3.1 CHARACTERIZATION OF HYDROGLYCOLIC EXTRACT OF *Calendula officinalis* flower

Hydroglycolic extract of *Calendula officinlias L.* flower (CFE) was prepared by Amine Pvt.Ltd., (Vadodara, Gujarat). CFE was characterized for physical appearance, pH, moisture content, DSC, FTIR, totol polyphenol content, total flavonoids content and antioxidant activity. List of materials used in experiments are shown in table 3.1.

TABLE 3.1: List of materials used in preliminary studies

Sr. no.	Materials	Source
1	Disodium tartrate	Merck, India
2	Methanol	Merck Millipore
3	Gallic acid	SRL Pvt Ltd, Mumbai, India
4	Sodium carbonate	SD fine chemical, Mumbai, India
5	Aluminum chloride	SD fine chemical, Mumbai, India
6	Quercetin dehydrate	SRL Pvt Ltd, Mumbai, India
7	FolinCiocalteu's phenol reagent	SRL Pvt Ltd, Mumbai, India
8	2,2-diphenyl-1-picryllhydrazyl	SRL Pvt Ltd, Mumbai, India
9	Sodium Hydroxide pellet	SD fine chemical, Mumbai, India

3.1.1 PHYSICAL APPEARANCE: CFE was pale yellow orange color powder.

3.1.2 pH: 1.0 g of CFE was weighed accurately on a single pan weighing balance (Shimadzu SCS, Japan) and transferred to 100.0 ml volumetric flask. To this, distilled water was added to dissolve CFE and volume was made up to 100.0 ml with distilled water. The pH of prepared CFE aqueous solution (1% w/v) was determined using digital pH meter (Lab India, India) at room temperature and it was 6.5 ± 0.2.

3.1.3 MOISTURE CONTENT BY KARL FISCHER TITRATION METHOD:

3.1.3.1 **Procedure:** Karl Fischer titration is commonly used method to find out water content in a variety of product (1). It is based on the Bunsen reaction between sulfur dioxide and iodine in an aqueous medium (2).

1) **Standardization of Karl Fischer reagent:** Disodium tartrate (DST) is used as a volumetric standard in Karl Fischer titration. 140.2 mg of disodium tartarate was taken and titrated by Karl Fischer reagent to the electrometric endpoint. At titration endpoint, Amount of reagent consumed was noted (B.R.). The water equivalence factor of Karl Fischer reagent (KFR factor) was calculated as per equation (3.1)

$$KFRfactor = \frac{wei\qquad of\ DST\ (mg)*0.1566}{B.R\ (ml)} \text{………. Equation (3.1)}$$

- The KFR factor was found to be 4.7271 ± 0.1264.

2) **Determination of water content in CFE**: 50.0 ml of methanol was taken in to the titration flask and neutralized with Karl Fischer reagent. After neutralization, about 100.0 mg of CFE was added to the titration flask and titrated with Karl Fischer reagent to the electrometric endpoint. Amount of reagent consumed was noted (B.R.). % Water content of CFE was calculated using equation 3.2.

$$\%\ water\ content = \frac{B.R.(ml)*KFRfactore}{Weightofsampl\quad (mg)} * 100 \text{ ……….….Equation (3.2)}$$

The % moisture content of CFE was found to be 4.945 ± 0.62 %

3.1.4 QUALITATIVE CHEMICAL EXAMINATION

Qualitative chemical examination was carried out for phytochemicals such as alkaloids, carbohydrates, glycosides, saponins, phenolic compounds, phytosterols, flavonoids and tannis (12).

3.1.4.1 Procedure:

1) Tannins: About 0.5 g of the sample was put in a test tube and 20 ml of distilled water was added and heated to boiling. The mixture was then filtered and 0.1 % of $FeCl_3$ was added to the filtrate and observations made. A brownish green color or a blue black coloration indicated the presence of tannins.
2) Alkaloids: About 0.5 g of the sample was mixed with 1% of HCl in a test tube. The test tube was then heated gently and filtered. To the filtrate, a few drops of Mayer’s and Wagner’s reagents were added by the side of the test tube. A resulting precipitate indicates the presence of alkaloids.
3) Saponins: About 0.5 g of the sample was mixed with 5 ml of water and vigorously shaken. The formation of stable foam indicated the presence of saponins.
4) Flavonoids: About 1 g of the CFE was mixed with a few fragments of magnesium ribbon (0.5 g) and few drops of concentrated hydrochloric acid were added. A pink or magenta red color development after 3 minutes indicate presence of flavonoids.
5) Terpenoids: About 0.5 g of CFE was taken in a clean test tube 2 ml of chloroform was added and vigorously shaken, then evaporated to dryness. To this, 2 ml of concentrated sulphuric acid was added and heated for about 2 minutes. A greyish color indicated the presence of terpenoids.
6) Glycosides: a Salkowsks’ test: CFE was mixed with 2 ml of chloroform and 2 ml of concentrated sulphuric acid was carefully added and shaken gently, then the observations were made. A red brown color indicated the presence of steroidal ring (glycone portion of glycoside)
7) Steroids: About 2 g of the extract was put in a test tube and 10 ml of chloroform added and filtered. Then 2 ml of the filtrate was mixed with 2 ml of a mixture of

