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Development of a Transdermal Formulation of *Chrysanthemum Indicum* Extract for Antimicrobial and Anti-Inflammatory Applications

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Abstract: ***Nowadays there is an increasing demand of the herbal medicines in worldwide. Present study describes that the formulation of the transdermal drug delivery that enhances the patient convenience and efficacy at the same time, as compared to the conventional drug delivery system like oral. This particular work aims at the transdermal drug delivery, the site of action being the inflammation at a particular part of the skin. For this we developed transdermal formulation (herbal gel) of an antimicrobial and anti-inflammatory agent extracts of flower of Chrysanthemum indicum. Chrysanthemum indicum is Ayurveda medicinal plant which has antimicrobial, anti-inflammatory, antioxidant activity and anticancer activity. The extraction of the Chrysanthemum indicum was done by the solvent extraction and Soxhlet extraction method and it is evaluated by various phytochemical and pharmacognostic studies. The extract was identified by the FTIR spectroscopic method which is known as the most preferred and superior technique for most analysis. The Chrysanthemum indicum extract was stabilized by using Carbopol, methyl paraben and propyl paraben. Stability studies were also performed on herbal gel that confirms that this formulation remains stable for 3 months. In-vivo and in vitro tests were also conducted for this herbal formulation. This herbal gel has been gone through the all necessary preformulation and evaluation studies that justifies that the efficacy of this transdermal drug delivery formulation (herbal gel) has shown its all activities that discussed above.***
Keywords: Chrysanthemum indicum extract, anti-inflammatory, FTIR spectroscopy, phytochemical study, Soxhlet extraction

I. INTRODUCTION

Herbal gels are used in various fields such as pharmaceuticals and cosmeceuticals to treat the skin disorder and for providing nourishment to the skin [1]. There are many products and formulations in the market which are used for the antimicrobial, anti-inflammatory, antioxidant and anticancer activity. Among which herbal products show the minimum or none side effect or harmful adverse effects [2,3]. To treat inflammation, purpose many peoples use the oral and conventional drug delivery system because that method has greater patience compliances but they also have some disadvantages like the drug metabolism and low bioavaibility of the drug at the site of injury or inflammation [4]. These disadvantages can be overcome by the use of transdermal drug delivery formulations like herbal gels because of their use the drug can be directly applied at the site of administration and it get protected from the metabolism so maximum bioavaibility of drug can be achieved at the site of injury. *Chrysanthemum indicum* belongs from the family of Asteraceae [5,6]. Flavonoids present in the chrysanthemum indicum extract is mainly responsible for the anti-inflammatory activity. Flavonoids help treat inflammation by acting as antioxidants and anti-inflammatory agents; they reduce oxidative stress, inhibit the production of pro-inflammatory mediators such as TNF-α, IL-6, and prostaglandins, block the NF-κB signalling pathway, and stabilize immune cell responses to prevent excessive activation. *Chrysanthemum indicum* extract contains a wide range of bioactive phytochemicals, including flavonoids (such as luteolin, apigenin, quercetin, and rutin), phenolic acids (notably chlorogenic acid and caffeoylquinic acid derivatives), terpenoids and essential oil components (such as camphor, borneol, 1,8-cineole, α-pinene, and chrysanthenone), glycosides (including linaria and other flavonoid glycosides), as well as sterols, saponins, and fatty acids like daucosterol and palmitic acid, all of which contribute to its antioxidant, anti-inflammatory, and antimicrobial activities [7,8]. *Chrysanthemum indicum* supports skin protection, calming, and anti-aging properties. Hence, the current work aims at transdermal drug delivery, the site of action being the inflammation at a particular site on the skin. The aim of the study is to developed transdermal formulation of an antimicrobial and anti-inflammatory agent extracts of flower of *Chrysanthemum indicum. Chrysanthemum indicum* is Ayurveda medicinal plant which has antimicrobial, anti-inflammatory, antioxidant activity and anticancer activity [9].

II. MATERIALS AND METHODS

A. Materials

Chrysanthemum indicum was collected from the nearby area of Sinhgad Institute of Pharmaceutical Science Kusgaon (BK) Lonavala. It was collected by Hand picking method and was cleaned and further processed for the study.

The plant was authenticated by Botanical survey of India, Pune.

1) Herbs And Chemicals

Sr. No	Material	Manufactures
1	Acetic acid	Research, Labfine Chem Industry., Mumbai
2	Acetonitrile	Pallav Chemicals & Solvents Pvt. Ltd., Mumbai
3	Ethanol	Pallav Chemicals & Solvents Pvt. Ltd., Mumbai
4	Carbapol 940	Research, Labfine Chem Industry., Mumbai
5	Propylene glycol	Research, Labfine Chem Industry., Mumbai
6	Propyl paraben	Research, Labfine Chem Industry., Mumbai
7	Methyl paraben	Research, Labfine Chem Industry., Mumbai
8	Thriethenlamne	Research, Labfine Chem Industry., Mumbai

Table: List of Drug and Chemicals

2) Equipment and Glasswares used.

Sr. No.	Glassware
1.	Beaker
2.	Glass rod
3.	Measuring cylinder
4.	Spatula
5.	Mortar & pestle
6.	funnel
7.	Test tube
8.	Petriplate
9.	Volumetric flask

3) Instrument Used

Table: List of Instruments

Sr. No.	Instrument Name	Model	Manufactures
1	Digital Balance	EL300	Shimadzu
2	pH Meter	EQ-610	Equitronic, India
4	Centrifuge	R8C	Remi
5.	Magnetic stirrer	IKA ETSD-5	IKATRON
6.	Ultra Turax	IKA T 18 basic	IKA, Germany
7.	Hot Air Oven	PEW-202/PEW-205	Pathak electronic work, Mumbai
8.	UV-vis spectrophotometer	UV-3000	Lab India, India
9.	FTIR spectrophotometer	V-530FT/IR-4100	Jasco, Japan
10.	Ultra Sonicator	Innovative 50	Innovative Instrument, New Delhi
11.	Particle size analyser	Zetasizer ver.7.12	Malvern Instrument Ltd.
12.	Brookfirld Viscometer	DV-E Viscometer	Brookfield Engineering Lab, Viscometer
13.	Digital Photometer	Digi 2- 1500	Labomed INC, USA

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III. METHOD OF PREPARATION

A. Collection of the Plant Material and Authentication:

Chrysanthemum indicum was collected from the nearby area of Sinhgad Institute of Pharmaceutical Science Kusgaon (BK) Lonavala. It was collected by Hand picking method and was cleaned and further processed for the study.
The plant was authenticated by Botanical survey of India, Pune.

B. Organoleptic Characterization

Organoleptic property as a chemical, taste, odor and color of the drug are studied out.

C. Extractive Value

Preparation of the Extract:

1) Firstly, the flower and leaf were dried then powdered using grinder.

Figure: Chrysanthemum flower and Flower Powder

2) The powdered flower & leaf sample (5g) was extracted in 100 mL 60% aqueous ethanol/ Water in a water bath at 80°C for 4 hrs. The extract was filtered through double layer muslin cloth and then cooled on ice [10]. The cold filtrate was centrifuged at 1200 rpm for 15 min. The supernatant was used for soluble solids [11].

Fig: Extract preparation of flower & leaf powder (*Chrysanthemum indicum*)

D. Determination of Soluble Solids

20 mL of the above supernatant was placed in a weighted dry glass dish (15 cm in diameter) and dried at 80°C overnight then at 103°C and cooled in a desiccator and finally weighted.

Figure: Soluble solid content of *Chrysanthemum indicum* Flower extract

$$\% \text{ yield} = \frac{\text{Weight of the residue obtain}}{\text{Weight of the plant material taken}} * 100$$

Soluble solids concentration was expressed as mg soluble solids per kg dry flower & leaf.

E. Loss on Drying (Moister content)

Accurately weighing 40 g of plant flower (Without preliminary drying) place in a tarred evaporating dish and kept in oven 1050 ^{0}C for 5 hours and weigh. The percentage loss on drying with reference to the air-dried plant flower calculated [11].

$$\text{Moisture content} = W_1 - W_2 / W_1 * 100$$

Where, W_1= Weight of flower before drying

W_2= Weight of flower after drying

F. Physico-chemical Analysis

The soluble solid plant extract of *Chrysanthemum indicum* was subjected to standard procedure for the determination of various physicochemical parameters.

G. Pharmacognostic Tests of Extracts

1) Determination of total ash value

Accurately weighing 2-3 g of extract powder drug in tared silica dish at a temperature not exceeding 4500 0C until free from carbon, cool and weight. If carbon free ash not obtained in this way, exhaust the charved mass with hot water and filter. Collected the residue on filter paper, incinerated the residue and filter, added ignited at temperature [12]. Calculated the % of ash with reference to the air-dried drug.

Wt. of the empty dish= X

Wt. of drug taken= Y

Wt. of the dish + ash (after complete incineration) = Z

Wt. of ash= (Z-X) g

'Y' g of the crude drug gives (Z-X) g of ash

Therefore 100 g of the crude drug gives 100/Y × (Z-X) g of ash

Total ash value of sample= 100 (Z-X)/Y %

2) Determination of acid- Insoluble ash Value

The crucible containing total ash, further added 25ml of dilute hydrochloric acid. Filter the insoluble matter (Whatman filter paper 41) and wash hot water until the filtrate was neutral [13,14]. Transferred the filter paper containing the insoluble matter to the original crucible, dry on a hot- plate and ignite to constant weight. Cool in desiccator for 30 minutes and immediate weighed. Calculated the content of acid insoluble ash with reference to the air- dried drug.

3) Water Soluble Ash

The ash obtained in total ash was boiled for 5 minutes with 25 ml of water. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited to constant weight at a low temperature. The weight of insoluble matter was subtracted from the weight of the ash. The difference in weights represents the water-soluble ash [15]. The percentage of water-soluble ash with reference to the air-dried drug was calculated.

H. Preformulation Studies

1) *Solubility:* Solubility was determined in ethanol, chloroform, propylene glycol, methanol, distilled water, dichloromethane. The flower extract powder drug (10mg) was placed in each test tube containing 1ml of solvent respectively, and mixed on the vortex for 10 minutes. Evaluated solubility by visually, if solid particle were still observed another milliliter of the same solvent was added. These procedures were repeated till clear solution was achieved [16].

2) Calibration curve of Drug (Extract Powder)

Stock solution I: It was prepared by dissolving 10mg of powder drug in 10 ml phosphate buffer of pH 7.4

Stock solution II: From stock I, 1 ml solution was pipette out and diluted up to 10 ml (100µg/ml).

Calibration curve: Calibration curve observed by prepared working solution concentration ranging from 2-10 µg/ml (Beers-Lambert range) from stock II. The absorbance of this solution was measured on UV- visible spectrometer (V-530 Jasco, Japan) at 510nm.

I. Preliminary Phytochemical Investigation

1) Test for Flavonoids:5 ml of dilute ammonia solution was added to the aqueous filtrate of the plant extract followed by the addition of concentrated H_2SO_4. Observed a yellow coloration indicated the presence of flavonoids. The yellow color disappeared on standing [17].

2) Alkaline reagent test: 5 ml of extract was added few drops of NaOH, a yellow coloration observed, and added Diluted Acid then the extract was colorless observed.

3) Lead Acetate test: 5 ml of extract was added few drops of Lead acetate; a yellow coloration observed in the extract indicated the presence of flavonoids.

4) Test for phenols: About 0.5 g of the dried powdered sample was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish green or a blue-black coloration. A few drops of alcohol and ferric chloride solution were mixed with the plant extract. A blue green or red color indicated the presence of phenol [18].

5) Detection of oil content (spot test): A small quantity of powder was pressed between two filter papers. Oil strains on the filter paper indicated the presence of fixed oils.

6) Test for cardio glycosides: 5 ml of the plant extract was treated with 2 ml of glacial acetic acid containing a drop of ferric chloride solution. Then it was underplayed with 1 ml concentrated sulphuric acid [17]. A brown ring of the interface indicates a deoxy sugar characteristic of cardio glycosides. A violet ring may appear below the ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

7) Test for Saponins: About 2 g of the powdered sample was boiled in 20 ml of distilled water bath and filtered. The 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a suitable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, and then the formation of emulsion was observed**.**

8) Test for Alkaloids: The plant extract was mixed with a few drops of acetic acid followed by dragendroff's reagent and mixed well. An orange red precipitate formed indicated the presence of alkaloid.

9) Test for steroids and sterols: 2 ml of acetic anhydride was added to 0.5 g of the plant extract of each sample with 2 ml of H_2SO_4. The color change from violet to blue green in the sample indicates the presence of steroids and sterols [17].

10) Test for tannins: A small quantity of the test sample was taken separately in water and test for the presence of phenol compounds and tannins was carried out with the following reagents:

- Violet color with Dilute Ferric chloride solution (5%)
- White precipitate with 10% lead acetate solution.

11) Test for proteins and free amino acids

Small quantities of the sample were dissolved in few ml of water and treated with following reagents:

- Million's reagent: Appearance red color
- Ninhydrin reagent: Appearance purple color

12) Tests for carbohydrates

Molisch's test: Sample was treated with 2-3 drops of 1% alcoholic - napthol solution and 2 ml of conc. sulphuric acid was added along the sides of the test tube [18].

Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.

