared films with PG were more transparent, flexible and easy to remove from mould after drying compared to film prepared without plasticizer.
- Folding endurance and %LOD were increased with increase in PG content.
- PG improved the flexibility of collagen film which was reflected in increased folding endurance. PG increases the polymer chain flexibility, resistance to fracture and increases optical clarity of film (29, 30). High concentration of plasticizer also effects negatively on the tensile properties (27).
- %LOD of film was increased from 10.5 % to 14.0% with increase in PG content from 1.0% to 1.5% respectively due to humectant properties of PG. Films prepared with 1.5% PG were very sticky and had high moisture content.
- Thus 1.0% of PG content was found to be optimized. Pepsin digestion time of film was not affected by change in PG content as it does not interfere in biodegradation of collagen.

TABLE 5.3: Effect of plasticizer on physicochemical parameters of film

Trial	PG	Thickness *	Folding endurance	Fluid absorption capacity (g/g) *	%LOD*	Pepsin digestion time (min)*	pH*
F4	0.0%	0.086 ± 0.004	>100	14.89 ± 1.43	10.5 ± 0.8	270	2.93 ± 0.2
F6	0.5%	0.098 ± 0.006	>150	15.0 ± 0.94	9.8± 1.15	270	3.16 ± 0.1
F7	1.0%	0.13± 0.01	>200	15.50 ± 1.3	10.8± 0.57	270	3.21± 0.1
F8	1.5%	0.16 ± 0.02	>200	16.0 ± 1.1	14.0± 0.98	270	3.9 ± 0.2

*±SD, n=3

- Pepsin digestion time was determined to check biodegradability of film in digestive enzyme. Developed films were completely biodegradable as all films

were digested in pepsin solution within 315 min. Biodegradation time can be increased by crosslinking of collagen. Here, we did not crosslink collagen because we aimed maximum amount of native collagen without any chemical irreversible modification which generally happen in crosslinking process and another reason was that we did aim sustained release of *Calendula* flower extract at site of application as crosslinking of collagen retard the release of extract.

- Fluid absorption capacity is one of most important parameter of dressing when they are used for wound healing. It is used to measure the capacity of film to absorb wound exudates. Prepared films had good water absorbing capacity due to hydrophilic groups such as carboxyl, hydroxyl and amidogen of collagen. Fluid absorption capacity of film had increased from 3.14± 0.44 to 16.0 ± 1.1g/g with collagen content from 0.25% w/w to 1.25%. There was slight increment in moisture content of film with collagen content due to hydrophilic fibril matrix of collagen.
- EDTA and sodium hyaluronate were used into formulation to enhance wound healing process. EDTA prevents and reduces biofilm formation, colonization and proliferation in wound by hindering the adhesion of bacteria at low concentration (32-34). Many commercially available products used for wound care contain EDTA. EDTA chelates cations, inhibits MMP activity and helps in wound healing. Sodium hyaluronate is biodegradable, nontoxic and biocompatible natural polymer. Hyaluronan binds with three major classes of surface receptor CD44, RHAMM and ICAM- 1 involved in the modulation of tissue repair and in various other functions which affects cell proliferation, motility and adhesion and help in wound healing process (35).

5.4.2 TOTAL POLYPHENOLIC, FLAVONOID CONTENT AND DPPH ASSAY:

- Standard calibration plot of gallic acid concentration vs. average absorbance was drawn from the absorbance value of the gallic acid solutions (2 – 12 ppm) which is shown in figure 5.4. Straight line equation of plot is mentioned in equation 5.5

  $y = 0.092x + 0.055$ ($R^2 = 0.099$) …….Equation (5.5)

  Using straight line equation (5.5) total polyphenolic content of CFE-collagen film was calculated and it was found to be 5.3510 ± 0.273 mg GAE/g

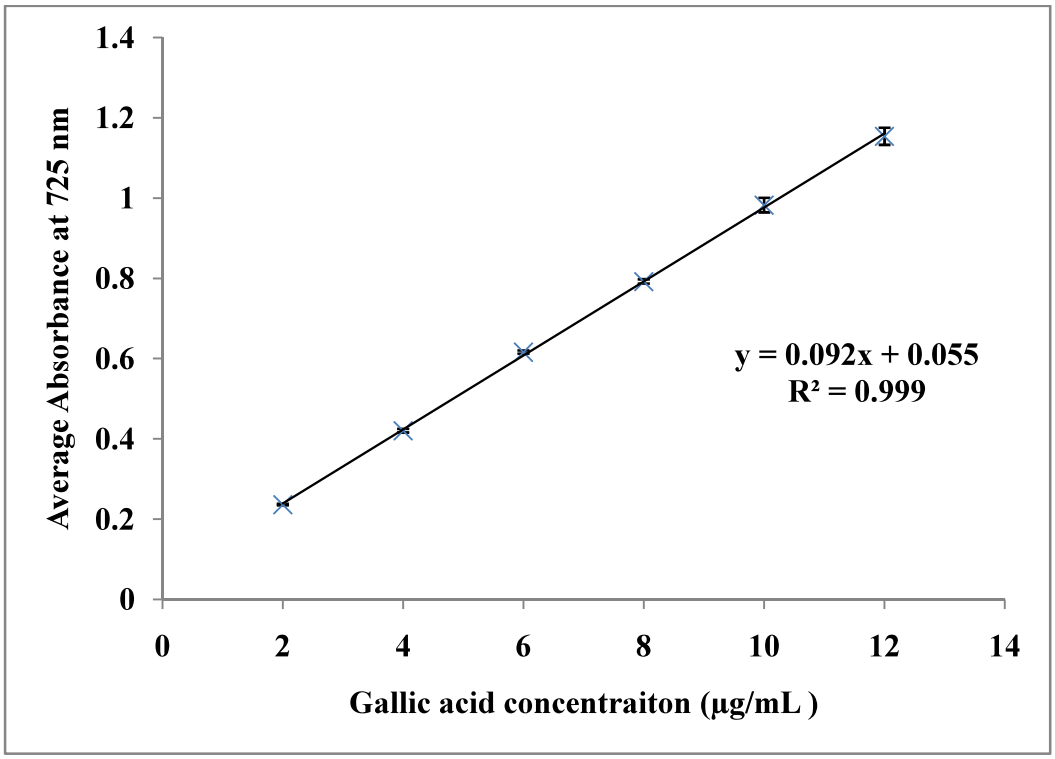


FIGURE 5.4: Standard calibration plot of gallic acid solution

- Standard calibration plot of quercetin concentration vs. average absorbance was drawn (figure 5.5) and straight line equation of plot was found to be