acetic acid and concentrated sulphuric acid. Bluish green ring indicated the presence of steroids.

8) Phenols: Sample was put in a test tube and treated with a few drops of 2% of FeCl3.Blue green or black coloration indicates the presence of phenols.

3.1.4.2 Result: Results of qualitative examination of Calendula flower extract are presented in table 3.2

TABLE 3.2: Qualitative analysis of phytochemical in CFE

Sr. No.	Phyto-chemical	Observation	Inferences
1	Tannis	Blue black colour	Present
2	Saponins	Formation of stable foam	Present
3	Terpenoids	Grey colour	Present
4	Flavonoids	Magenta-red colour	Present
5	Phenols	Black coloration	Present
6	Alkaloids	No precipitation	Absent
7	Steroids	No bluish green ring	Absent
8	Glycosides	Formation of brown ring	Absent
9	Steroidal ring	Red-brown colour	Absent

3.1.5 DSC STUDY: DSC analysis was carried out using a Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan). CFE was 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. DSC thermogram of CFE is presented in figure 3.1. A broad endothermic peak indicated that the CFE exists in a completely amorphous form.

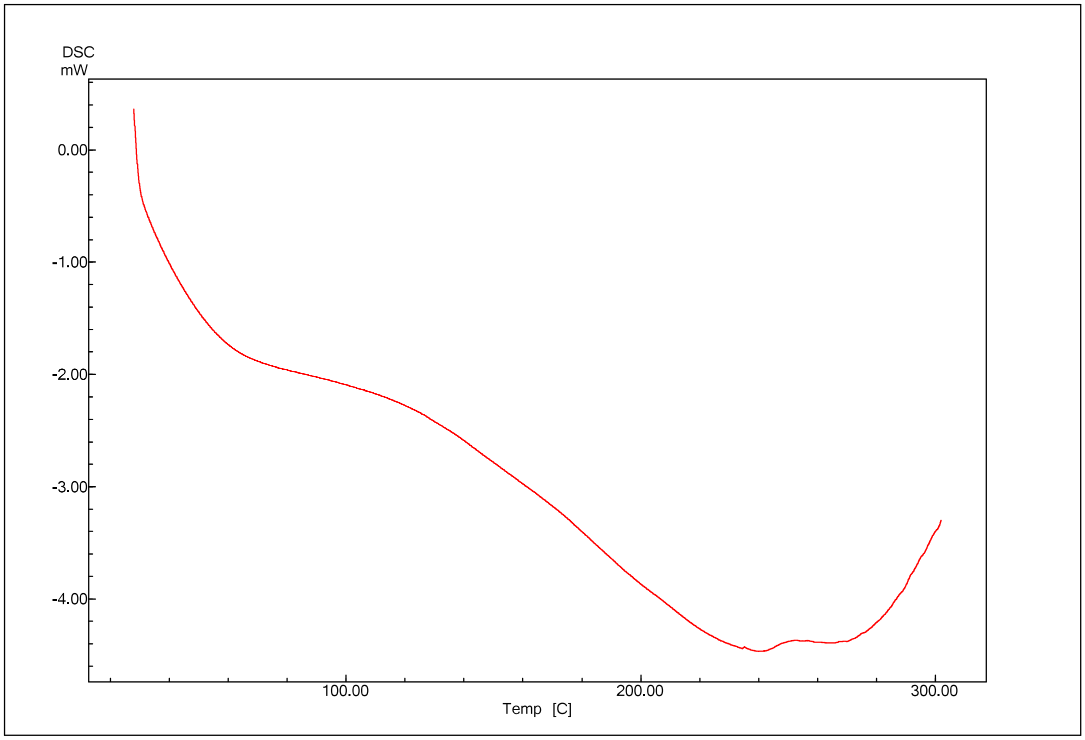

FIGURE 3.1: DSC thermogram of *Calendula flower* extract

3.1.6 FT-IR STUDY: FT-IR-spectrum of CFE was 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).

3.1.6.1 Result: FTIR spectrum of CFE (figure 3.2) proved presence of alkyl halides, alcohols, phenols, saturated amine and aliphatic amine groups which was reported by Al-Mussawi et. al., 2019 (3).