13) Content of flavonoids

The spectrophotometer assay for the quantitative determination of flavonoid content by, the extract was diluted with 4 ml distilled water. At zero-time, 0.3 ml 5% NaNO2 was added to the mixture. After 5 min, 3 ml 10% AlCl3 was added. After another 6 min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. Immediately, the solution was mixed well again and the absorbance of the mixture, pink in color, was determined at 510 nm versus prepared water blank. The blank was performed using distilled water. Quercetin was used as standard. The samples were performed in triplicates. The calibration curve was plotted using standard quercetin. The data of total flavonoids of polyherbal formulation were expressed as mg of quercetin equivalents/ 100 g of dry mass.

14) Thin layer chromatography (TLC)

Thin layer Preparation: 25 g of silica gel-G was mixed with 50 ml of distilled water and the slurry formed was uniformly spread over TLC plates with a thickness of 0.25 mm using the spreader. The plates were allowed to dry a t room temperature and heated in an oven at 1000 C for 2 h.

Standardization of solvent system: Each sample of the crude extract of *C. indicum* leaves was diluted in distilled water. The prepared TLC plates were marked 1 cm from bottom and 10µl each sample was applied on TLC plates at equal distance with the help of capillary tubes. For separation of bands on TLC plates in solvent systems were used according to polarity and selected as standard solvent system [17,18].

Phase A: Acetonitrile/Acetic acid/Water (3/.05/96.5 v/v/v)

Phase B: Acetonitrile/Acetic acid/Water (50/.05/49.5 v/v/v)

Extract of *C. indicum* were screened for preliminary phytochemical analysis and thin layer chromatography of *C. indicum*. Observations were recorded for presence or absence of phytochemicals and Rf values of bands (compounds) present in extract.

Rf values Calculated by formula;

$$\text{Rf values} = \frac{\textbf{Distance travel by comnpound}}{\textbf{Distance travel by solvent}}$$

IV. FORMULATION AND DEVELOPMENT OF TRANSDERMAL HERBAL GEL:

During formulation gelling agents used at two different concentrations (1.0-1.5), resulting in six different batches of gels for six batches for *C indicum* extract powder.

A. Drug-excipient interaction should be investigated by FT-IR

Drug-excipients compatibility studies were carried out using FT-IR spectroscopy (V-530 FT/IR- 4100, Japan) in range between 600-3800 cm-1 in dry KBr pellet. The study was carried out on individual pure drug and its physical mixture with the excipients used in the study, were Dry KBr (50 mg) was finely ground in mortar and pure drug with the excipients (1-2 mg) were subsequently added and softly mixed in order to avoid of the crystals. FTIR was used for determination of functional group in the sample of pure drug and mixture [19].

B. Optimization Formulation of Herbal Gel

During formulation gelling agents used at two different concentrations (1.0-1.5) and three drug concentrations (500mg-2.0g), resulting in six different batches of gels for *C indicum* extract powder.

Table: Composition of herbal gel formulation

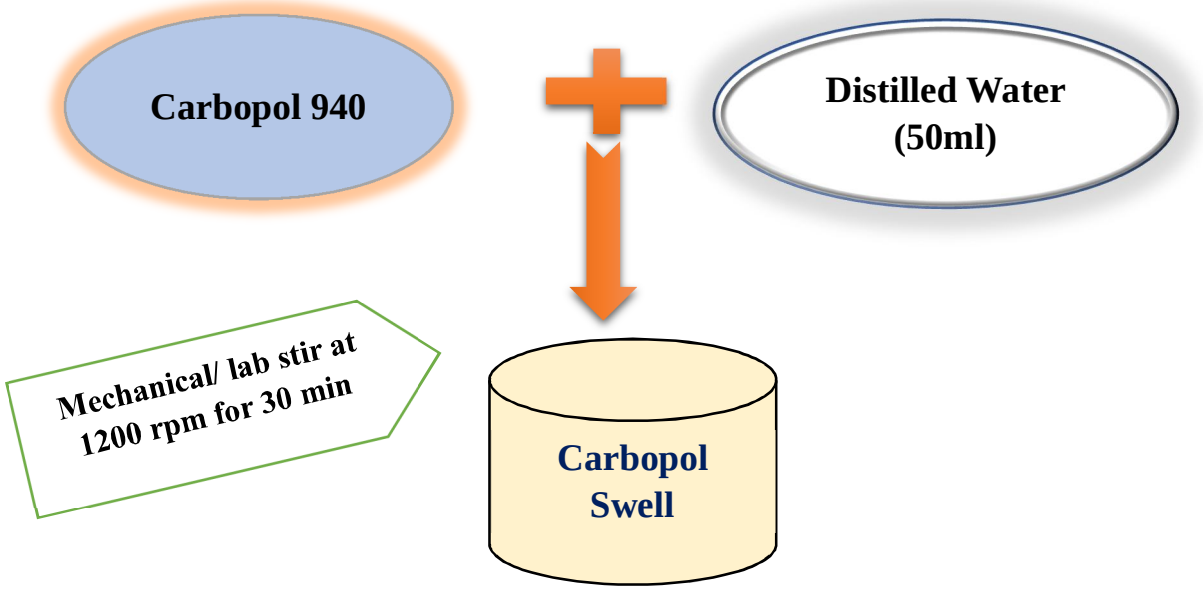
INGREDIENTS	F1	F2	F3	F4	F5	F6
Carbopol 940 (gm)	1.0	1.5	1.0	1.5	1.0	1.5
Propylene glycol (ml)	5	5	5	5	5	5
Propyl paraben (gm)	1.0	0.2	0.1	0.2	0.1	0.2
methyl paraben (gm)	0.2	0.2	0.2	0.2	0.2	0.2
Triethanolamine (ml)	1.5	1.5	1.5	1.5	1.5	1.5
Extract Powder	500mg	1.0g	2.0g	500mg	1.0g	2.0g
Distilled water (ml)	q.s	q.s	q.s	q.s	q.s	q.s

All the ingredients weighed accurately and all batches were prepared according to the experimental design.

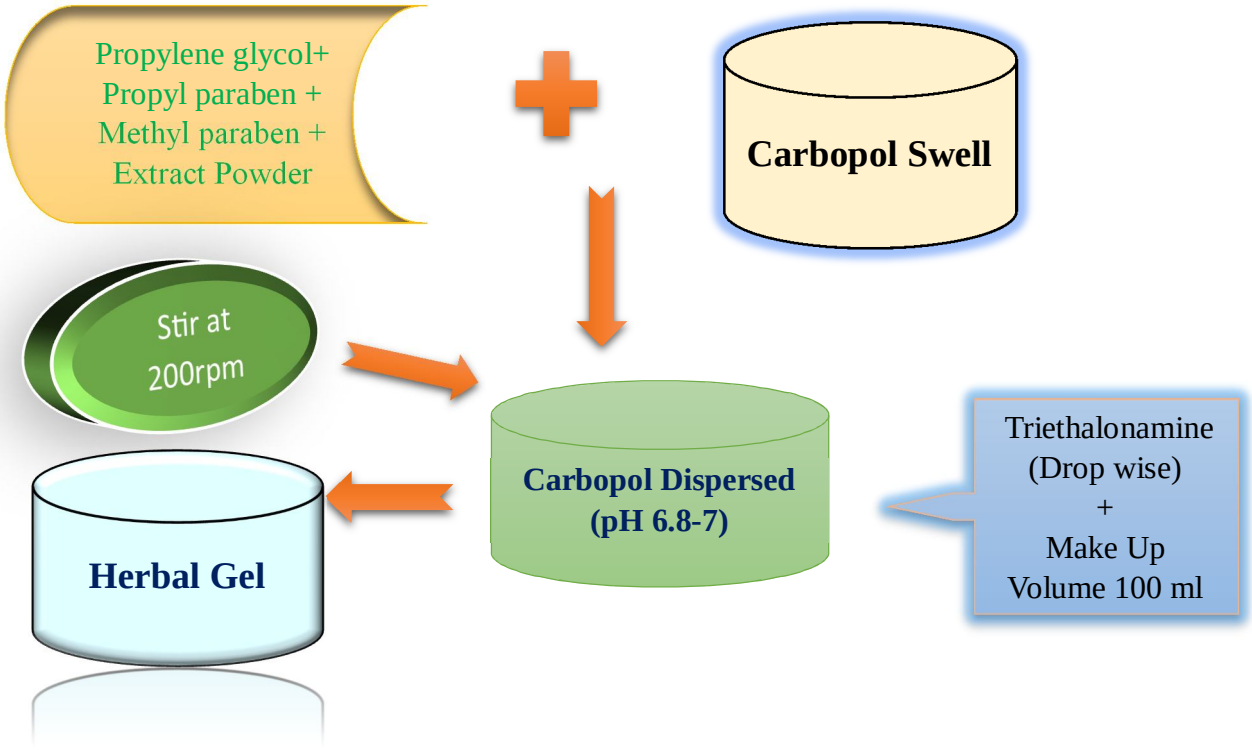
C. Formulation of Herbal Gel

During formulation gelling agents used at two different concentrations (1.0-1.5), resulting in six different batches of gels for six batches for *C indicum* extract powder [20].

STEP-I:



STEP-II:



V. CHARACTERIZATIONS OF HERBAL GEL

All prepared formulations of gel were characterized for:

A. Appearance

Color and appearance of all formulation herbal gel batch were observed manually.

B. pH determination

The pH of all gel formulations was determined by using digital pH meter. 1 gram of gel was dissolved in 50 ml distilled water and stored for two hours [21,22]. The measurement of pH of each formulation was done in triplicate and average value was calculated. *(Pawar DP, et al., 2013)*

C. Grittiness

All the gel formulations were evaluated microscopically for the presence of any appreciable particulate matter which was seen under light microscope. Hence the gel preparation fulfills the requirement of freedom from particular matter and form grittiness as desired for any topical preparation [23].

D. Viscosity

The viscosity of the gel formulations was determined using Brookfield viscometer with spindle no 64 at rpm 50, 60,100.

E. Spredability

The 1 g of herbal gel formulation was placed over one of the slides (20 cm × 20 cm). The other slide was placed on the top of the, 75gm weight was placed upon the upper slides so that the herbal gel between the two slides was pressed uniformly to form a thin layer. The two slides in position were fixed to a stand without slightest disturbance and in such a way that only the upper slide to slip off freely by the force of weight tied to it. A 75-gm. weight was tied to the upper slide carefully. The time taken for the upper slide to travel the distance in cm and separated away from the lower slide under the influence of the weight was noted. The experiment was repeated by three times and the mean time was taken for calculation.

Spreadability was calculated using formula:

$$S = M.\ L / T$$

Where- M= weight tied to upper slide

L = length of glass slide

T = Time taken to separate the slide

F. Drug Content

1 g of the prepared gel was mixed with 100ml of phosphate buffer pH 7.4. Aliquots of different concentration were prepared by suitable dilutions after filtering the stock solution and the drug content was determined measuring the absorbance at 510 nm using UV/Vis spectrophotometer (UV-3000, Lab India) [21,23].

Drug content= $\frac{\text{Absorbance}}{\text{slope}} * \text{dilution factor} * \frac{1}{1000}$

G. Extrudability

The all-gel formulations were filled in standard capped collapsible aluminum tubes and sealed by crimping to the end. Weights of the tubes were recorded. The tubes were placed between two glass slides and were clamped. 500 gm. Weights was placed over the slides and then the cap was removed. The amount of the extruded gel was collected and weighed. The percentage of the extruded gel was calculated.

% Extrudability = $\frac{\text{Weight of extrude gel}}{\text{Weight of formulation gel}} * 100$

Where, (>90% extrudability: excellent, >80% extrudability: good, >70% extrudability: fair).

H. Homogeneity

All the developed gels were tested for homogeneity by visual inspection after setting the gels in the test tube and observed for their appearance and presence of any aggregates.

I. Washable Test

The all formulation of gel was applied on hand and observes washability under running water.

J. Swelling Index

Swelling of the polymer depends on the concentration of the polymer, ionic strength and the presence of water. The determination of the swelling index of prepared topical gel, weighed 1gm of gel was taken on porous aluminum foil and then placed separately in a 50 ml beaker containing 10 ml 0.1 N NaOH. Then samples were removed from beakers at different time intervals and put it on dry place for some time after it reweighed [21,23].

Swelling index was calculated by the formula:

Swelling Index (SW) % = [(Wt. – Wo) / Wo] × 100.

Where,

(SW) % = Equilibrium percent swelling,

Wt. = Weight of swollen gel after time t,

Wo = Original weight of gel at zero time.

K. Antimicrobial Test

1) In-Vitro Antibacterial Activity

20mL of sterile, molten Muller Hinton (MH) agar butts & cooled to 45^{o}C were added to sterile petri dishes containing 0.625mL of the 18-24hrous old Bacteria and the plates were rotated clockwise and anticlockwise so as to distribute the culture uniformly in the medium. The plates were kept undistributed for 10 to 15mins. Then using a sterile steel bore with internal diameter 6mm and external diameter 8mm; four wells were punched in the medium one in each quarter of the plates. Added herbal gel, control, was then added to each of the well and the plates were kept in the refrigerator for 30min for prediffusion of the sample. Then the plates were incubated at 37^{0}C for 24 hours [21].