  $y = 0.089x + 0.270$ ($R^2 = 0.998$) …….Equation 5.6

- From the straight line equation of standard calibration plot of quercetin dehydrate solution (4-20 ppm), total flavonoid content of CFE-collagen film was calculated and it was found to be 5.14 ± 0.92 mg QUE/g.

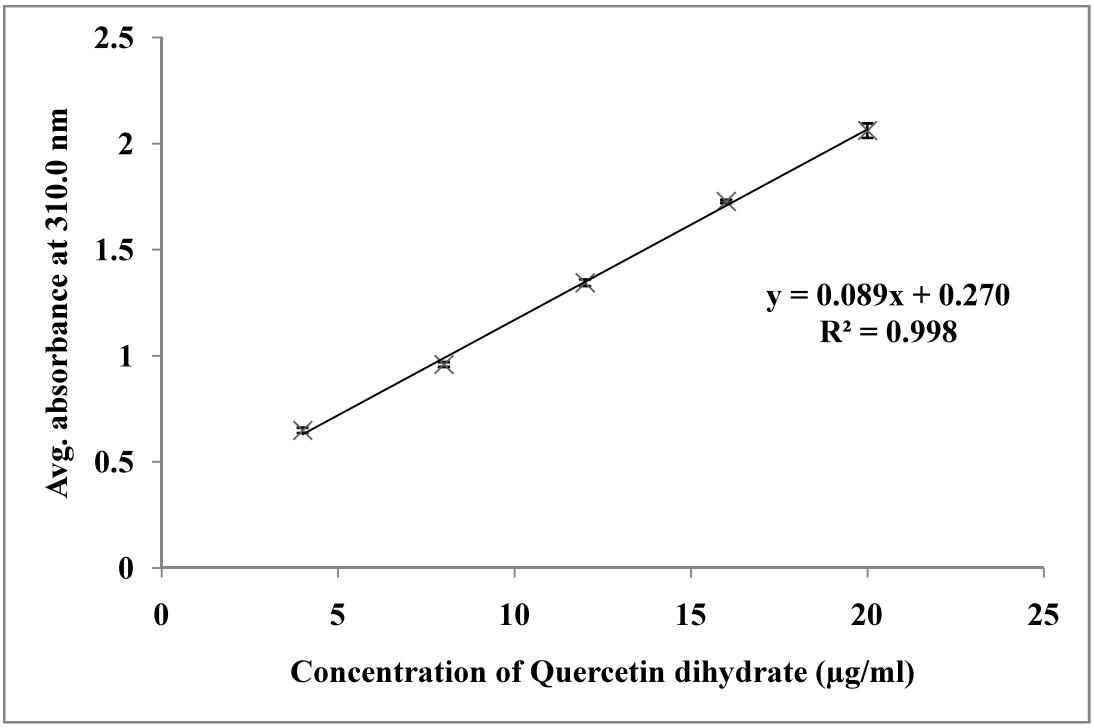


FIGURE 5.5: Standard curve of quercetin dehydrate solution

- The standard calibration curve of %Inhibition of free DPPH radical vs. Concentration of quercetin solution (µg/mL) was prepared from the % inhibition activity of quercetin solution which is shown in figure 5.6. Free radical scavenging capacity of CFE-collagen film was calculated from the straight line equation of standard calibration curve (equation 5.7) which was found to be 0.8987 ± 0.057 µmol QUE/g

$$y = 14.05x + 0.950 \quad (R^2 = 0.993) \ldots\ldots\ldots\ldots\ldots\text{Equation (5.7)}$$