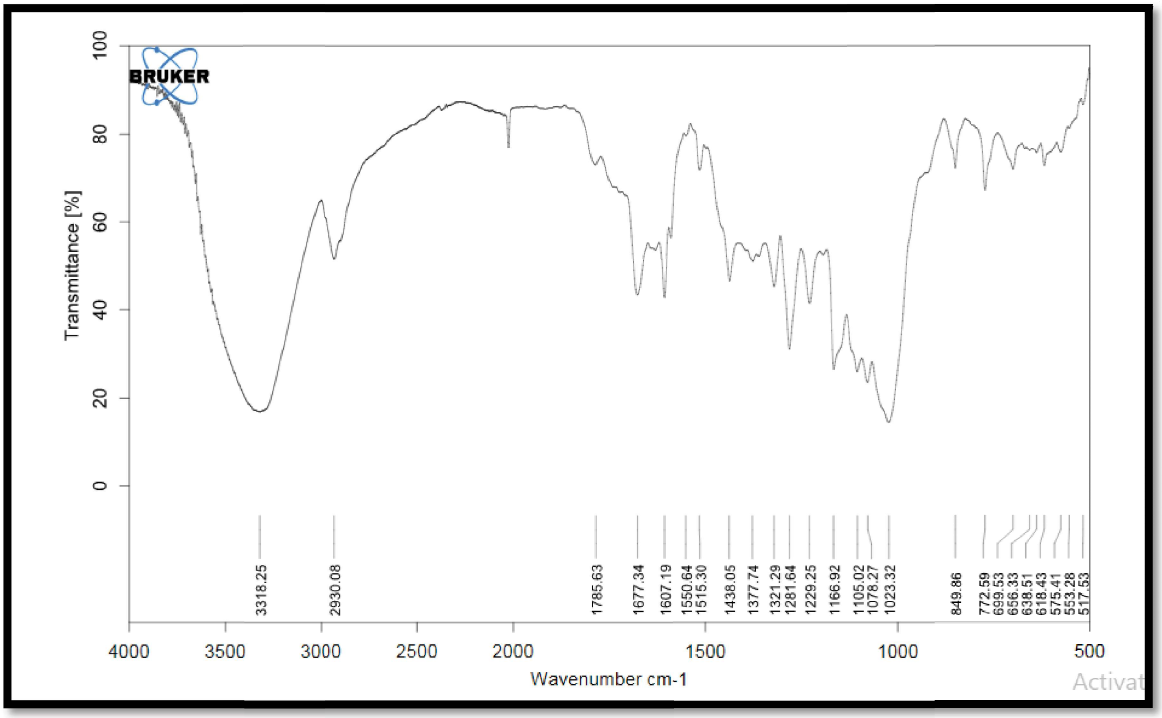

FIGURE 3.2: FT-IR spectra of *Calendula flower* extract

TABLE 3.3: FT-IR peaks of *Calendula officinalis* flower extract

Peak area (cm^{-1})	**Bond**	**Functional group**
517.53	C-Br stretch	Alkyl halides
656.33	C-Br stretch	Alkyl halides
1078.27	C-N stretch	Aliphatic amine
1105.02	C-N stretch	Aliphatic amine
1228.25	C-H wag (-Ch2X)	Alkyl halides
1166.92	C-O stretch	Ether bonds
1438.05	C-H bend	Alkanes
1677.34	N-H bend	Amines
1785.63	C=o stretch	Ketones, saturated aliphatic
2930.08	O-H	Carboxylic acid
3318.25	O-H alkanes, H-Bonded	Alcohols, phenols

3.1.7 TOTAL POLYPHENOLIC CONTENT BY FOLIN-CIOCALTEU METHOD

3.1.7.1 Principle:

The total polyphenol content of CFE was determined using colorimetric method based on Folin Ciocalteu reagent which contains phosphomolybdic/ phosphotungstic acid complexes (4). The method relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds (5). Reduction reaction was independent of the structures of phenolic compounds. The reduced Folin-Ciocalteu reagent is detectable with a spectrophotometer in the range of 710 to 750 nm. The total concentration of phenolic compound in the extract was calculated using calibration curve of gallic acid prepared in a similar way.