2) In-Vitro Antifungal Activity

Accurately weighed 16.25gm of Sabouraud dextrose agar transfer into 500 ml of sterile conical flask. Added 250ml of distilled water and applied heat for completely dissolved. Then the conical flask was sterilized for 15minutes at 120 ^{0}C (15lb) in autoclave. Cooled at room temperature. The media was poured into petri dish and the fungal strain (*Candida albicans*) was dispersed in media plate, cooled at room temperature for completely solidifying. Then using a sterile steel bore with internal diameter 6mm; four wells punched in the medium one in each quarter of the plates. Added herbal gel, control, standard drug was added to each of the plates were kept in the incubated at 37^{0}C for 72 hours [24,25].

L. In-vitro Anti-inflammatory Test

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate-buffered saline (phosphate buffer saline, pH 6.4) and 2 ml of gel solution so that final concentrations was (31.25, 62.5, 125, 250, 500, 1000µ g/mL) used. A similar volume of double distilled water served as the control.

Next, the mixture was incubated at 37±2^{0}C in a BOD incubator for 15 minutes and then heated at 70^{0}C for 5 minutes. After cooling, their absorbance was measured at 510nm by using the vehicle as a blank. Diclofenac sodium in the final concentration of (78.125, 156.25, 312.5, 625, 1250, 2500µ g/mL) was used as the reference drug and treated similarly for the determination of absorbance [26].

The percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibition= 100× [Vt/Vc-1]

Where, Vt = absorbance of the test sample

Vc = absorbance of control

M. IN-Vivo Studies

Animal Preparation

Mice, weighing 20-30g, were obtained from the Sinhgad institute of pharmaceutical sciences (SIPS), Lonavala. Housing conditions and in vivo experiments were carried out in accordance with the CPSCC (SIPS/IAEC/2018-2019/06) for the care and use of laboratory animals in scientific research and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals issued by the SPPU, Pune University.

1) Skin Irritation Test on Mice

The aim of present study was evaluation of skin irritation activity of herbal gel formulation. The different concentration of gel will be prepared by using carbapol 940 as a gel base *Chrysanthemum indicum* gel will be used for skin irritation study in mice.

All animals will be divided into 4 groups. Group I will be control (1% Carbopol 940 base gel), Group II will be treated with standard (Diclofenac gel), Group III will be treated with 2.5% extract soluble solid in 1% gel base and group IV will be treated with 5% extract soluble solid in 1% gel base shows in Table. The animal was treated daily up to seven days and finally treated skin was examined visually for erythema and edema [25].

2) In vivo Anti-Inflammatory Test

Mice will be randomly divided in 4 groups and to all the groups, hind paw edema will be induced by injecting 0.1 ml of 1% (w/v) carrageenan subcutaneously into the planter region of the hind paw of mice. Herbal gel will be applied 1 hour prior to carrageenan injection. One group of mice will be served as treated with 1% Carbopol 940 gel base treated control shows in Table.

The hind paw edema volume will be measured by volume displacement method using plethysmometer by immersing the paw till the level of lateral malleolus at various time interval (0, 1, 2, 3, 4, 5, 6, hrs.) after carrageenan injection. Results will be expressed as percentage inhibition of edema by comparing with treated with gel base treated [27,28,29].

The percentage inhibition in paw volume was calculated by using the formula:

% inhibition $\frac{\boldsymbol{Paw\ volume\ (Control) - Paw\ volume(Test)}}{\boldsymbol{Paw\ volume\ (Control)}}$=*100

Table: Animal study divided group for skin irritation and anti-inflammatory test

FOR SKIN IRRITATION TEST: Group of Mice: Four Groups (n=4)	
Animal Group	Treatment
Group I	Gel base (1%w/w of Carbopol 940) for 7days (control group)
Group II	5% of Diclofenac gel for 7 days. (Standard)
Group III	2.5% using extract of *chrysanthemum indicum* soluble solid in 1% gel base for 7 days. (Test 1)
Group IV	5% using extract of *chrysanthemum indicum* soluble solid in 1% gel base for 7 days. (Test 2)
FOR ANTIINFLAMMATORY TEST: Group of Mice: Four Groups (n=4)	
Group I	0.1ml of 1% carrageenan induced paw- edema
Group II	Gel base (1% w/w of Carbopol 940) (control group)
Group III	5% using extract of *chrysanthemum indicum* soluble solid in 1% gel base
Group IV	Diclofenac sodium (10mg/kg) (Standard)

N. Stability Studies

The stability study was performed as per ICH guidelines. The formulated gel was filled in collapsible tubes and stored at different temperatures and humidity conditions,

25±2 ºC / 60±5% RH,

30±2 ºC / 65±5% RH,

40±2 ºC / 75±5% RH

For a period of three months and studied for appearance, pH, spreadability, extrudability, drug content and *In-Vitro* drug diffusion study [30,31,32,33,34].

VI. RESULT & DISCUSSION

A. Preformulation Studied

Preformulation parameters were identified for organoleptic properties

1) Organoleptic properties of plant flower shown in Table.

Table: Organoleptic properties of *Chrysanthemum indicum* flower

ORGANOLEPTIC PROPERTIES	FLOWER	Inference
Test	Observation	
Color	Yellowish	Pass
Oduor	Aromatic	Pass
Taste	Bitter	Pass
Morphology		
Size	0.5-1 cm	Pass
Shape	Oval, straight pellets	Pass

2) Extractive value

Extractive value of flower of *Chrysanthemum indicum* by solvent and Soxhlet extraction shown in (Table)

Table: Extractive value of flower of *Chrysanthemum indicum*

Sr. No	Extraction process	Solvent	Extractive Value	Inference
1	Solvent extraction	Ethanol	15.660%	Excellent
2	Soxhlet extraction	Ethanol	13.33%	Good

3) Determination of soluble solid and loss of drying

The determination of soluble solid content and loss of drying of *Chrysanthemum indicum* Flower extract shown in Table:

Table: The determination of soluble solid content and loss of drying of *Chrysanthemum indicum* Flower extract

Sr. No	Test	Observed value (%)	Inference
1	Soluble solid content	46.5%	-
2	Loss of drying	11%	-

4) Determination of ash value

The determination of ash value *Chrysanthemum indicum* extract soluble solid for identification exhausted drug, low grade product and earthy matter was shown in Table:

Table: Ash value of *Chrysanthemum indicum* extract soluble solid

Sr. No	Test	Observed value (%)	Inference (Compare with reference)
1	Total ash value	10	Good
2	Acid insoluble ash	1.064	Good
3	Water soluble ash	1.56	Good

5) Preformulation Studies

A. Determination of Solubility: Solubility of extract soluble solid in various medium shown in Table:

Table: Solubility of Extract soluble solid

Sr. No	Medium	Extract soluble solid	Inference
1	Water	Soluble	From 10 to 30 parts
2	Ethanol	Freely soluble	From 1 to 10 parts.
3	Chloroform	Freely soluble	From 1 to 10 parts
4	Methanol	Freely soluble	From 1 to 10 parts
5	Propylene glycol	Freely soluble	From 1 to 10 parts

B. Calibration curve of Extract Soluble Solid

The extract soluble solid in solution was found to be obeying Beer's Lambert's law with concentration range of 2-10 µg/ml in phosphate buffer of pH 7.4. The absorbance was measured at 510 nm. The different absorbance of each concentration mention in Table and calibration curve was shown in Figure**.** The correlation coefficients value (R^2) for the calibration curve was found to be R^2=0.9937.

Table: Calibration curve of extract soluble solid

Calibration curve of Extract soluble solid		
Sr. No	**Concentration (µg/ml)**	**Absorbance**
1	0	0.0488
2	2	0.1199
3	4	0.2398
4	6	0.3644
5	8	0.4625
6	10	0.5409

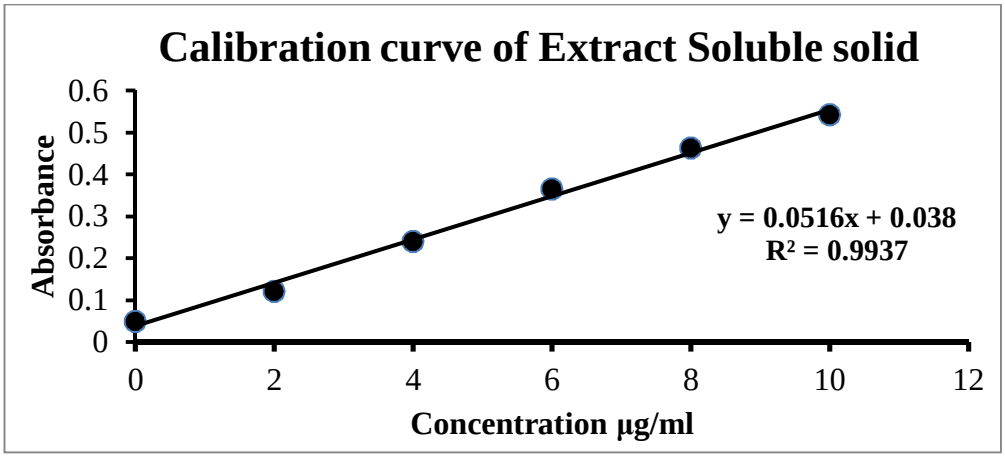
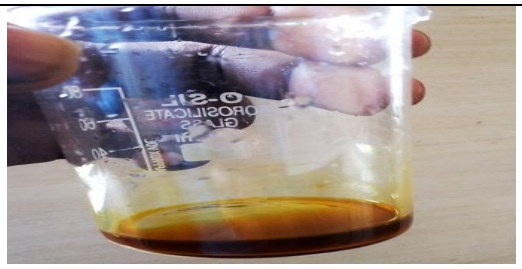


Figure: Calibration curve of extract soluble solid

6.1.6 Preliminary Phytochemical Investigation

6.1.6.1 Flavonoids test:

Table: Flavonoids Test

Ammonia test: Observation- A yellow coloration observed in the extract. Result- Confirm the presence of flavonoids.	 Figure: Yellow coloration observed
Alkaline reagent test: Observation- A yellow coloration observed in the extract Result- Confirm the presence of flavonoids.	 Figure: Yellow coloration observed
Lead Acetate test: Observation- A yellow coloration observed in the extract. Result: Flavonoids Present	 Figure: Yellow coloration observed

6.1.6.2 Test for phenols:

Observation- A brownish green or a blue-black coloration observed in the extract

Result- Confirm the presence of Phenols in leaf extract.

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6.1.6.3 Detection of oil content (spot test):
Observation- Oil strains on the filter paper indicated the presence of fixed oils in flower powder.
Result- Confirm the presence of oil content in flower powder.

Figure: Oil content

6.1.6.4 Test for Cardio Glycosides
Observation- A violet ring was appearing below the ring, and not formed thin layer.
Result- Cardio glycoside is present in flower extract powder.

6.1.6.5 Test for Saponins
Observation- Emulsion was not formed and not observed foam.
Result- Saponins are not present this extract.

6.1.6.6 Test for alkaloids
Observation- An orange red precipitate was formed in extract.
Result- Indicated the presence of alkaloid in extract.

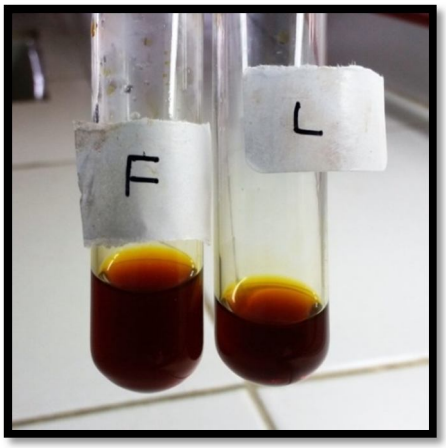

Figure: Orange red precipitate

6.1.6.7 Test for steroids and sterols
Observation- The colour was not changed from violet to blue green in the extract.
Result- Indicates the absence of steroids and sterols

6.1.6.8 Test for tannins
Observation- The extract with dilute ferric chloride solution (5%) observes violet colour and White precipitate with 10% lead acetate solution
Result- Indicates the presence of tannins.

6.1.6.9 Test for proteins and free amino acids
Observation- Million's reagent: Appearance red colour and Ninhydrin reagent Appearance purple colour with sample.
Result- Indicates the presence of proteins and amino acid.

6.1.6.10 Tests for carbohydrates
Observation: Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.
Result- Indicates the presence of carbohydrates.
Observation presence or absence of phytochemicals namely, cardio glycosides, saponins, fixe d oils and fats, alkaloids, steroids and sterols, flavonoids, tannins, protein, free amine, carbohydrate and phenolic compounds in *Chrysanthemum indicum* extract were noted as + for presence and – sign for absence and are presented in Table.

Table: Preliminary phytochemical analysis of extract of *C. indicum* Flower

Sr. No.	**Test**	**Presence (+) / Absence (-)**
1	Flavonoids	+
2	Phenol	+
3	Cardiac glycoside	+
4	Oil content	+
5	Alkaloids	+
6	Saponins	-
7	Steroids & Sterols	-
8	Tannins	+
9	Carbohydrates	+
10	Proteins and free amino acids	+

6.1.6.11 Content of flavonoids:
The spectrophotometer assay for the quantitative determination of flavonoid content of the extract was calculated by the help of calibration curve of the quercetin (used as standard). The quercetin solution of concentration (2-10µg/ml) conformed to Beer's Law at 510 nm. The different concentration absorbance shown in Table, with a regression co-efficient (R2) = 0.9927. The plot has a slope (m) = 0.0371 and intercept = 0.0199. The equation of standard curve is y = 0.0371x+ 0.0199 (Figure).