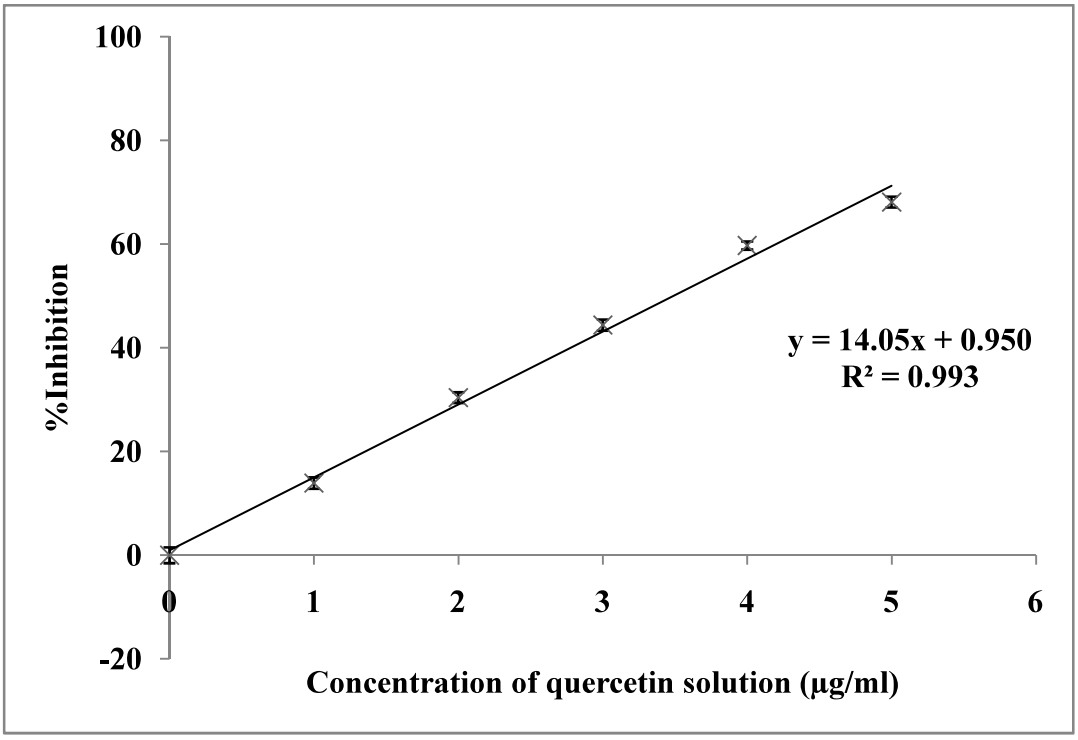


FIGURE 5.6: Standard calibration curve of %Inhibition vs. Concentration of quercetin solution

Phenolic compounds play an important role in the capture and neutralization of free radicals due to their chemical structures (25). Free radical scavenging abilities of CFE-collagen film using the DPPH assays were investigated. DPPH is used to find out the antioxidant activity of substance by its ability to scavenge free DPPH radical. Free radical scavenging activity of collagen film loaded with CFE was observed from its extract. Calendula flower extract contain polyphenols, carotenoids and flavonoids which scavenge free radicals –DPPH which help in wound healing (11, 36). Non-phagocytic cells of wound generate free radicals which break DNA, lipid peroxidation and enzyme inactivation that retard the normal wound healing process (37, 38). CFE-collagen film prevents breakdown of DNA, lipid peroxidation and enzyme inactivation by scavenging free radicals and hence enhance wound healing process (39).

5.4.3 *IN-VITRO* HAEMOLYSIS STUDY: Haemolysis in body can lead to jaundice, anemia and other pathological conditions. In many wounds there is loss of partial or full skin, in that case dressing will be in direct contact of blood during treatment. Hence it must be evaluated for its safety in terms of haemolysis. There was less than 1.0% haemolysis observed with 500 ppm solution of CFE and CFE-collagen film extract equivalent to about 500 ppm CFE which indicated safety of CFE and developed formulation as less than 5% haemolysis consider as a hemocompatible (table 5.4).

TABLE 5.4: Result of *in-vitro* haemolysis study with CFE-collagen film

Sr. No.	Sample Name	UV absorbance at λmax = 540 nm	% Haemolysis
1	Netative control (150 µl erythrocyte + 850 µl sterile saline solution)	0.441 ± 0.002	0 %
2	Positive control (150 µl erythrocyte + 100 µl of 1% Triton solution + 750 µl sterile saline solution)	0.840 ± 0.002	100 ± 0.238 %
3	Sample 1 (500 ppm CFE)	0.437 ± 0.004	1.002 ± 0.009 %
4	Sample 2 (500 ppm CFE)	0.436 ± 0.004	1.253 ± 0.015 %

5.4.4 SDS-PAGE: The SDS PAGE images (figure 5.7) of collagen and CFE-collagen film shows two bands with the molecular weight about 95 to 116 kDa and one band with molecular weight about 220 kDa. This result indicates that no degradation of collagen occurred during film formation process as collagen and collagen film had similar SDS PAGE profile. The SDS PAGE results also revealed that the collagen is in its native form by displaying one β band and two α bands which were the unfolding polypeptide chains of collagen triple helix (40-42).