3.1.7.2 Reagents:

- Preparation of stock solution of gallc acid (1000 µg/mL): 100.0 mg of gallic acid was weighed accurately on a single pan weighing balance (Shimadzu SCS, Japan) and transferred to 100.0 ml volumetric flask. To this, distilled water was added to dissolve gallic acid and volume was made up to 100.0 ml with distilled water.
- Preparation of stock of gallic acid (100 µg/mL): 10.0 ml of 1° stock solution of gallic acid (1000 µg/mL) was taken into 100.0 ml volumetric flask and diluted upto 100.0 ml with distilled water
- Preparation of 20%w/v sodium carbonate solution: 20.0 g of sodium carbonate was weighed accurately on a single pan weighing balance (Shimadzu, Japan) and transferred to 100 volumetric flasks. To this, distilled water was added to dissolve sodium carbonate and volume was made up to 100 ml with distilled water.
- 10 fold diluted Folin Ciocalteu’s solution: 10.0 ml of Folin Ciocalteu’s reagent was transferred to 100 ml volumetric flask and diluted upto100.0 ml with distilled water.

3.1.7.3 Procedure: It was determined using colorimetric method*(4)*. Aliquot of CFE solutions were assayed with 2.0 ml of Folin Ciocalteau reagent and 2.0 ml of 20%w/v sodium carbonate solution. The mixture was vortexed and diluted upto 10.0 ml with distilled water. The mixture was further incubated for 30 min at room temperature. Absorbance of the solution was measured at 725.0 nm using UV-Visible spectrophotometer (SHIMADZU SCS-1700, Japan). The total phenol content of extract was expressed as gallic acid equivalent (mg GAE/g), using a calibration curve of a freshly prepared gallic acid solution.

❖ **Preparation of standard calibration curve of gallic acid solution:**

1) Aliquots of 0.2, 0.4, 0.8, 10 and 1.2 ml of gallic acid stock solution (100 μg/mL) were accurately withdrawn using calibrated pipettes and transferred to different five 10.0 ml volumetric flask labbed as sample 1 to 5.Final concentration of gallic acid solution in each volumetric flask was 2, 4, 6, 8, 10 and 12 μg/mL respectively.
2) 2.0 ml of 10fold diluted Folin–Ciocalteau reagent was added into every flask. The mixture was vortexed for 30 sec.
3) After that, 1.5 mL of aqueous sodium carbonate (20%, w/v) was added.
4) The mixture was again vortexed for 30 sec and diluted upto 10.0 ml with distilled water.
5) Resulted solutions were incubated for 30 min at room temperature in dark condition.
6) λmax determined by scanning 10 μg/mL solution against the reagent blank using UV-Visible spectrophotometer (SHIMADZU SCS-1700, Japan)was found to be 725.0 nm. Absorbance of the solutions ranging from 2-12 μg/mL was measured at 725.0 nm.

3.1.7.4 Result: Absorbance values and spectra of the gallic acid solutions are shown in table 3.3. Standard calibration plot of gallic acid concentration vs. average absorbance was drawn (figure 3.3). Straight line equation of plot is mention in equation 3.3

$$y = 0.092x + 0.055 \quad (R^2 = 0.099) \ldots\ldots\text{Equation (3.3)}$$

Using straight line equation (3.3) total polyphenolic content of CFE was calculated and it was found to be 9.649 ± 0.6339 mg GAE/g.

TABLE 3.4: UV-Visible absorbance values at 725 nm of Standard gallic acid solutions

Sample no.	Concentration of Gallic acid (µg/mL)	Absorbance at 725 nm			Average Absorbance at 725 nm	±SD, n=3	%RSD
Std 1	2	0.2341	0.2370	0.2380	0.2363	0.0020	0.8570
Std 2	4	0.4241	0.4220	0.4164	0.4205	0.0045	1.0796
Std 3	6	0.6127	0.6205	0.6158	0.6163	0.0039	0.6372
Std 4	8	0.7868	0.7944	0.7972	0.7928	0.0053	0.6788
Std5	10	0.9627	0.9980	0.9872	0.9826	0.0181	0.1845
Std6	12	1.1725	1.1305	1.1592	1.1540	0.0214	1.8580