Table: Calibration curve of Standard Quercetin

Sr. No	Concentration (µg/ml)	Absorbance
1	0	0.2161
2	2	0.4201
3	4	0.6003
4	6	0.8201
5	8	1.0087
6	10	1.1744

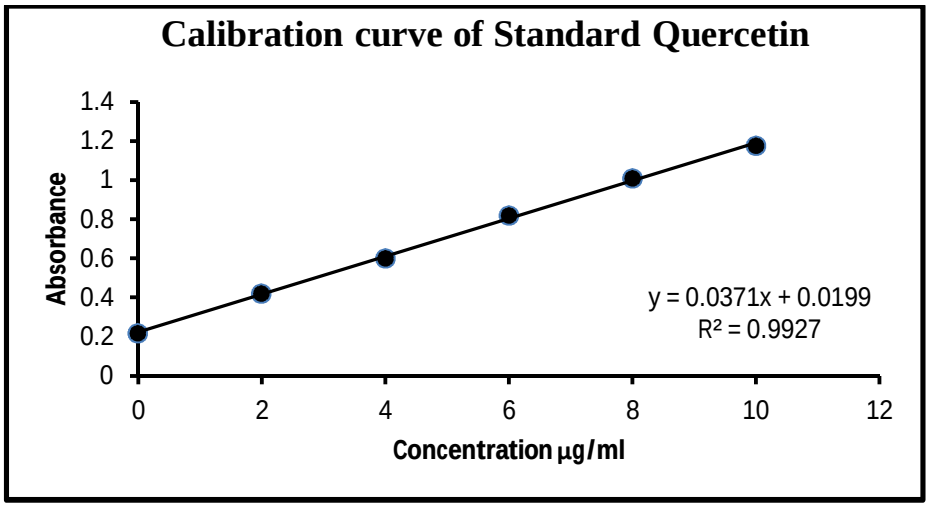

Figure: Calibration curve of standard Quercetin

Table: Results of total flavonoid content for *C. indicum* herbal gel formulation

Sr. no	Concentration of extract Powder	Absorbance	Flavonoid content (mg of quercetin equivalent/ g dry material)
1	Ethanol, 5 mg/ml	2.1039	0.37 mg/g
2	Ethanol, 10 mg/ml	2.8392	0.501mg/g
3	Ethanol, 15 mg/ml	3.9994	0.550 /g

6.1.6.12 Thin layer chromatography (TLC)

It is observed from data presented that, different solvent systems showed differences in number of bands and their Rf values in extract of *C. indicum* Among all the tested solvent systems Phase A & Phase B solvent.

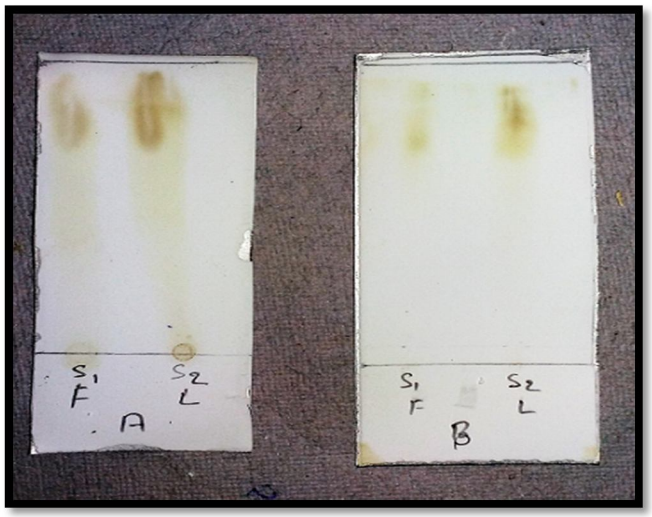

Figure: TLC plate of *C. indicum* flower (A- Phase A solvent, B- Phase B solvent, S_1- Flower extract & S_2- Soluble solid)

- Chromatography of *C. indicum* flower and leaf extract the Rf in Phase A std. solvent- Flower extract: 0.90.
- Chromatography of *C. indicum* flower and leaf extract the Rf in Phase B std. solvent -Flower extract: 0.95.

VII. EXPERIMENT DESIGN

A. Formulation And Development

7.1.1 Drug-excipient interaction should be investigated by FTIR:

For evaluation of compatibility between drug and excipient FTIR spectra were recorded. FTIR spectra of extract soluble solid with Carbopol 940, Methyl paraben, Propyl paraben, Triethanolamine, Propylene glycol was observed. The results showed important functional group in the spectra of drug sample which confirmed that the analysed sample was extracting soluble solid Figure and peak of the spectra described in Table.

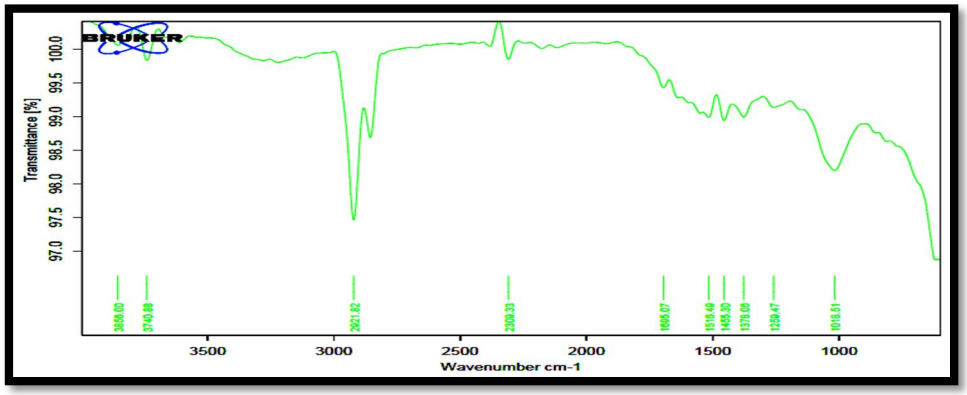

Figure: FTIR spectra of soluble solid

Table: FTIR interpretation of extract soluble solid

Sr. no	Functional group	Frequency (cm^{-1})
1	OH Stretch	3886
2	OH Stretch	3740.88
3	-C-H stretch	2921.82
4	C=C alkene	1696.07
5	NO2 stretch	1518.48
6	CH2 bend	1455.30

The FTIR spectra of Carbopol 940 with soluble solid (Figure) and peak of spectra found are described in the Table.

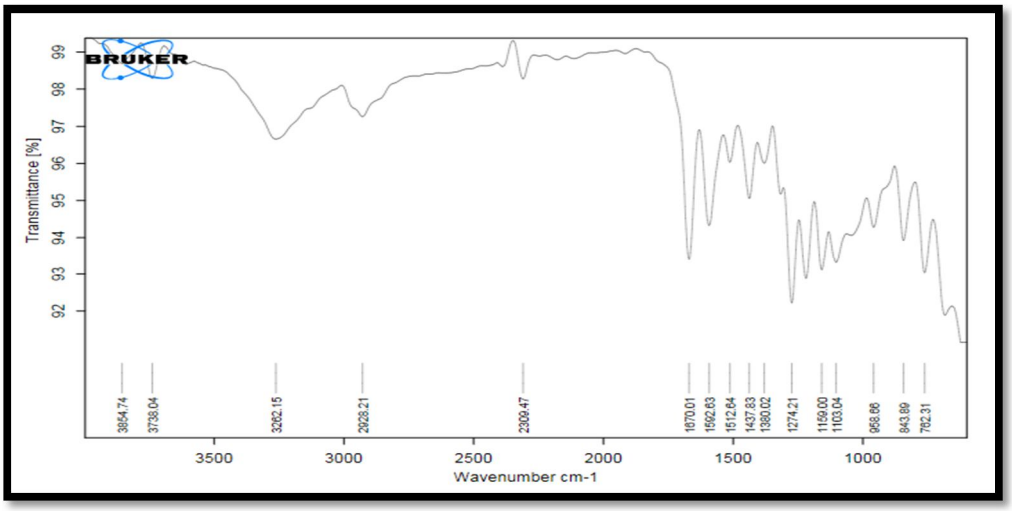

Figure: FTIR spectra of soluble solid with Carbopol 940

Table: FTIR interpretation of extract soluble solid with Carbopol 940

Sr. no	**Functional group**	**Frequency (cm^{-1})**
1	OH Stretch	3854.74
2	OH Stretch	3738.04
3	-C-H stretch	2928.21
4	C=O amide	1592.63
5	CH2 bend	1437.83
6	CH_3 bend	1380.02
7	C-O-C stretch	1274.21

The FTIR spectra of Methyl paraben with soluble solid (Figure) and peak of spectra found are described in the Table.

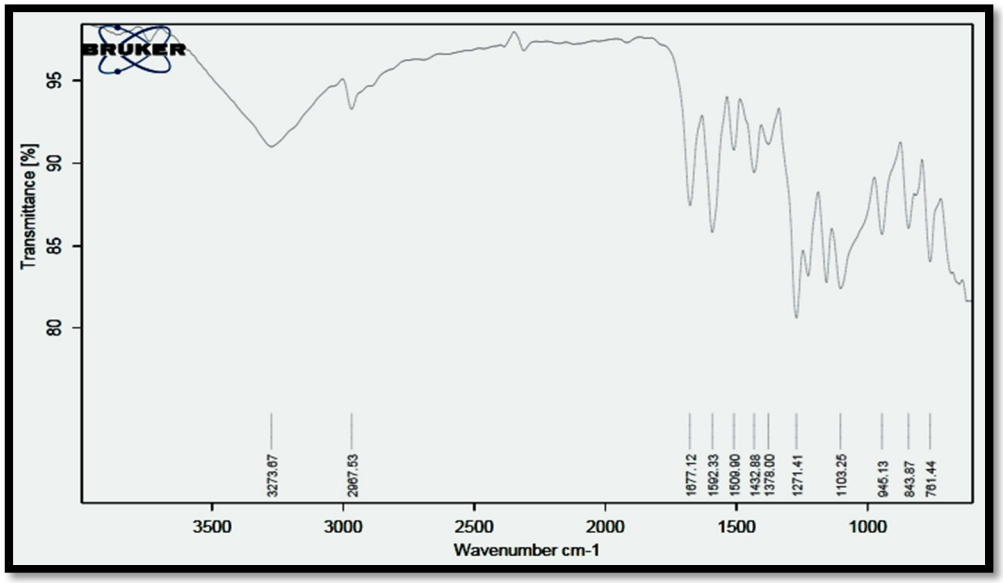

Figure: FTIR spectra of soluble solid with Methyl paraben

Table: FTIR interpretation of extract soluble solid with Methyl paraben

Sr. no	Functional group	Frequency (cm^{-1})
1	OH stretch	3273.87
2	-C-H stretch	2987.53
3	C=O anhydride	1871.12
4	C=O amide	1592.33
5	CH2 bend	1432.88
6	C-O-C stretch	1271.41

The FTIR spectra of Propyl paraben with soluble solid (Figure) and peak of spectra found are described in the Table.

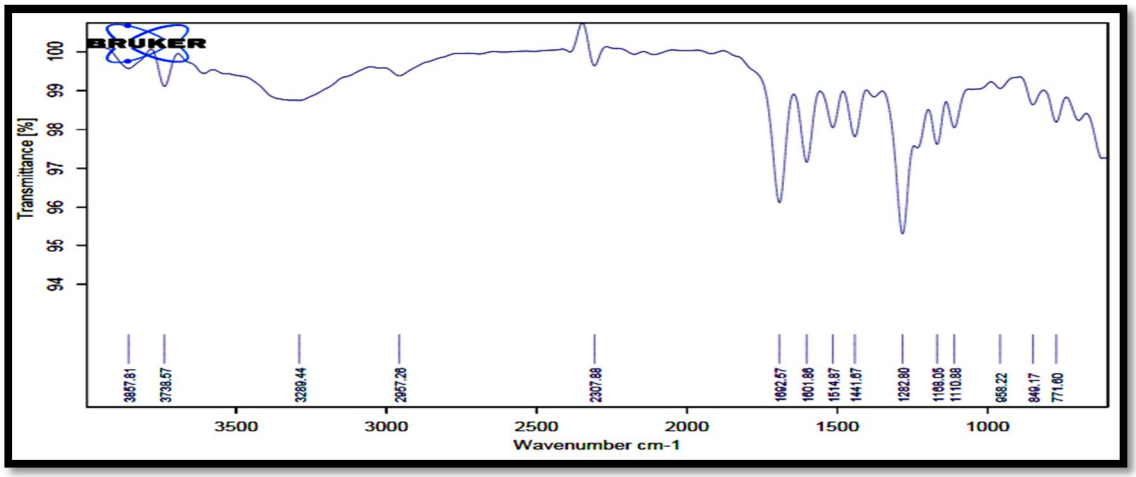

Figure: FTIR spectra of soluble solid with Propyl paraben

Table: FTIR interpretation of extract soluble solid with Propyl paraben

Sr. no	Functional group	Frequency (cm^{-1})
1	OH stretch	3857.81
2	OH stretch	3738.57
3	OH stretch	3288.44
4	-C-H stretch	2957.28
5	C=C alkene	1695.07
6	C=O amide	1516.49
7	C-F	1018.51

The FTIR spectra of Triethanolamine with soluble solid (Figure) and peak of spectra found are described in the Table.