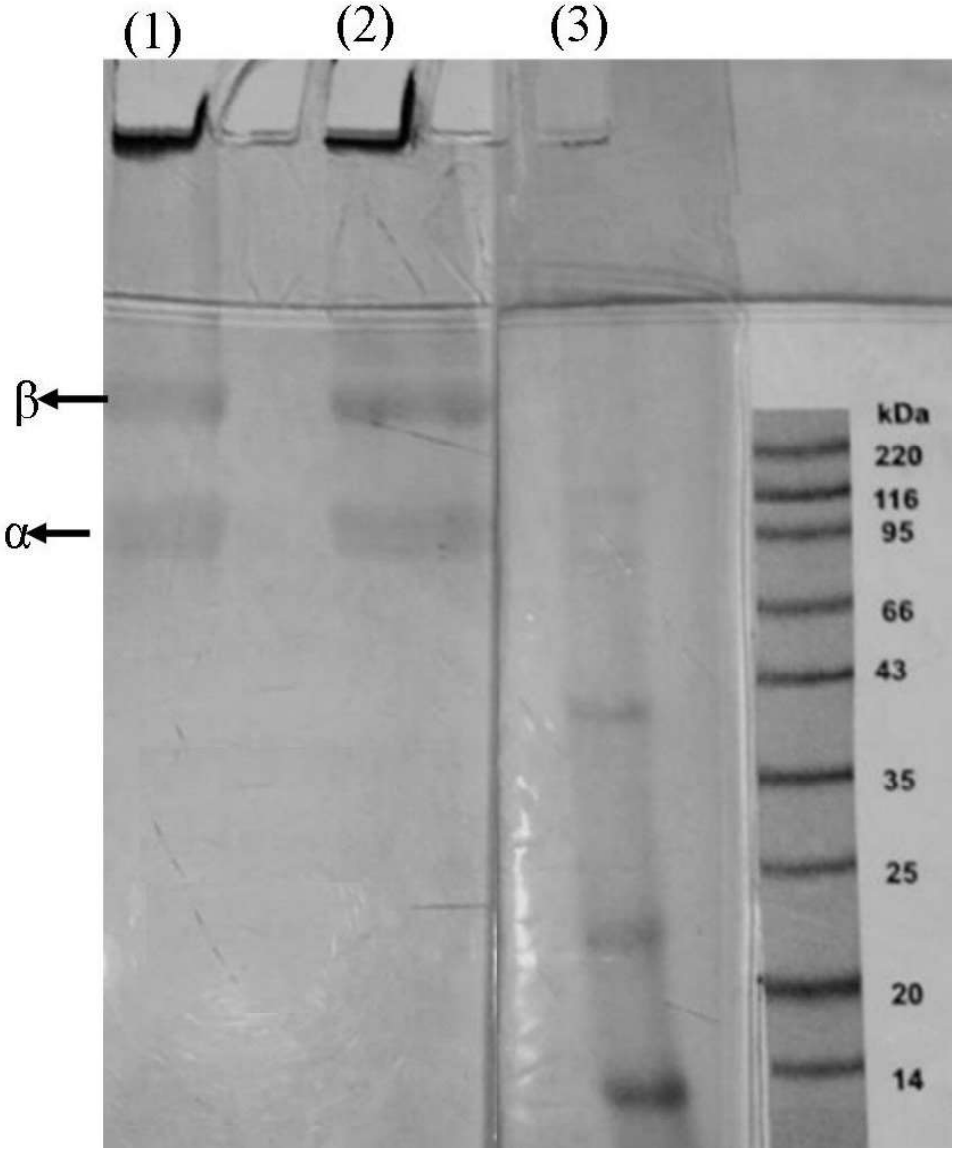


FIGURE 5.7: SDS-PAGE profile of (1) collagen, (2) collagen film and (3) marker protein

5.4.5 OPTICAL MICROSCOPY & AFM STUDY: Clumps of several fibrils of collagen can be seen when viewed by optical microscope (Nikon Eclipse TS100, Japan) as shown in figure 5.8. 3D helical structure of collagen fibril cannot be confirmed by optical microscopy. To further explore the surface microstructure, the film was analyzed by AFM. AFM study is providing the information of surface morphology and 3D helical structure of fibril for the sample film. 2D and 3D AFM images of collagen film (fig. 5.9) demonstrate the triple helix conformation of collagen fibrils which is a characteristic of native collagen. Characteristic periodic banding of native collagen is also apparent in AFM images of collagen film. Banding periodically can be determined by simply measuring and averaging the longitudinal distance between peaks or valleys. In fig. 5.9 (iii) shown a Height–profile taken with dashed line of fig 5.9 (i) that clearly shows periodic banding pattern with periodicity of 40 -70 nm which is due to special micro structural "quarter stagger" arrangement of collagen molecule which result in "gap" and "overlap" regions (43-45). Native collagen (type I) fibrils have characteristic nano-topographical periodic patterns of 40-60 nm which are believed to be beneficial for cell growth and cell migration (43-47). Native collagen also provides the tripeptide Arg-Cly-Asp (RGD) binding sites for cell adhesion. It may be noted that type I collagen is superior to other types of collagen (48)(49, 50).

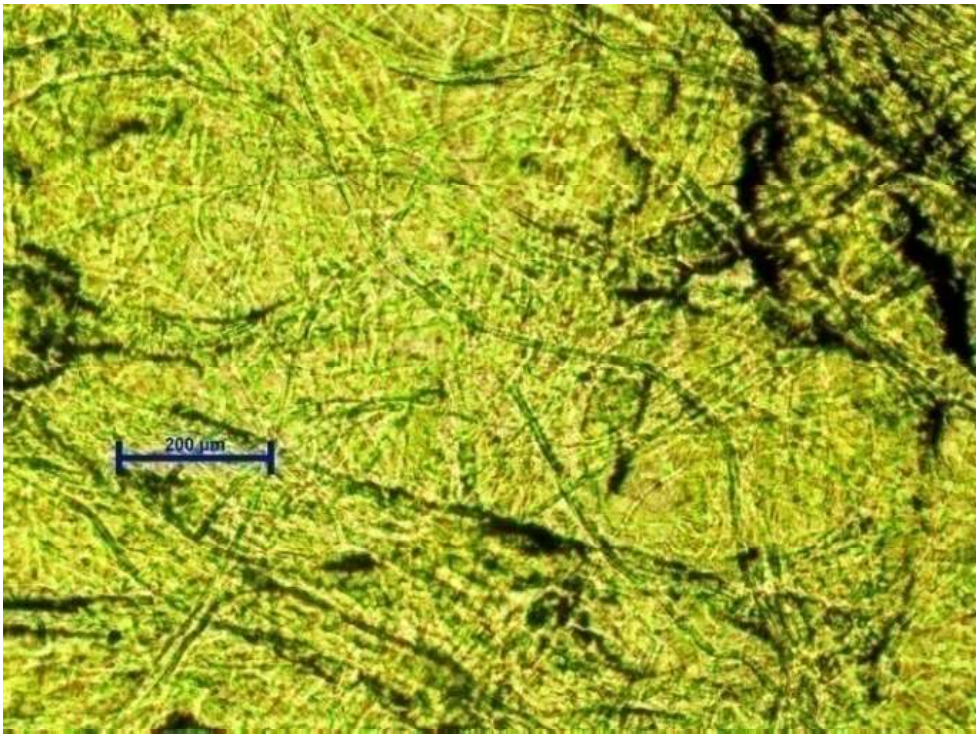


FIGURE 5.8: Optical microscopy of CFE-collagen film (magnification 20x)