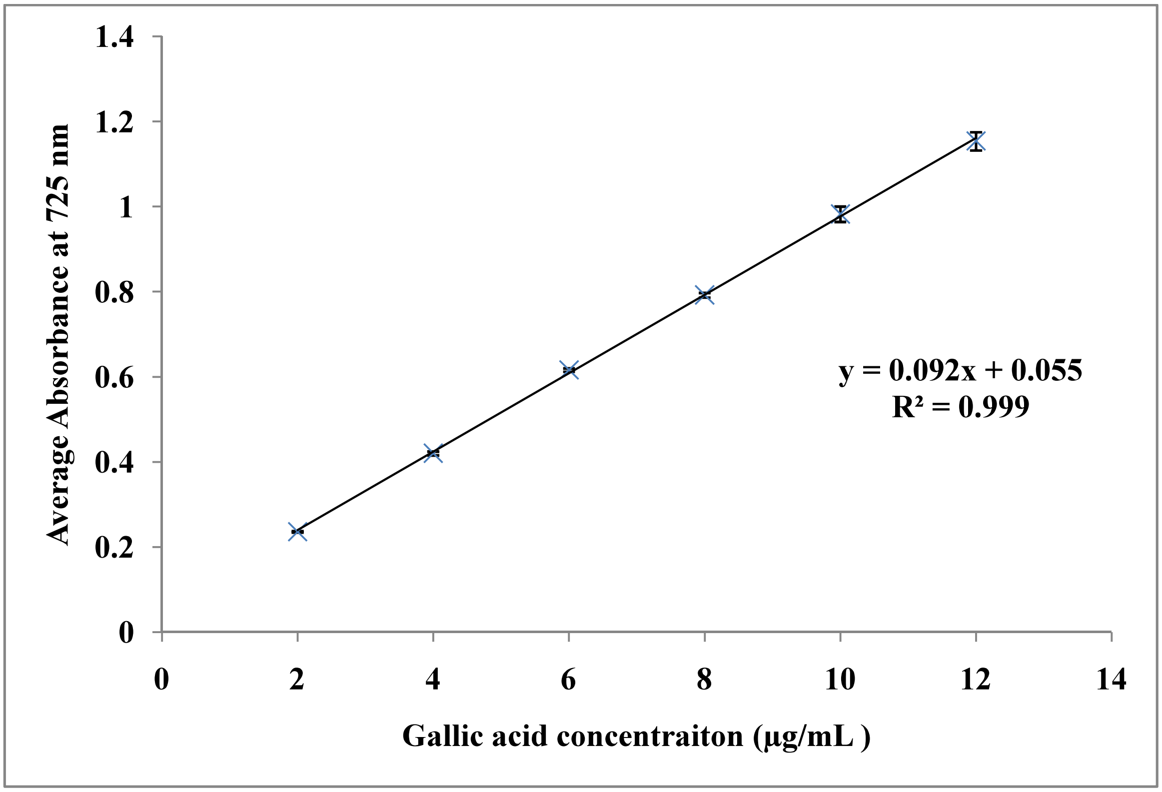

FIGURE 3.3: Standard calibration plot of gallic acid solution

3.1.8. TOTAL FLAVONOID CONTENT BY ALUMINUM CHLORIDE METHOD

3.1.8.1 Principle: Total flavonoid content was determined by aluminum colorimetric method in which Al(III) ion forms yellow colored acid stable complexes with carbonyl and hydroxyl groups of flavones and flavonols (6).

3.1.8.2 Reagents:

- Solvent: - 1:9 methanol : water
- 10% Al_2Cl_3 solution: 10.0 g of Al_2Cl_3 was weighed accurately and transferred to 100 ml volumetric flask. To that, distilled water was added to dissolve Al_2Cl_3 and volume made up to 100 ml with distilled water.
- 5% sodium nitrite solution: 5 g of sodium nitrite was weighed accurately and transferred to 100 ml volumetric flask. To that, distilled water was added to dissolve sodium nitrite and volume made up to 100 ml with distilled water.
- 1 M NaOH solution: 2.0 g of NaOH pellet was taken into 50 ml volumetric flask, dissolve and diluted upto 50.0 ml with distilled water.
- Stock solution of quercetin dihydrate (1000 µg/mL): 100.0 mg of quercetin dihydrate was weighed accurately and transferred to 100 ml volumetric flask. To that, methanol was added to dissolve quercetin dehydrate and diluted upto 100.0 ml with same solvent.