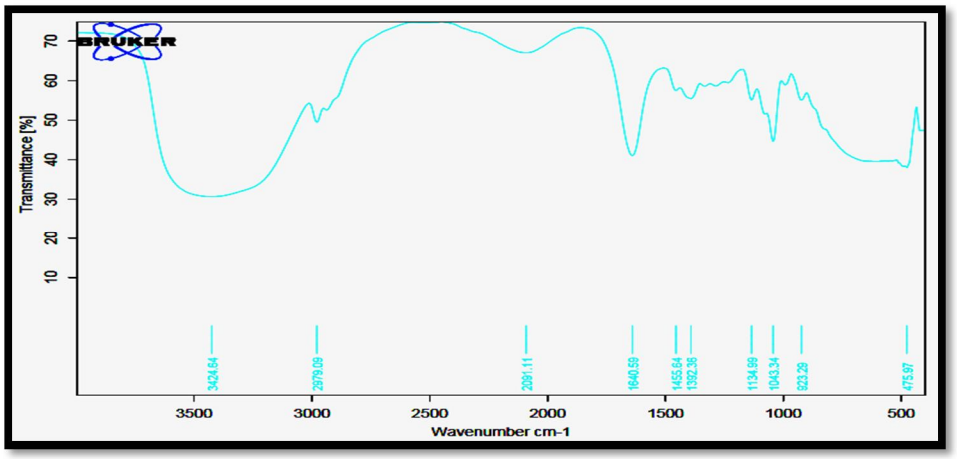

Figure: FTIR spectra of soluble solid with Triethanolamine

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Table: FTIR interpretation of extract soluble solid with Triethanolamine

Sr. no	Functional group	Frequency (cm^{-1})
1	OH stretch	3424.64
2	-C-H stretch	2979.09
3	C=C alkene	1640.59
4	CH_2 bend	1455.64
5	C-F	1043.34

The FTIR spectra of Propylene glycol with soluble solid (Figure) and peak of spectra found are described in the Table.

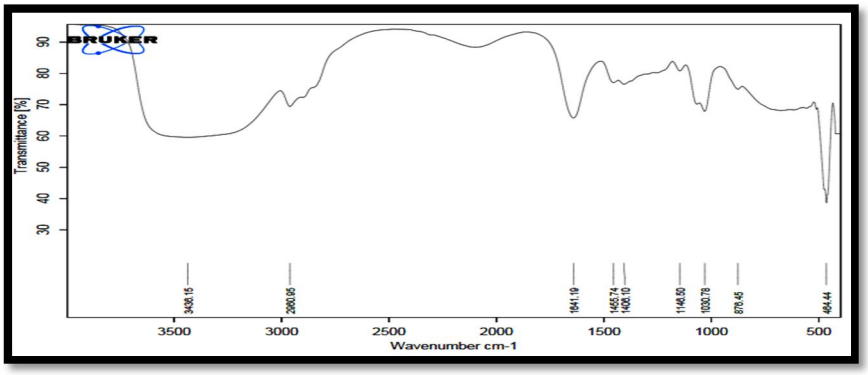


Figure: FTIR spectra of soluble solid with Propylene glycol

Table: FTIR interpretation of extract soluble solid with Propylene glycol

Sr. no	**Functional group**	**Frequency (cm^{-1})**
1	OH stretch	**3435**
2	-C-H stretch	**2990.95**
3	C=C alkene	**1641.19**
4	CH_2 bend	**1455.74**
5	C-F	**1030.78**

Hence, the whole excipient is compatible with extract soluble solid, shows similar frequency.

7.1.2 Optimization Formulation of herbal gel: Optimization of gelling agent with different drug concentration shown in Table and formulated six batches with drug concentration and polymer concentration (F1, F2, F3, F4, F5, F6).

7.1.3 CHARACTERIZATION OF HERBAL GEL:

7.1.3.1 Appearance:

All herbal gel formulation batches containing *Chrysanthemum indicum* extract soluble solid were found to be homogeneous or color shown in Table:

Table: Appearance of gel formulation batches

Sr. no.	**Formulations**	**Appearance**
1	F1	Light yellowish
2	F2	Light yellowish

3	F3	Yellowish
4	F4	Light yellowish
5	F5	Dark yellowish
6	F6	Yellowish

7.1.3.2pH determination:

All herbal gel formulation batches containing *Chrysanthemum indicum* extract soluble solid were found to be pH shown in Table and their graphical evaluation shown in figure:

Table: Measurement of pH value of formulations

Sr. No	Formulation	pH value
1	F1	7.4
2	F2	7.3
3	F3	7.2
4	F4	7.4
5	F5	7.1
6	F6	7.4

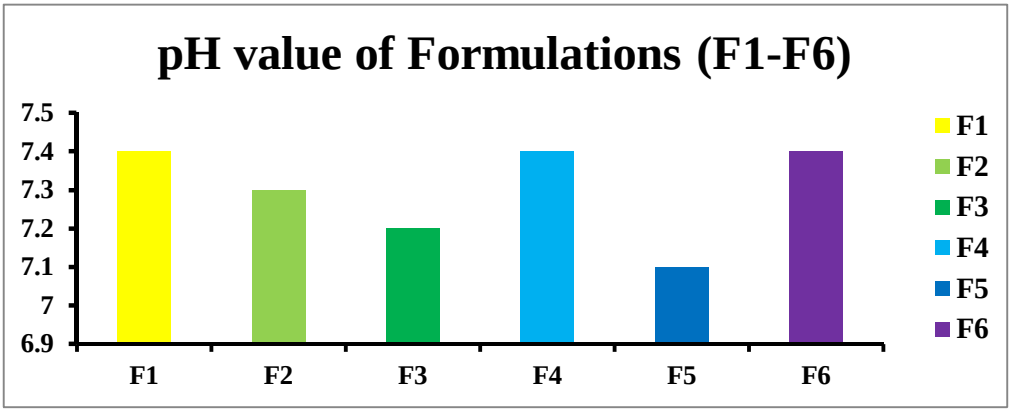


Figure: Measurement of pH value of formulation (F1-F6)

7.1.3.3 Grittiness:

All the gel formulations were evaluated microscopically for determination of presence of any appreciable particulate matter which was seen under light microscope shown in Figure.

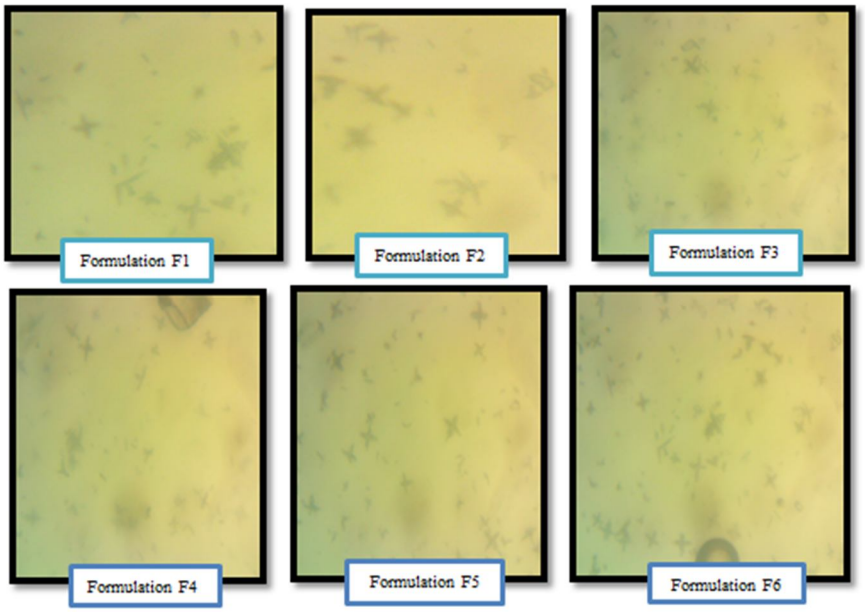


Figure: Grittiness test of gel formulation batches

7.1.3.4 Viscosity:

The determination of viscosity of herbal gel formulations using Brookfield viscometer with spindle no 64 at rpm 50, 60,100 was shown in Table:

Table: Measurement of viscosity of gel formulation (F1-F6)

FORMULATION	RPM	cP	% Viscosity
F1	50	9930	97.5
	60	9798	96.5
	100	5838	93.4
F2	50	11828	97.2
	60	18292	94.1
	100	38148	97.6
F3	50	1138	99.1
	60	8484	84.0
	100	5438	95.3
F4	50	8382	83.2
	60	1019	98.3
	100	18091	93.9
F5	50	29701	99.6
	60	18460	94.2
	100	8210	80.9
F6	50	5283	94.3
	60	10193	99.1
	100	19281	94.3

7.1.3.5 Spredibility: (6.2.11.5)

The spreadability of *Chrysanthemum indicum* extract soluble solid containing gel formulation was depicted in Table from the combined graph of all formulation it was concluded that all the developed formulation showed acceptable spreadability (Figure). All formulation compare with marketed gel formulation shows good spreadability.

Table: Measurement of spredibility (gm.cm/sec) of formulation

Sr. No	Formulation	Spredibility (gm.cm/sec)
1	F1	23.07
2	F2	19.23
3	F3	20
4	F4	22.72
5	F5	23.43
6	F6	21.12
7	Standard (Diclofenac gel)	24.59

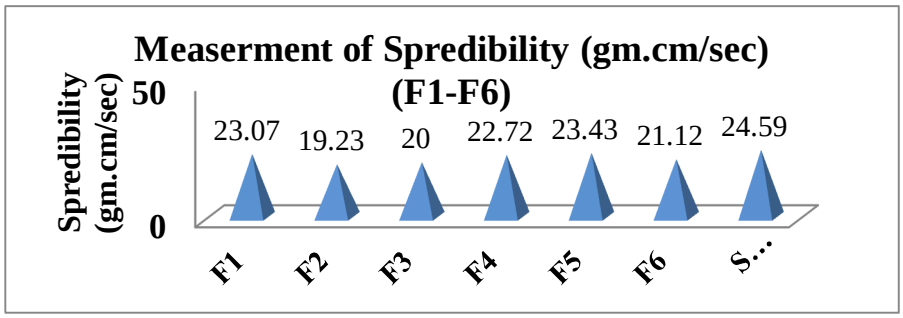


Figure: Spreadability of gel compare with Standard gel formulation

7.1.3.6 Drug content:

Determination of drug content in all herbal gel formulation spectrophotometer assays for the quantitative determination of Drug content of the herbal gel formulation was calculated by the help of calibration curve of the soluble solid solution in phosphate buffer pH 7.4. The soluble solid solution of concentration (2-10µg/ml) conformed to Beer's Law at 510 nm. The different concentration absorbance shown in Table no 8.21, with a regression co-efficient (R^2) = 0.9937. The plot has a slope (m) = 0.0516 and intercept = 0.038. The equation of standard curve is y = 0.0516x+ 0.038 (Fig. 8.19). The drug content of gel formulation shown in Table no. 8.22 and graph show in Fig no. 8.20:

Table: Calibration curve of soluble solid of extract

Sr. No	Concentration (ug. /ml)	Absorbance
1	0	0.0488
2	2	0.1199
3	4	0.2398
4	6	0.3644
5	8	0.4625
6	10	0.5409

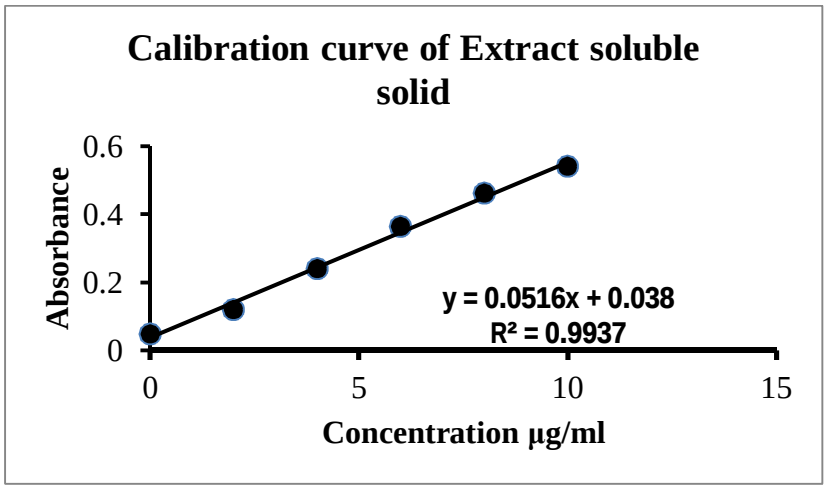


Figure: Calibration curve of soluble solid of extract

Table: Measurement of % Drug Content (F1-F6)

Sr. No	Formulation	Drug content (%)
1	F1	97.32
2	F2	91.07
3	F3	96.87
4	F4	94.78
5	F5	96.62
6	F6	92.82

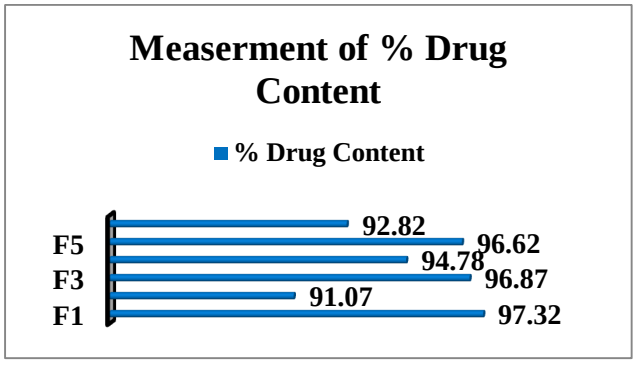


Figure: Graphical presentation of measurement of % drug content

7.1.3.7 Extrudability:

Gels with high consistency may not extrude from tube whereas, low viscous gels may flow quickly and good consistency is required in order to extrude the gel. The extrusion of the all-formulated gel from the tube is an important during its topical application and good patient acceptance. The measurement of extrudability value shown in Table and the graphical presentation shows in Figure.