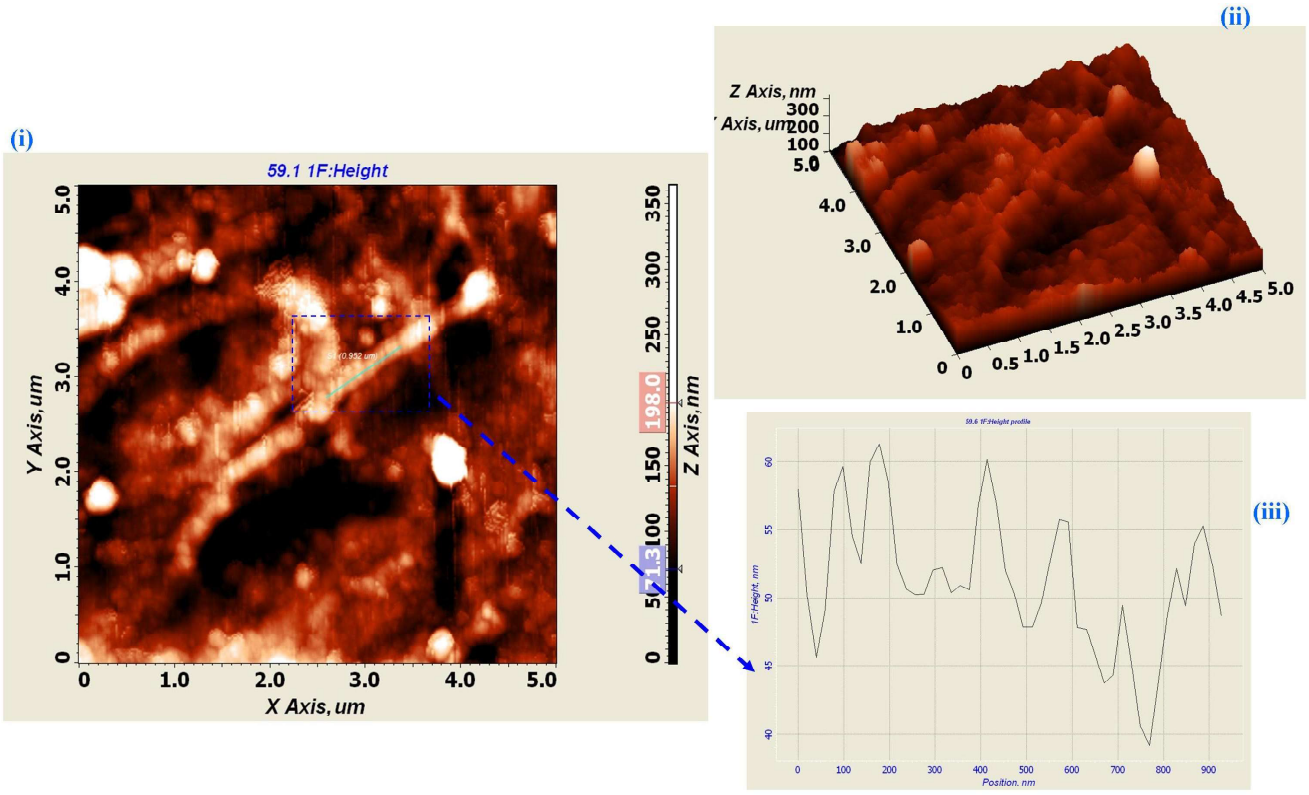


FIGURE 5.9: Atomic force microscope topography images of collagen film (i) 2D images (ii) 3D images and (iii) Height-profile taken of the dashes line part shown in figure 5.9 (i) 2D image

5.4.6 DSC STUDY: The DSC thermograms of CFE figure 5.10. CFE is completely amorphous in nature confirmed from broad endothermic peak in its thermogram. Endothermic peak at 86.29°C observed in collagen's DSC thermogram (figure 5.11) that shift to higher temperature at 133° C in CFE-collagen film (figure 5.12). It suggests the thermal stability of the film has been enhanced. It might be due to polyphenols which improves the stability of collagen matrix and increase the shrinkage temperature of film (70-71). There is one endothermic peak at 208° C in CFE-collagen film which is due to EDTA. There was no exothermic peak in collagen film thermogram which confirms absence of chemical reaction between the ingredients.