3.1.8.3 Procedure: The flavonoids content was determined spectrophotometrically according to Rigane*et al.* (2011), based on the formation of complex flavonoid aluminum, having the maximum absorption at 510 nm (7). The sample solutions were mixed with 0.3 mL of 10% solution of Al_2Cl_3, 0.3 ml of 5% sodium nitrite solution, 2 ml of 1 M NaOH solution and diluted upto 10.0 mL. After 30 min, samples were analyzed using UV-Visible spectrophotometer (Shimadzu, Japan) and total flavonoids content was calculated using calibration curve of quercetin solution (2 -20 µg/mL) as a standard. The flavonoids content was expressed in mg of quercetin equivalent per gram of dry weight extract (mg QE/g).

- Preparation of calibration curve of quercetin dehydrate (4 – 20 µg/mL): Aliquots of 0.4, 0.8, 1.2, 1.6 and 2.0 ml of quercetin stock solution (100 µg/mL) were accurately withdrawn using calibrated pipettes and transferred to different five 10.0 ml volumetric flask labeled as Std 1 to 5. Final concentration of quercetin solution in each volumetric flask was 4, 8, 12, 16 and 20 µg/mL respectively. Solutions were mixed with 0.3 ml of 10% solution of Al_2Cl_3, 0.3 ml of 5% sodium nitrite solution, 2 ml of 1 M NaOH solution and diluted upto 10.0 ml. After 30 min, samples were analyzed using UV-Visible spectrophotometer at λmax (310 nm).

3.1.8.4 Results: Absorbance values of the quercetin solutions measured at 310.0 nm using UV-Visible spectrophotometer are shown in table 3.4. Absorbance values of CFE solutions measured at 310.0 nm are mentioned in table 3.5. Standard calibration plot of quercetin concentration vs. average absorbance was drawn (figure 3.4). Straight line equation of plot is mentioned in equation 3.4

$$y = 0.089x + 0.270 \quad (R^2 = 0.998) \text{ ....Equation 3.4}$$

Total flavonoid content of CFE was found to be 10.386 ± 0.275mg QUE/g that it 1.0386% which compiles wit WHO monograph of *Calendula* flower**.** As per WHO monograph, *Calendula* flower should not contains less than 0.4% flavonoids (13).

TABLE 3.5: UV-Visible absorbance values at 310.0 nm of quercetin solution

Solution name	Con. of quercetin solution (µg/mL)	Average absorbance	SD (n=3)	%RSD
Std 1	4	0.6494	0.01248	1.9218
Std 2	8	0.9596	0.01146	1.1951
Std 3	12	1.3452	0.01585	1.1787
Std 4	16	1.7280	0.00771	0.4466
Std 5	20	2.0618	0.03423	1.6603