Table: Measurement of % Extrudability of all formulations (F1-F6)

Sr. No	Formulations	% Extrudability
1	F1	90.41
2	F2	90.2
3	F3	87.45

4	F4	72.64
5	F5	84.52
6	F6	89.81

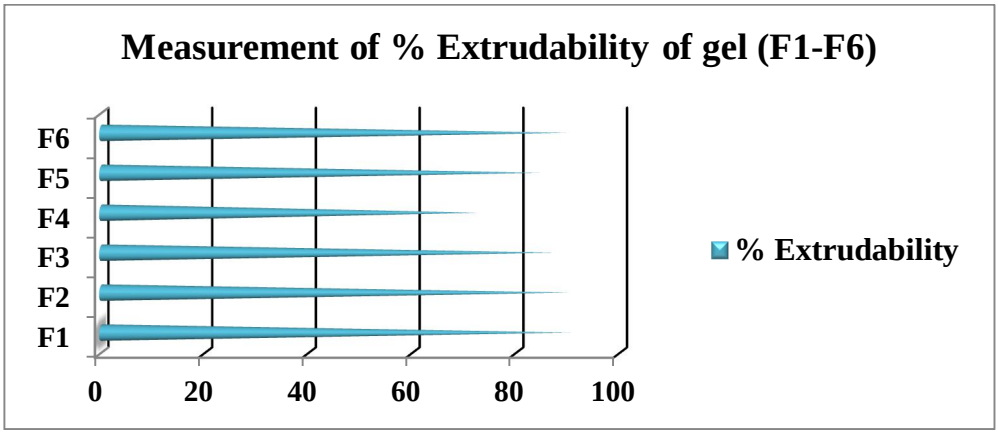


Figure: Measurement of % Extrudability of all gel formulation

7.1.3.8 Homogeneity:
All the developed gels were tested for homogeneity by visual inspection after setting the gels in the test tube. Result showed that the developed herbal gel was yellowish in colour, translucent in appearance and showed good homogeneity with absence of lumps.

7.1.3.9Washable test:
The washability study of all gel formulation was observed on the hand; Hence the gel was easily washed with running water.
The evaluation of all developed gel was optimized by studying physical evaluation, drug content, spredibility, pH, extrudability, viscosity and grittiness. Batch F1 showed good result based on above mentioned parameter. Hence, it was selected for further study.

7.1.3.10Swelling index (F1):
The swelling index study of formulation (F1) gel was observed. The calculation data shown in Table and graphical presentation shown in figure.

Table: Measurement of swelling index %

Sr. No	Time (Hrs)	Swelling index %
1	1	6.87
2	2	10.68
3	3	12.21
4	4	18.32
5	5	29.00
6	6	37.40
7	7	49.40
8	8	60.30

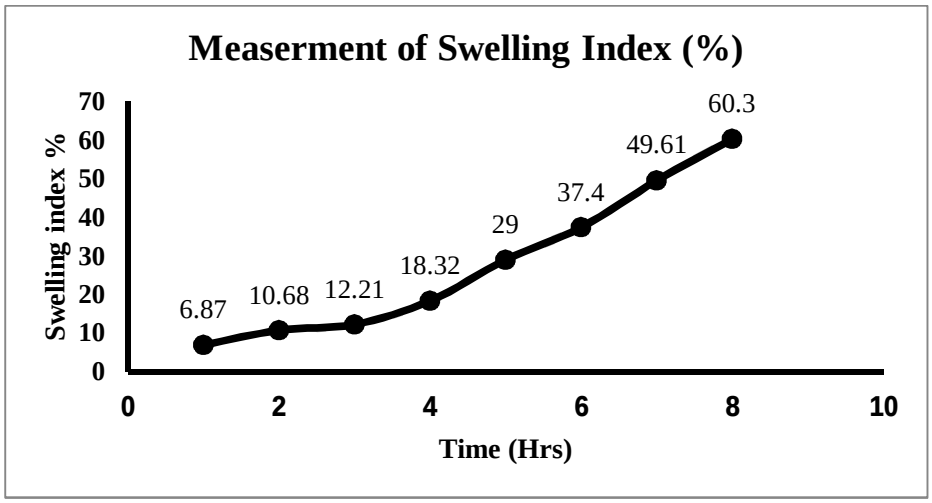


Figure: Graphical presentation of swelling index %

7.1.3.11 In-vitro drug diffusion study:

For determination of drug diffusion in herbal gel formulation spectrophotometer assays for %CDR of the herbal gel formulation was calculated with the help of calibration curve of the soluble solid in phosphate buffer pH 7.4. The soluble solid solution of concentration (2-10µg/ml) conformed to Beer's Law at 510 nm. The different concentration absorbance shown in Table, with a regression co-efficient (R2) = 0.9937. The plot has a slope (m) = 0.0516 and intercept = 0.038. The equation of standard curve is y = 0.0516x+ 0.038 (Fig. 7.14). The % CDR of gel formulation shown in Table and graph show in Figure:

Table: In–vitro Drug Diffusion Study of Topical Gel

Sr. No.	Time (Hrs.)	% CDR of Herbal gel	% CDR of Diclo. gel
1	1	0.71	0.87
2	2	0.93	1.01
3	3	1.09	1.39
4	4	1.37	1.40
5	5	1.51	1.75
6	6	1.65	1.80
7	7	1.73	1.87
8	8	1.77	2.14

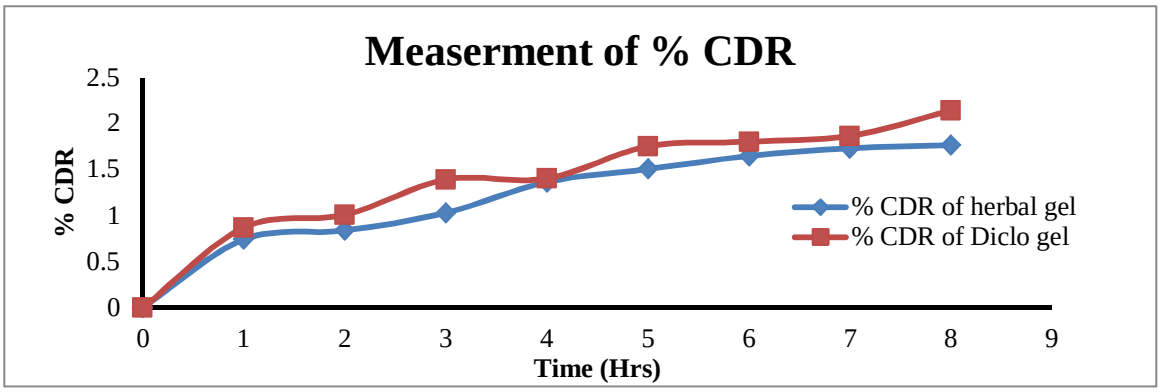


Figure: Graph showing comparison between % CDR in herbal gel and diclo gel

In-vitro diffusion profile of herbal gel and diclofenac gel, the cumulative amount of drug release was found to be 1.77% and 2.14% after period of 8hr for herbal gel and diclo gel respectively as shown in Table and graph show in Figure:

- Drug Release Kinetic

Based on kinetic released study F1 batch showed better release (98.4%) and it was fitted to the model shown in Figure.

Table: Kinetics release studies for best fit model

Herbal gel code	Zero Order Kinetics	First Order Kinetics	Higuchi kinetics	Kors-Peppas	Hixson-Crowell	Best fit Model
	r	r	r	r	r	
F1	0.9151	0.9165	0.9958	0.8632	0.9105	Higuchi
Marketed Gel	0.8779	0.8796	0.983	0.8246	0.874	Higuchi

The study of drug release kinetics showed that the formulation is governed by Higuchi's model. The curve was obtained after plotting the cumulative amount of drug released from formulation vs. time given in Table and Figure

Table: Higuchi's plot square root time Vs. Formulation

Sr. No	Square root of time (hrs.)	Formulation
		F1
1	0.000	0
2	1.000	0.71
3	1.414	0.93
4	1.732	1.09
5	2.000	1.37
6	2.236	1.51
7	2.449	1.65
8	2.646	1.73
9	2.828	1.77

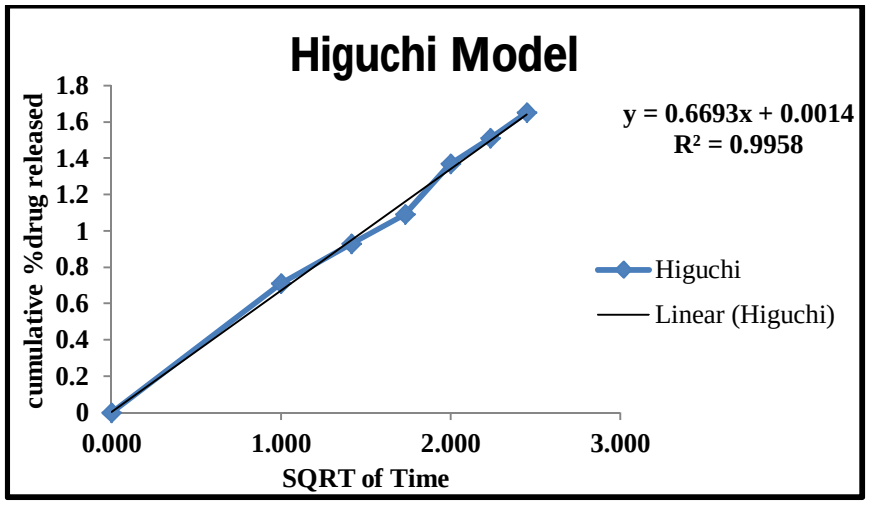
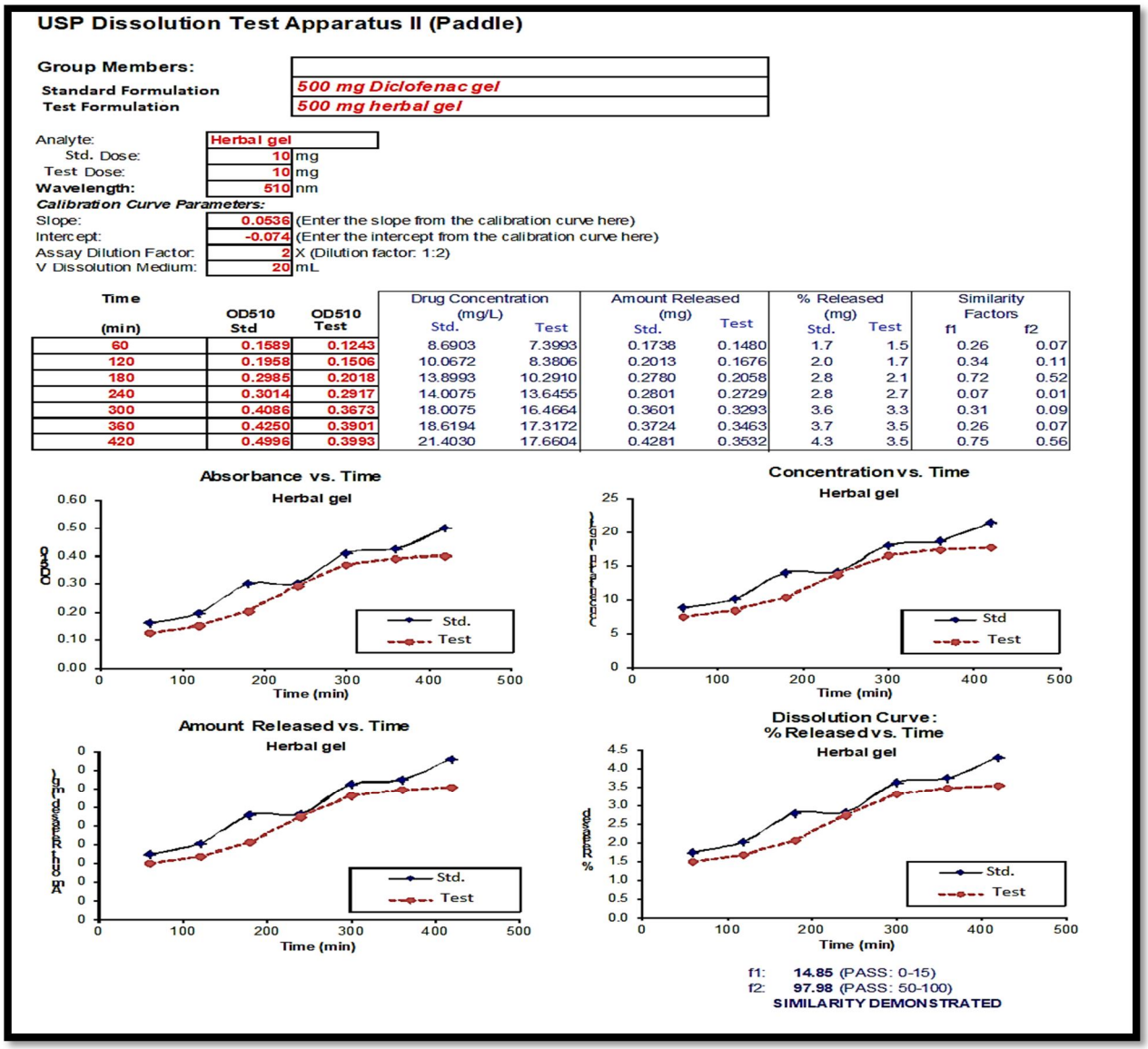