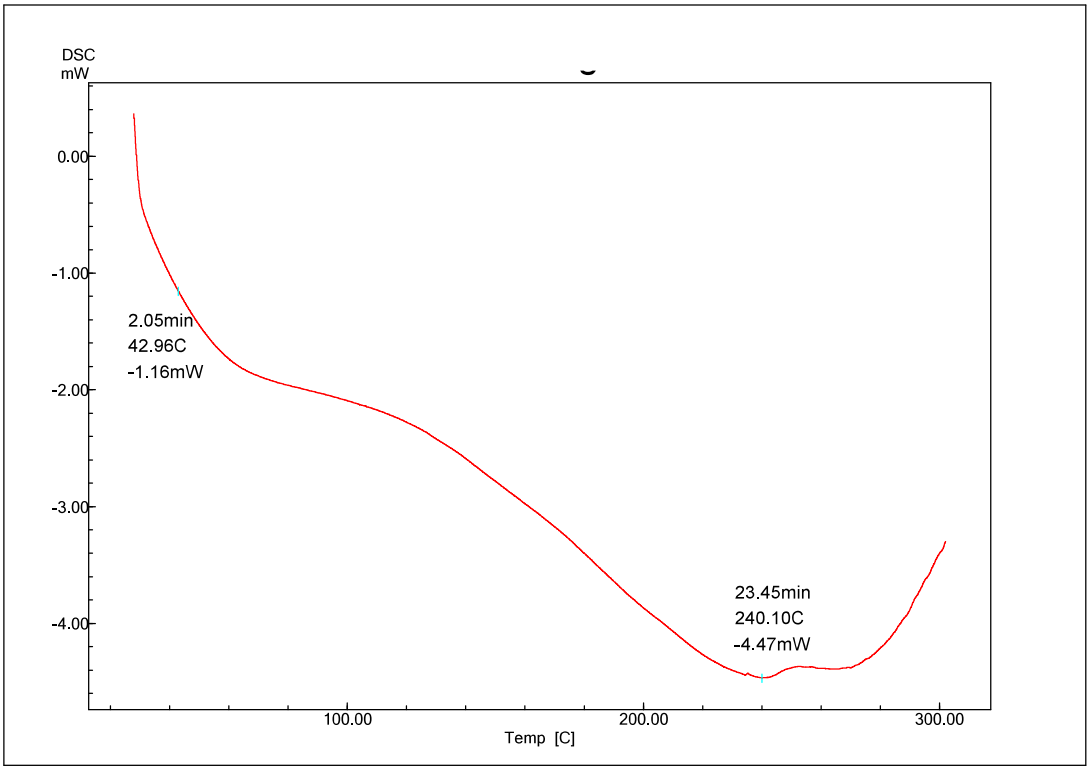


FIGURE 5.10 : DSC thermogram of CFE

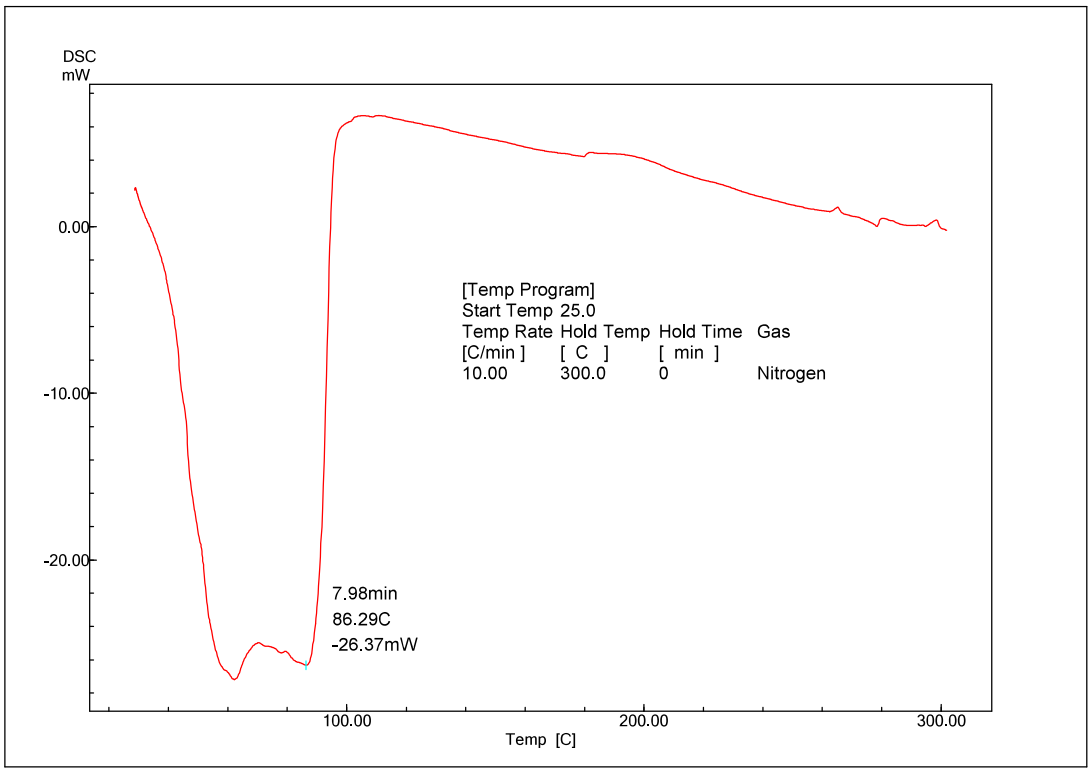


FIGURE 5.11: DSC thermogram of Collagen

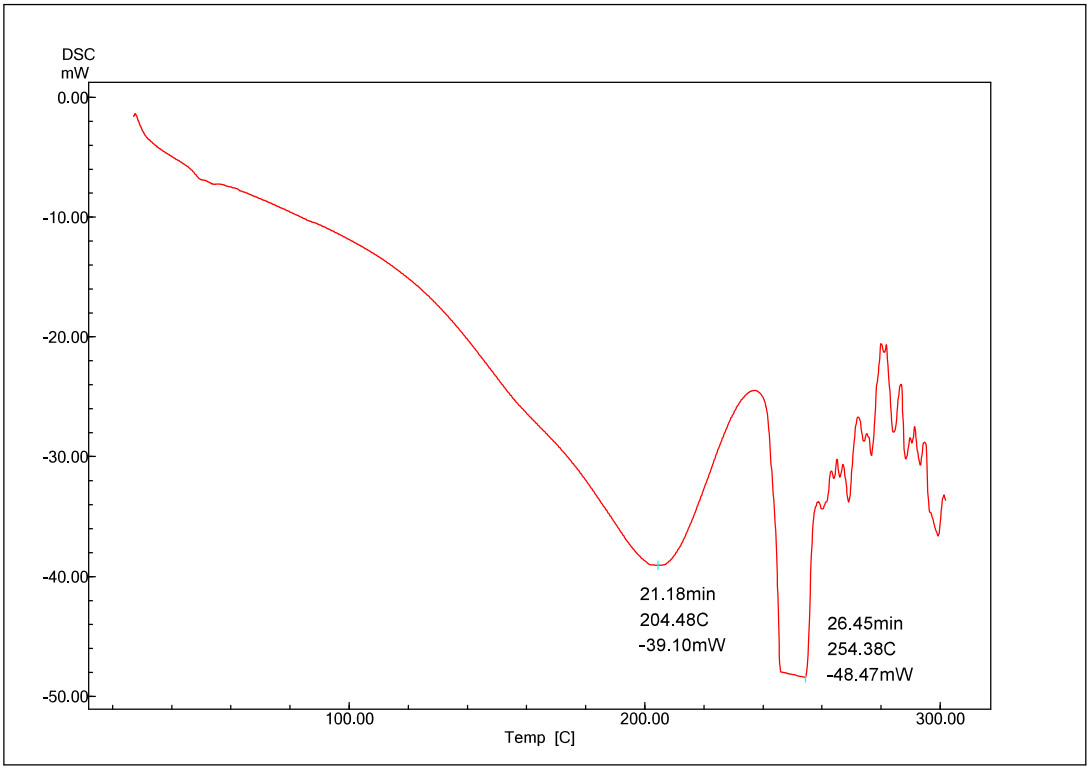


FIGURE 5.12: DSC thermogram of EDTA

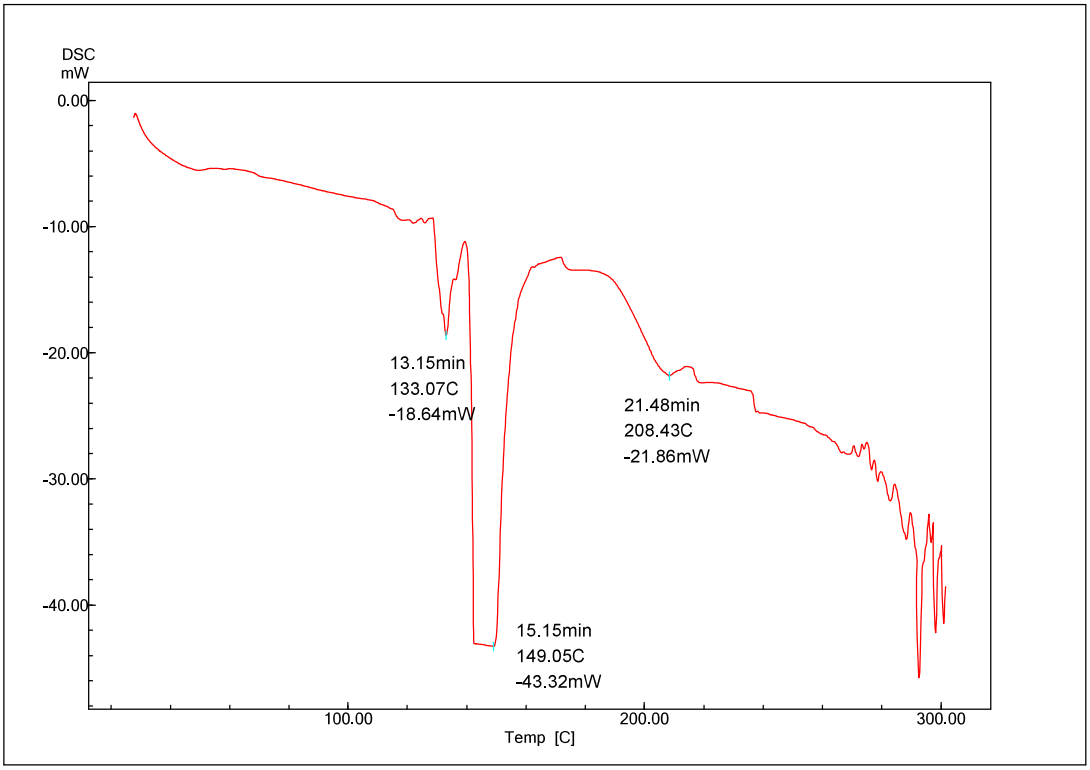


FIGURE 5.13 : DSC thermogram of CFE-collagen film

5.4.7 FT-IR STUDY:

- FT-IR spectra of CFE is depicted in fig. 5.14. FT-IR analysis of CFE proved presence of phenolic alcohols (3318.25 cm^{-1}), ketones group (1785.63 cm^{-1}), alkanes (1438.05 cm^{-1}), saturated amine and aliphatic amine groups (1078.27 cm^{-1}, 1105.02 cm^{-1}), and alkyl halides (517.53 cm^{-1}, 565.33 cm^{-1}) which were also reported by Al-Mussawi et. al., 2019 (51).

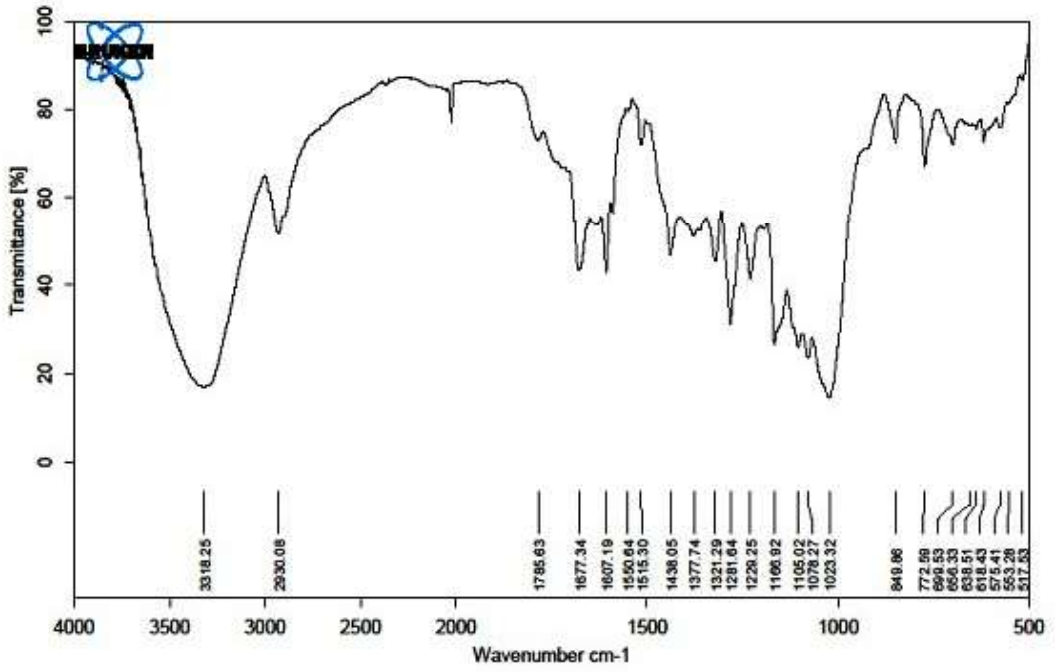


FIGURE 5.14 : FT-IR spectra of CFE

- A FT-IR spectrum of collagen is shown in figure 5.15. Collagen exhibited four characteristic absorption bands at 3326, 1658, 1554 and 1240 cm-1 for amide A band, amide I band, amide II band and amide III band respectively.