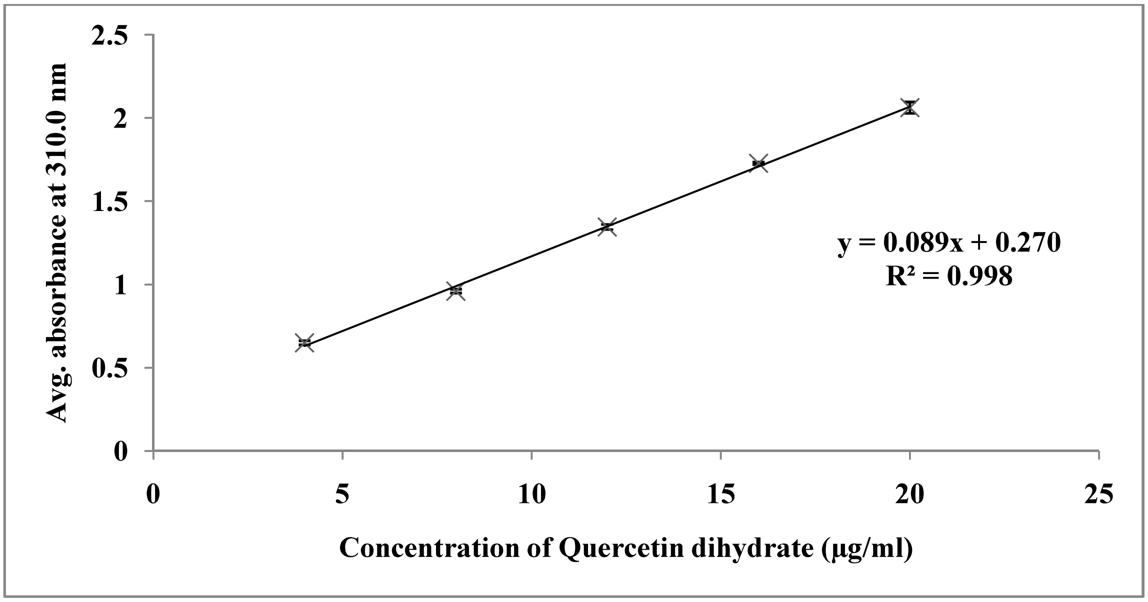

FIGURE 3.4: Standard calibration curve of quercetin dehydrate solution

Table 3.6: Total flavonoid content in *Calendula* flower extract

Wt. of sample (wt)	Absorbance (y)	Equivalent quercetin concentration (x) $= \frac{y-0.072}{0.089}$µg/ml	Total Phenolic content in terms of equivalent quercetin concentration (mg/g) $= \frac{x*10*1000}{wt*1000}$	Average total Phenolic content mg QUE/g *
6.5 mg	0.8529	6.54943	10.076	10.386 ± 0.275
10.1 mg	1.2123	10.5876	10.483	
8.5 mg	1.0718	9.00898	10.599	

*±SD, n=3

3.1.9 ANTIOXIDANT ACTIVITY BY DPPH ASSAY:

3.1.9.1 Principle: The radical scavenging capacity of CFE was examined by the colorimetric assay using 2,2-diphenyl-1-picryllhydrazyl (DPPH)(8). DPPH is a stable free radical which produces a violet solution in alcohol at room temperature. It is reduced by antioxidant molecule and forms yellow colour solution. This method is widely used because it is a precise, accurate and easy method (9).

3.1.9.2 Reagent:

- Stock solution of DPPH: 5.7 mg of DPPH was weighed accurately, methanol was added to dissolve DPPH and volume was made up to 25 ml.
- Stock solution of Quercetin (10 µg/mL): 10.0 mg of quercetin was weighed accurately and transferred to 100.0 ml volumetric flask. To this, methanol was added to dissolve and volume was made up to 100.0 ml with methanol. From that 10.0 ml of solution was taken into 100.0 ml volumetric flask and diluted upto 100 ml with distilled water.
- CFE solution: 16.0 mg of CFE was weighed accurately into 10 ml volumetric flask and dissolved and diluted upto 10.0 with distilled water.

3.1.9.3 Procedure: The free radical scavenging activities of the extracts were determined by using 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (10). Aliquots of 1, 2, 3, 4 and 5 mL of 10 µg/mL quercetin solution were accurately withdrawn using calibrated pipettes and transferred to different five 10.0 mL volumetric flasks labeled as std. 1 to 5. Final concentration of quercetin solution in each volumetric flask was 1, 2, 3, 4 and 5 µg/mL respectively. 1.0 mL, 0.2 mM DPPH methanol solution was added into every flask. The mixture was vortexed for 30 sec and diluted upto 10.0 mL with methanol. Resulted solutions were incubated for 30 min at room temperature in dark condition. Absorbance of the solution was measured at 517.0 nm using UV visible spectrophotometer (SHIMADZU SCS-1700).The inhibitory percentage of DPPH was calculated according to equation (3.5).

$$\%\,Inhibition = \frac{A\,(std) - A(sam)}{A\,(std)} * 100 \text{..............................Equation (3.5)}$$

Standard calibration curve % Inhibition vs. concentration of quercetin solution (0 – 5µg/mL) was drawn and straight line equation of calibration curve was determined. Same experiment was carried out with sample of CFE solution. The antioxidant activity of extract was expressed as quercetin antioxidant activity equivalent (QE) µmol per 100 ml of the solution (extract). All the experiments were performed in triplicate

3.1.9.4 Result: The absorbance and %inhibition activity of quercetin solution is shown in table 3.6. The standard calibration curve of %Inhibition vs. Concentration of quercetin solution (µg/mL) and regression equation are shown in figure 3.5. Free radical scavenging capacity of CFE solution was calculated from the straight line equation of standard calibration curve (equation 3.6) which is shown in table 3.7

$$y = 14.05x + 0.950 \quad (R^2 = 0.993) \text{ ……………Equation (3.6)}$$

Free radical scavenging activity of CFE was found to be 4.604 mg QUE/g extract.