Figure: Higuchi model release kinetics of herbal gel (F1)

❖ Similarity factor of herbal gel formulation:



Time (min)	OD510 Std	OD510 Test	Drug Concentration (mg/L) Std.	Drug Concentration (mg/L) Test	Amount Released (mg) Std.	Amount Released (mg) Test	% Released (mg) Std.	% Released (mg) Test	Similarity Factors f1	Similarity Factors f2
60	0.1589	0.1243	8.6903	7.3993	0.1738	0.1480	1.7	1.5	0.26	0.07
120	0.1958	0.1506	10.0672	8.3806	0.2013	0.1676	2.0	1.7	0.34	0.11
180	0.2985	0.2018	13.8993	10.2910	0.2780	0.2058	2.8	2.1	0.72	0.52
240	0.3014	0.2917	14.0075	13.6455	0.2801	0.2729	2.8	2.7	0.07	0.01
300	0.4086	0.3673	18.0075	16.4664	0.3601	0.3293	3.6	3.3	0.31	0.09
360	0.4250	0.3901	18.6194	17.3172	0.3724	0.3463	3.7	3.5	0.26	0.07
420	0.4996	0.3993	21.4030	17.6604	0.4281	0.3532	4.3	3.5	0.75	0.56

Figure: Evaluation of Similarity factor of herbal gel formulation (F1)

7.1.3.12 Antimicrobial test:

1.*In-Vitro* Antibacterial activity:

- Minimum inhibitory concentration (MIC):

The MIC of herbal gel standard drug (Amoxicillin) was determined and compares with carbopol 940 (Vehicle base gel) gel and the results obtained are shown in Table:

Table: MIC of Herbal gel, Std. Drug and Vehicle base gel

Sr. no	Microorganism	Herbal Gel (T)	Carbapol 940 (C)	Std. Drug (S)
1.	***Staphylococcus aureus (S)***	0.96gm	0.0gml	1.06 mg/ml
2.	***Escherichia coli (E)***	0.94gm	-	1.05mg/ml

- Zone of inhibition:

Antibacterial activity of herbal gel was studied on the *S. aureus* and *E coli*. The results obtained were shown are Table and Figure:

Table: *In- Vitro* antibacterial activity of herbal gel

Sr. no	**Formulation**	**Zone of inhibition (mm)**	
		S. aureus (S)	***E. coli (E)***
1.	Herbal Gel (T)	13mm	9mm
2.	Carbapol 940 (C)	0.00mm	0.00mm
3.	Std. Drug (S)	16mm	13mm

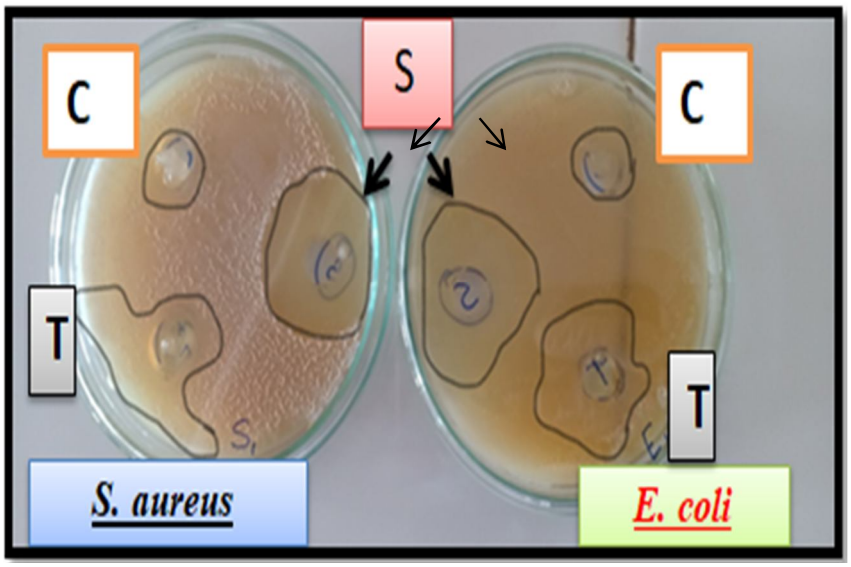


Figure: *In-Vitro* Antibacterial activity of herbal gel drug on *S. aureus* and *E. coli*

2. In- Vitro Antifungal activity:

- Minimum inhibitory concentration (MIC):

MIC of the herbal gel and standard drug (ketoconazole) was determined and compare with Carbopol 940 gel (Base) and the results obtained are shown in Table. The Herbal gel obtained was 0.59gm and ketoconazole 1.05mg/ml.

Table: MIC of Herbal gel and ketoconazole compare with Carbopol 940 gels

Micro-organisms	Herbal gel (T)	Carbopol 940 (C)	Std. drug (S)
Candida albicans	0.59gm	0.00gm	1.05mg/ml

- Zone of inhibition:

In-Vitro antifungal activity for herbal gel was studied on the *Candida albicans*. The results obtained are shown in Table and Figure:

Table: *In- Vitro* antifungal activity of herbal gel

Sr. no	Formulation	Zone of inhibition (mm)
1.	Herbal Gel (T)	15
2.	Carbopol 940 (C)	0
3.	Std. Drug (S)	17

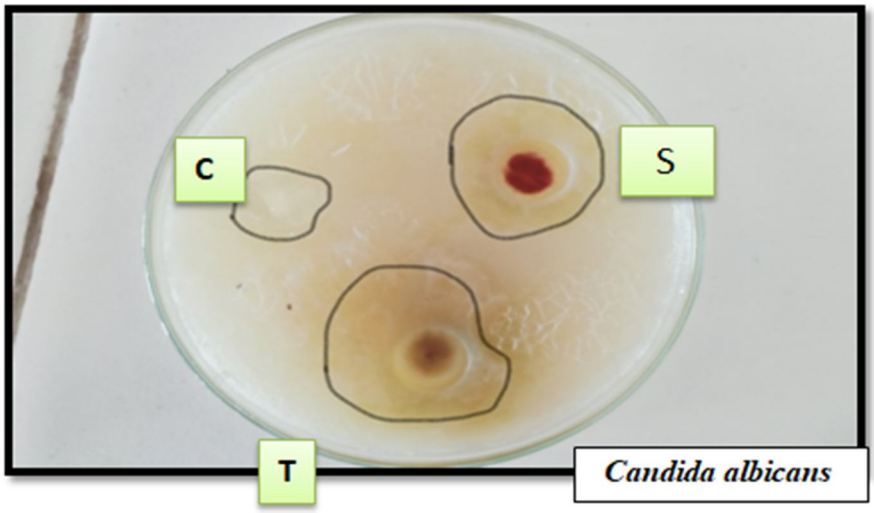


Figure: *In-Vitro* Antifungal activity of herbal gel drug on *Candida albicans*

7.1.3.13 *In-vitro* Anti-inflammatory test:

The *In-Vitro* anti-inflammatory activity of herbal gel on egg albumin was observed with the % inhibition is shown in Table and Graphical presentation shown in Figure. Here the herbal gel shows good result as compare to control formulation.

Table: In- Vitro anti-inflammatory study of herbal gel:

Sr. No	Test Solution		Control Solution		% Inhibition
	Concentration (µg /ml)	Absorbance	Concentration (µg/ml)	Absorbance	
1	31.5	0.1796	78.125	0.2895	37.9
2	62.5	0.1887	156.25	0.2919	35.35
3	125	0.2039	312.5	0.3912	47.99
4	250	0.2373	625	0.4332	45.22
5	500	0.2661	1250	0.4813	44.71
6	1000	0.3991	2500	0.5391	25.96

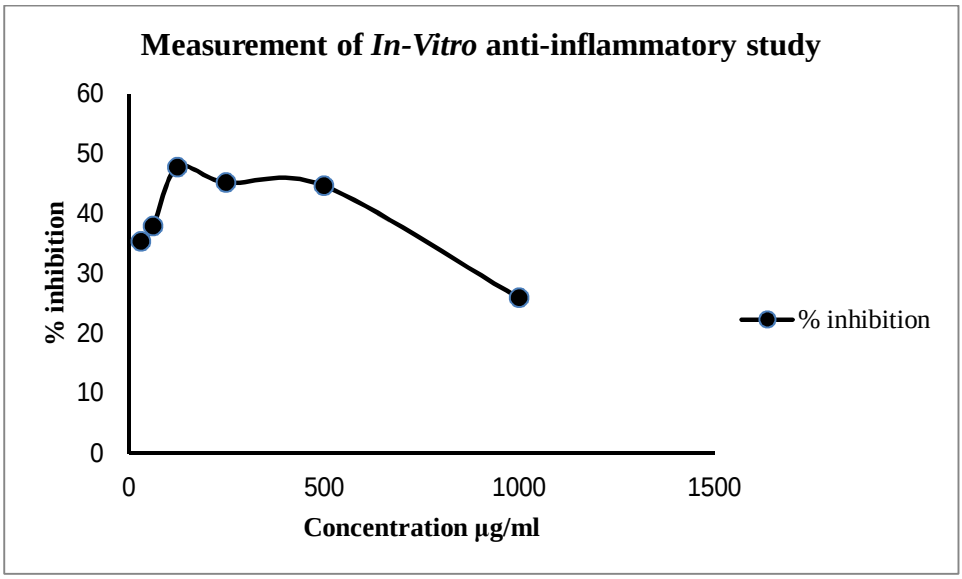


Figure: Measurement of *In-Vitro* anti-inflammatory activity of herbal gel

7.1.4*In- Vivo* Study:

7.1.4.1 Skin irritation study:

Skin irritation study on mice of herbal gel F1 showed No redness, inflammation or Escher formation during the observation period of 7 days. Oedema was not present after observations for 5 days; formulations did not indicate any skin irritation symptoms such as redness of skin or inflammation (erythema). Thus, it is safe use formulation F1and results are shown in Table and figure.

Table: Skin irritation study of optimized gel formulation (F1)

Group (N=4)	Score						
	Day1	Day2	Day3	Day4	Day5	Day6	Day7
Group I (Control)	0	0	0	0	0	0	0
Group II (Standard)	0	0	0	0	0	0	0
Group III (2.5% soluble solid extract gel)	0	0	0	0	0	0	0
Group IV (5% soluble solid extract gel)	0	0	0	0	0.5	0.5	0.5

Where, 0 = No irritation.

0.5 =Faint, barely perceptible and slight dryness.

1 =Defined erythema but no eruption or broken skin.

1.5 = Well defined erythema with dryness and epidermal fissuring.

2 = Moderate erythema.

2.5 = Moderate erythema with barely perceptible edema.

3 = Sever erythema.

3.5 = Moderate to severe erythema with eschar formation.

4= Moderate to severe erythema with eschar formation and edema extending the applied area.