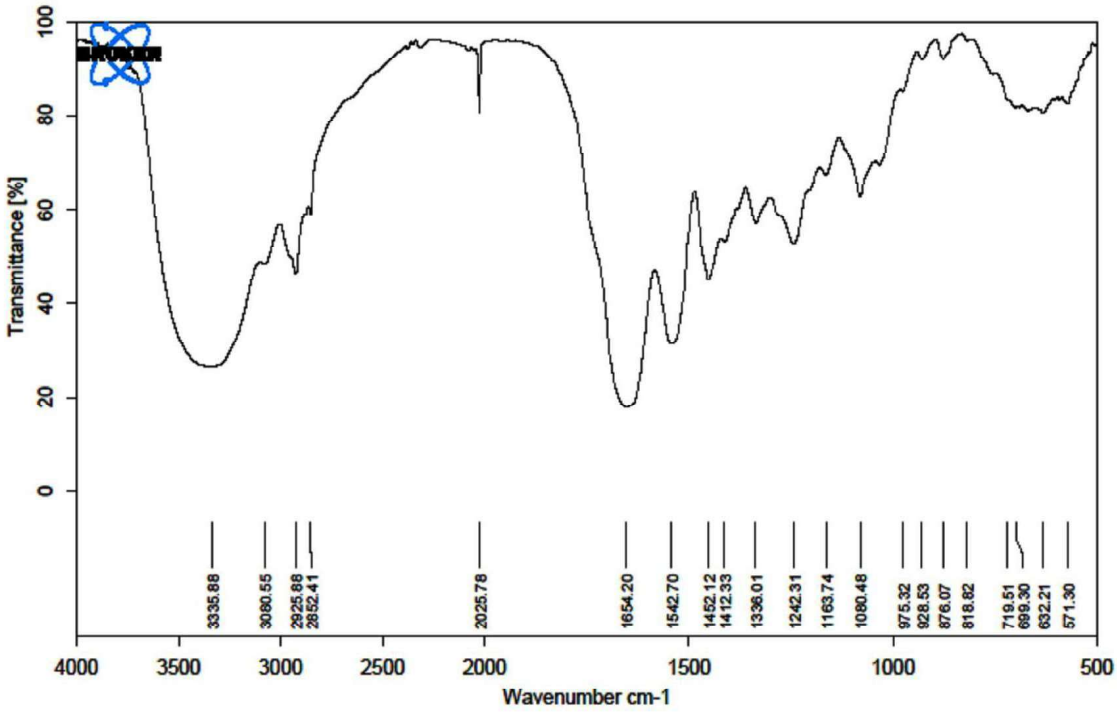


FIGURE 5.15 : FT-IR spectra of Collagen

- A FT-IR spectrum of CFE- collagen film is shown in fig. 5.16. Characteristic peaks of CFE [phenolic alcohols (3318.25 cm^{-1}), ketones group (1785.63 cm^{-1}), alkanes (1438.05 cm^{-1}), saturated amine and aliphatic amine groups (1078.27 cm^{-1}, 1105.02 cm^{-1}), and alkyl halides (517.53 cm^{-1}, 565.33 cm^{-1})] are present in the FTIR spectra of CFE-collagen film. Both collagen and collagen film showed similar IR absorbance patterns. CFE-collagen film exhibited four characteristic absorption bands at 3326, 1658, 1554 and 1240 cm-1 for amide A band, amide I band, amide II band and amide III band respectively which are also observed into FTIR spectra of collagen. For film, the OH stretches (3318.25 cm^{-1}) and asymmetric COO stretching (3081.76 cm^{-1}) shifted to a lower wave number which indicates the existence of N-H stretching vibration, coupled with hydrogen bonding (72).

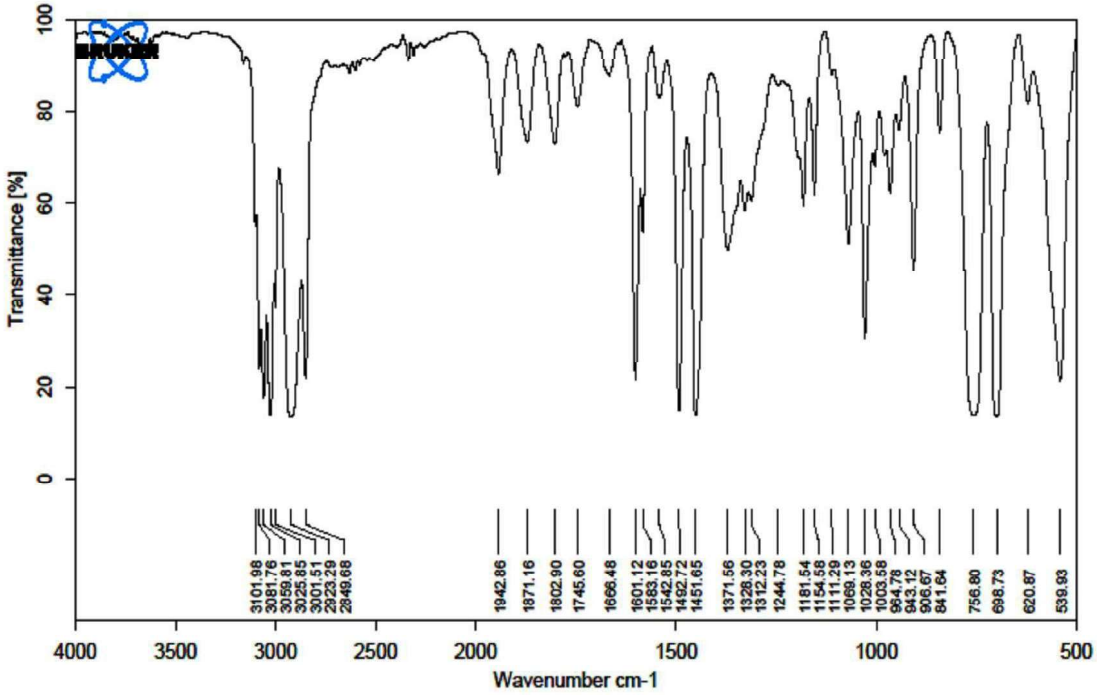


FIGURE 5.16 : FT-IR spectra of CFE-Collagen film

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