TABLE 3.7: UV-Visible absorbance values at 517.0 nm and % inhibition activity of quercetin solution

Sample Name	Concentration of quercetin solution (µg/mL)	Avg. Absorbance at 517.0 nm	±SD, n=3	%RSD	%Inhibition
Std 1	1	0.936	0.012	1.282051	13.9178%
Std 2	2	0.756667	0.011015	1.455746	30.4107%
Std 3	3	0.604667	0.01159	1.916796	44.3899%
Std 4	4	0.437667	0.008505	1.943237	59.7485%
Std 4	5	0.346667	0.011504	1.318353	68.1176%
Blank	0	1.087333	0.016623	1.528811	0.0 %

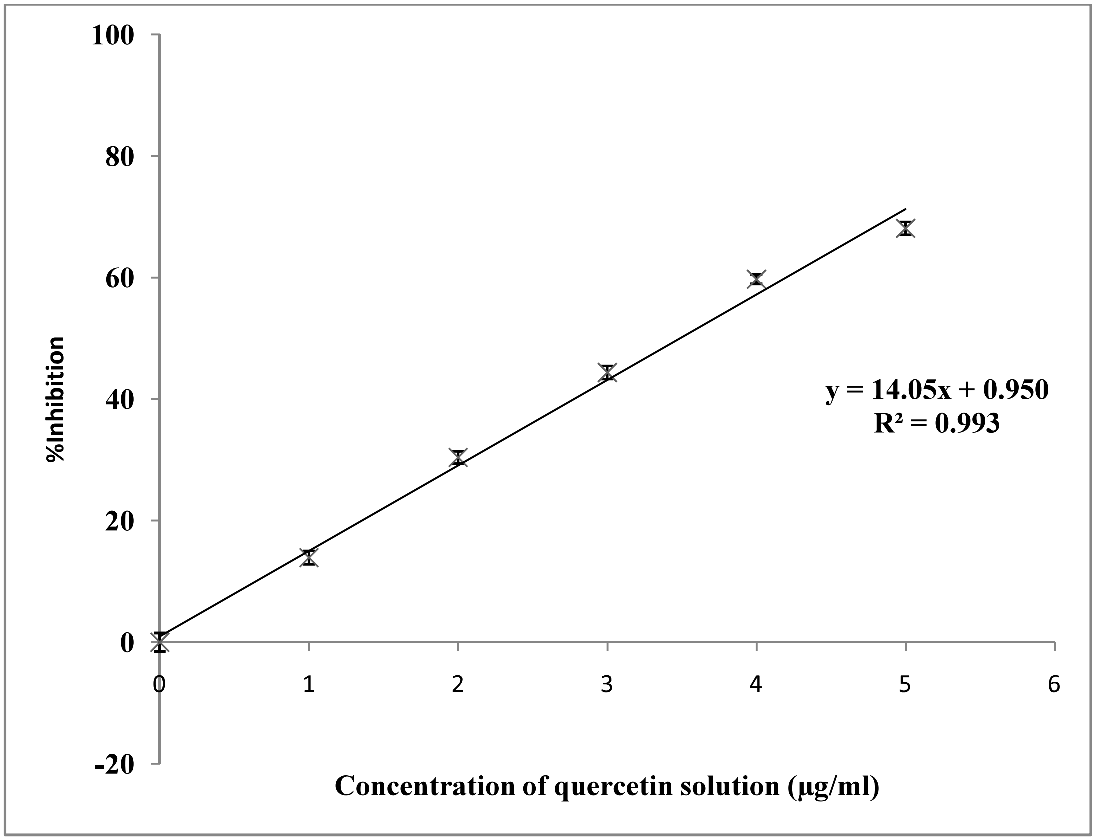

FIGURE 3.5: Standard calibration curve of % inhibition vs. quercetin solution concentration

TABLE 3.8: UV-Visible absorbance values at 517.0 nm and % inhibition activity of CFE solution

Sample Name	Absorbance at 517.0 nm	Avg.	±SD, n=3	%RSD	%Inhibition
Sample 1	0.731	0.739	0.0073	0.9969	32%
Sample 2	0.742				
Sample 3	0.745				

DPPH free radical scavenging method was used to determine the concentrations of extracts at which they scavenge the 50% of the DPPH solution termed as IC50 values.

Quercetin was used as standard for this purpose. The lower the IC50 value of an antioxidant the higher would be its free radical scavenging power. The mean IC50 of quercetin and CFE were 3.4862 µg/ml and 0.7323 mg/ml respectively. Calendula flower extract contain polyphenols, carotenoids and flavonoids which scavenge free radicals – DPPH (10, 11). Reactive oxygen species are formed during inflammation which damage and regard the normal wound healing process. Antioxidant activity of formulation reduces the oxidative damage to tissue and help in wound healing process.

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