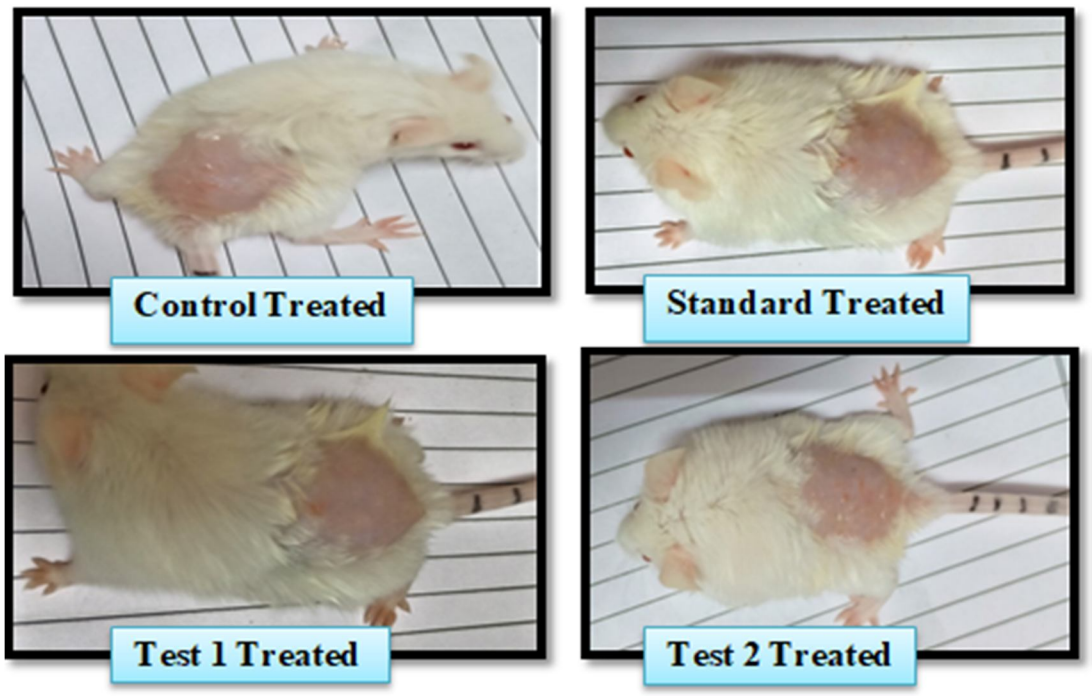


Figure: Skin irritation study of herbal gel formulation F1

7.1.4.2 *In –Vivo* anti-inflammatory activity:

Anti-inflammatory activity of selected gel formulation was investigated and results obtained are shown in Table and Figure:

Table: In-Vivo anti-inflammatory activity of herbal gel formulation F1

Treatment	Paw volume (ml)[a] % inhibition					
	1Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.
Control Gel base	0.1933 ±0.00272	0.2033 ±0.00272	0.2133 ±0.0072	0.2233 ±0.0144	0.27 ±0.00471	0.27 ±0.00816
Diclofenac gel	0.1533 ±0.00272* (21.7%)	0.13 ±0.00471* (35%)	0.1033 ±0.0072 * (54.54%)	0.08333 ±0.00544* (63.63%)	0.07 ±0.00471 * (74.07%)	0.05 ±0.00471 * (81.48%)
F1 Gel Formulation	0.1567 ±0.0072 * (21%)	0.14 ±0.00471* (30%)	0.11 ±0.00471* (47%)	0.1067 ±0.00544* (54.54%)	0.08333 ±0.0072 * (70.37%)	0.07333 ±0.00544 * (74.07%)

[a] =Values are expressed as mean±SD (Number of animals, n=4); one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability values of 0.05 ($p<0.05$) or less were considered statistically significant; p*<0.01 p**<0.001 vs. control.

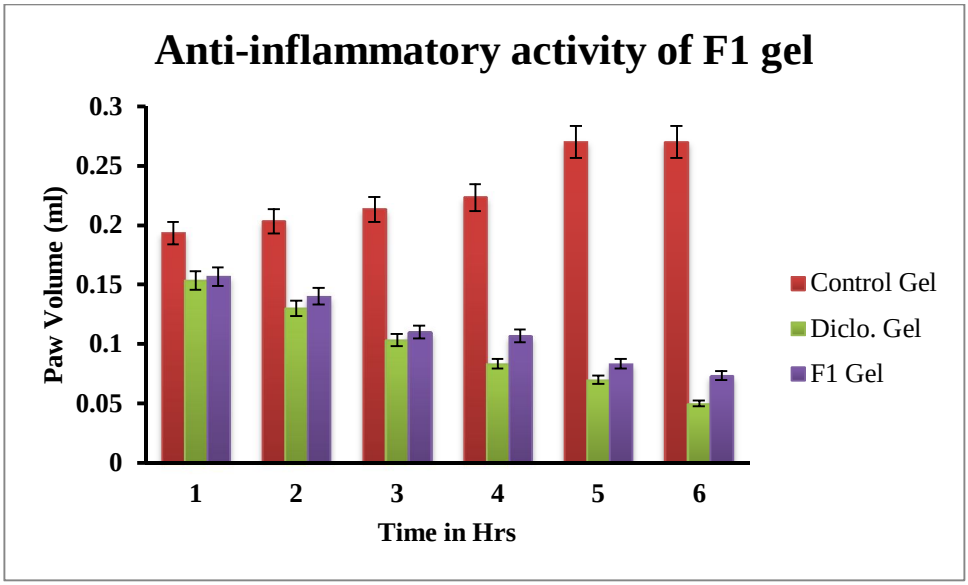


Figure: Graphical presentation of Anti-inflammatory activity of F1 gel

Treatment with formulation F1 showed 74.07% reduction of paw edema when compared to the control topical base group after 6 h from carrageenan injection. Gel formulation of *Chrysanthemum indicum* flower extract soluble solid significantly reduced the paw oedema throughout the entire period of observation in comparison to control ($p<0.01$). Fenugreek gel formulation containing combination 1% Carbopol 940 was found to be best for topical application as anti-inflammatory agents.

7.1.5 Stability Studies:

Accelerated stability studies according to ICH guidelines indicated that the physical appearance, rheological properties, extrudability, spreadability, drug content and drug diffusion study of the prepared gel remained unchanged upon storage for 3 months. The pH observed of prepared gel through 3-month storage was in between 6-7. Rheological properties and spreadability was showed uniformly. The observation of herbal gel formulation F1 shown in Table:

Table: Stability study of Herbal gel formulation F1

Time	pH	Appearances	Spredibility (gm/cm/sec)	Excrudability	Drug content
After 1 month					
	No changes				
After 2 months					
25±2 ºC / 60±5% RH	No changes				
30±2 ºC / 65±5% RH					
40±2 ºC / 75±5% RH					
After 3 months					
25±2 ºC / 60±5% RH	7.4	Light Yellowish	24.19	90.54%	96.67%
30±2 ºC / 65±5% RH	7.4		25	90%	97.06%
40±2 ºC / 75±5% RH	7.27	Yellowish	23.80	90.31%	96.75%

In-vitro diffusion profile of herbal gel formulation F1 (After 3 month), the cumulative amount of drug release was found to be 3.26%, 2.33 and 3.54% after period of 8hr at different Temperature such as 25±2 ºC / 60±5% RH, 30±2 ºC / 65±5% RH, 40±2 ºC / 75±5% RH respectively as shown in Table and curve showed in Figure:

Table: % CDR of herbal gel formulation F1 after 3 months

Time in Hrs.	% CDR of Formulation F1 at		
	25±2 ºC / 60±5% RH (A)	30±2 ºC / 65±5% RH (B)	40±2 ºC / 75±5% RH (C)
0	0	0	0
1	1.82	1.14	2.20
2	2.14	1.36	2.40
3	2.16	1.63	2.52
4	2.39	1.70	2.78
5	2.82	1.81	2.90
6	2.90	2.11	3.14
7	3.23	2.15	3.35
8	3.26	2.33	3.54

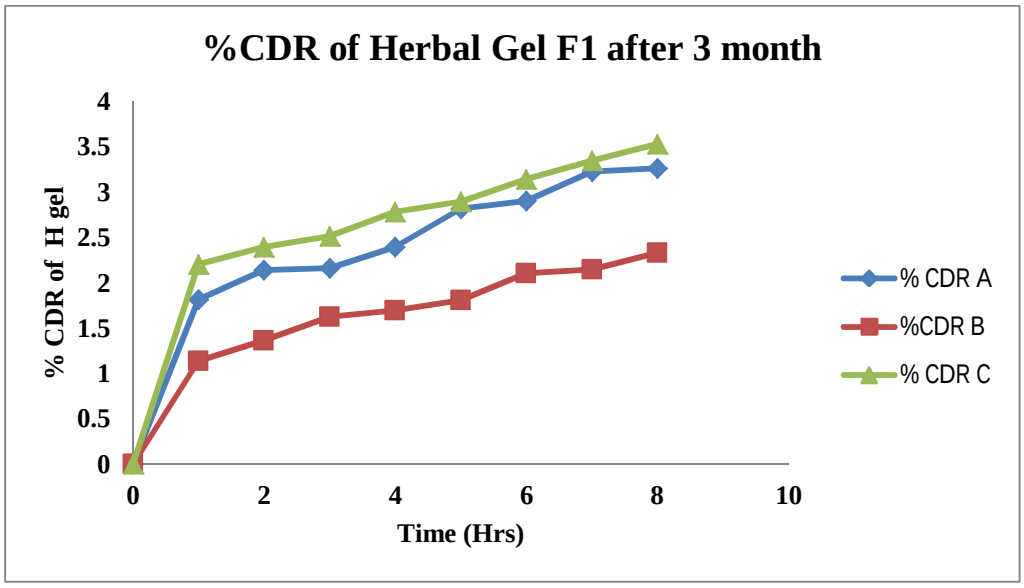


Figure: Measurements of % CDR oh herbal gel formulation F1 after 3 months

The most satisfactory formulation-F1 did showed any significant change in drug content, In vitro drug diffusion studies pattern after stability studies at 30 ± 2°C and at 65 ± 5 RH for 3 months. Thus, the objective of the present work of formulation and evaluating of herbal topical gel containing *Chrysanthemum indicum* has been achieved with success.

VIII. CONCLUSION

Seeing the results of phytochemical analysis it can be concluded that *C. indicum* produces many secondary metabolites of medicinal value. Present study confirms the presence of many important phytochemicals in the unexplored plant *C. indicum* (flower) extract. It can be concluded from the present investigation that proper selection of polymers and drug is a prerequisite for designing and developing a transdermal drug delivery. The physical compatibility studies (Viscosity) suggest that polymers selected. Carbopol 940 was found to be compatible with drug *C. indicum*. The varying concentration (1-1.5 gm) of the polymers was found to affect the gel parameters like viscosity and spreadability. Gel formulations prepared with Carbopol 940 showed good homogeneity, no skin irritation, good stability and anti-inflammatory activity. However, the Carbopol 940 based gel proved to the formula of choice, since it showed the highest percentage of extrudability, good spreadability and rheological properties, drug content, grittiness, appearance, pH, swelling index etc. Formulation F1 with 500mg extract soluble solid of *Chrysanthemum indicum* showed the best formulation with significant anti-inflammatory activity.

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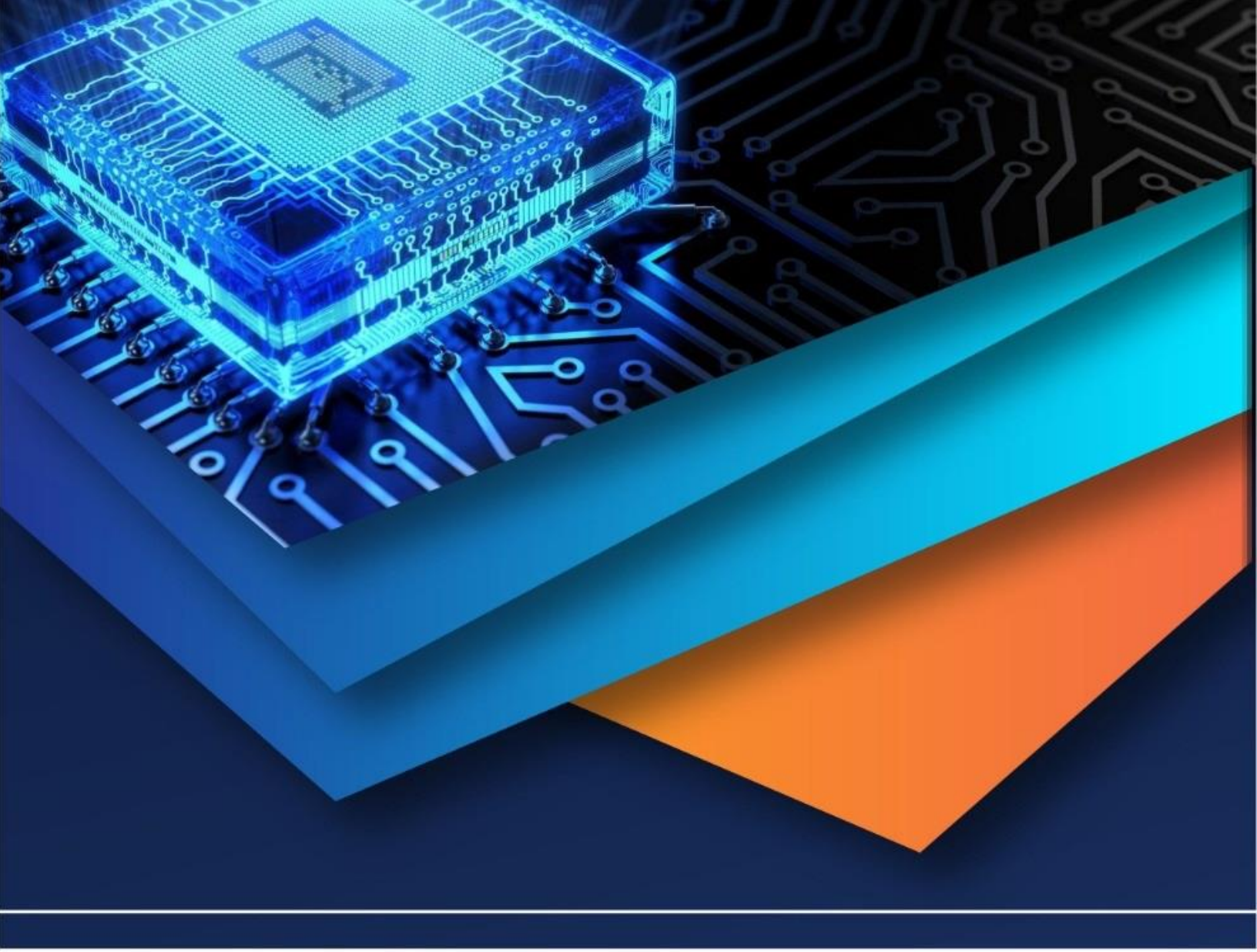

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