TOPICAL DELIVERY OF PAPAIN FOR MANAGEMENT OF PSORIASIS: FORMULATION DEVELOPMENT AND ASSESSMENT OF ANTI-PROLIFERATIVE ACTIVITY USING HaCaT CELL LINE

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ABSTRACT

The study was aimed at developing topical gel containing papain as the drug and assessing its anti-psoriatic activity. Carbopol was used as the polymer and drug-excipient compatibility was ascertained by infrared spectroscopy (Fourier transform) and differential scanning calorimetry. All prepared formulations were evaluated for viscosity, pH, homogeneity, extrudability, spreadability, *in vitro* diffusion and release kinetics. Anti-proliferative activity was determined on HaCaT cell line. The optimized formulation, F2 exhibited pH 6.8, optimum viscosity, extrudability and spreadability for convenient use. The optimized formulation followed Higuchi kinetic model with release mechanism being super-case two transport. The anti-proliferative activity of optimized formulation on HaCaT cell line showed only 3.9 % cells to be viable with respect to control confirming its efficacy in treating psoriasis. The gel exhibited stability under accelerated stability conditions. It can be inferred from the study that papain gel can be effectively used for managing psoriasis and its single application would give prolonged action.

Keywords: Papain, psoriasis, topical therapy, HaCaT cell line, anti-proliferative, Carbopol

INTRODUCTION

Human skin is one of the important organs for drug targeting and administering of drugs in various skin disorders like alopecia (hair loss, baldness), acne (vulgaris), skin cancer and psoriasis[1].

Psoriasis is a chronic autoimmune skin disease, commonly appearing on the skin of the knees, gluteal cleft, and areas of lower-back, scalp, penis and elbows. It is identified by plaques of thick, scaling skin. The active addition of cells of skin, created by pro-inflammatory agents which are made by T-lymphocytes stems in the development of dry flakes of skin. Different types of biomarkers like Western blotting, bioplex assay, immunohistochemistry and ELISA are used to analyze the status of the disease. Psoriasis affects 2% people worldwide[2,3].

Papain or papaya proteinase belongs to the class of cysteine protease enzymes and is found in papaya (*Carica papaya*). Papain is a globular protein consisting of a lone chain having a molecular weight of 23,406 DA and is made up of 212 amino acids comprising of four disulphide bridges[4,5].

Papain encompasses a family of closely linked proteins possessing a plethora of activities. It includes enzymes having both exo- and endo-peptidase activities. Members belonging to this family are distributed extensively in baculoviruses, eubacteria, yeast and almost all protozoa, plants and animals. These proteins are usually lysosomal or released and cleavaged for activation of enzyme, although bleomycin hydrolase is cytosolic.

Papain-like proteinases of class cysteine are basically synthesized as proenzymes which are inactive (zymogens) with *N*-terminal propeptide sections[5,6].

Mechanism of action of papain: When topically applied, papain actuates an allergen-like proactive reaction by means of enrolling neutrophils, pole cells, and CD3 (bunch of separation type 3) positive cells and by enlistment of a TH2 (T partner cell type 2) - one-sided counter acting agent reaction. *In vitro* treatment of papain bring about the breakdown of tight intersections of essential human keratinocytes that keep up the epithelial boundary upright.

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These tight intersection proteins incorporate zonula occludens-1, claudin-4, and occluding-1. It is suggested that papain prompts hypersensitive reactions through enactment of TLR4 (toll like receptor 4), prompting an expansion in neutrophils, CD3+ cells, pole cells, and CCL8 (chemokine ligand 8) positive cells[7].

Use of papain in treating psoriasis

It has been reported that papain has been used to treat psoriasis[8]. It is applied directly to the skin, to the infected wounds, patches and pustules and, because of enzyme specificity, no harmful effects on sound tissues have been seen. This is because it lacks the α1- antitrypsin plasmatic antiprotease that prevents breakdown of proteins in tissues that are healthy[8]. However, no formal study has been conducted to confirm its effectiveness in psoriasis. Also, no topical formulation of papain has been developed and tested for its activity in psoriasis. Therefore, taking into consideration these facts, the present work was undertaken to assess the potential of papain in management of psoriasis and to develop its suitable formulation.

MATERIALS AND METHODS

Materials

Papain was purchased from Vital Herbs, Delhi which was obtained from latex of raw papaya. All other chemicals were of analytical grade. Methyl paraben, propyl paraben, propylene glycol and triethanolamine were procured from SDFCL (SD Fine Chem. Limited). Carbopol 934 was procured from Burgoyne Urbidges and Co. HaCat cell line was procured from NCCS, Pune. All polymers and chemicals were obtained from authorized agents.

Methods

Formulation of gel

For formulation of gel, 1 g Carbopol 934 was dipped in 60 mL distilled water and allowed to hydrate overnight. In another beaker, papain and 10.3 g of propylene glycol were added, sonicated for 5 min and then 0.2 g and 0.02 g methyl and propyl paraben, respectively, were put into the solution. Sonication was continued until they dissolved. The mixture so formed was combined with hydrated carbopol dispersion with stirring. Eventually, triethanolamine was added drop-by-drop to adjust pH to approximately 6.8[9]. The weight was finally made upto 100 g by adding distilled water, if required. Three formulations F1, F2 and F3 were formulated by varying the percentage of papain from 2.5- 4.5 % (Table I). Papain used had an assay/extract ratio of at least 500 TU mg^{-1} and conformed to heavy metal and microbiological specifications.

Table I: Composition of papain gels

Ingredient	F1	F2	F3
Papain (g)	2.5	3.5	4.5
Carbopol 934 (g)	1	1	1
Methyl paraben (g)	0.2	0.2	0.2
Propyl paraben (g)	0.02	0.02	0.02
Propylene glycol (g)	10.3	10.3	10.3
Triethanolamine	Upto 6.8 pH	Upto 6.8 pH	Upto 6.8 pH
Distilled water (q.s) (g)	100	100	100

The prepared gels were assessed for the parameters given below:

1) Physical parameters like color and appearance were inspected visually.
2) Viscosity: Brookfield Digital Viscometer (NDJ-5S) was employed to determine the viscosity (mPas) of formulated gels. Spindle number 2 was rotated in the samples taken in a beaker and measurements were done in triplicate.
3) pH: To measure the pH, each gel formulation was subjected to pH determination in triplicate with the help of a digital pH meter (ME 962P).
4) Spreadability: The spreading capacity of the gel was assessed by spreading 0.5 g gel on a circle of 2 cm diameter marked on a glass plate. Another glass plate was then put over this plate. A weight of 500 g was made to rest on the top plate for 5 min. The area of the circle in terms of diameter, after spreading of the gel, was then measured.
5) Extrudability: A capped collapsible tube having approximately 20 g of gel was squeezed at the crimped end by applying a pressure of 1 kg cm^{-2} for 30 s by Monsanto hardness tester. Roll back was prevented by using a clamp. The tube was uncapped and the gel was allowed to extrude. The gel that extruded was amassed and weighed. The measurements were done in triplicate and the mean of three readings was documented[10-13].
6) Homogeneity: The formulated gels were checked for homogeneity by visual observation after allowing the formulation to set in a container and then evaluated for appearance and presence of lumps.

7) *In vitro* diffusion study: All formulations were subjected to *in vitro* diffusion studies using Franz diffusion cell. The studies were carried out by spreading 1 g of the gel evenly onto the cellophane membrane. Cellophane membrane was pretreated by dipping in phosphate buffer (pH 6.8) overnight. The membrane was set up between the compartments of Franz diffusion cell. The receptor compartment contained 25 mL of phosphate buffer (pH 6.8). The experiment was conducted at (37±2) °C. 5 mL samples were removed from reservoir compartment at 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 12 h. At each sample withdrawal, an equal quantity of phosphate buffer (pH 6.8) was replenished in the reservoir compartment to maintain sink condition[14].

Spectrophotometric analysis of the withdrawn samples was done at a wavelength of 278 nm against phosphate buffer pH 6.8 as blank.

Release kinetic study

Results obtained from *in vitro* diffusion studies of papain gels were fitted to different kinetic models such as zero order, First order, Higuchi model and Korsmeyer-Peppas model.

8) Study of anti-proliferative activity of formulated gels on HaCaT cell line: MTT Assay : Anti-proliferative activity of the 2.5 %, 3.5 %, 4.5 % w/w and blank gel samples on HaCaT cell line was assessed by MTT Assay with respect to control. The cells (10000 cells/well) were grown in 96 well plates for 24 h in RPMI medium which was augmented with 10 % FBS and 1 % (Streptomycin, amphotericin, penicillin) antibiotic solution at 37 °C with 5 % CO_2. The following day, all cells were given treatment with gel samples which were prepared by diluting 1000 mg gel to 1000 mL to give concentration of papain in dilutions as 25, 35, 45 mcg mL^{-1} (different concentrations were prepared in complete medium). Cells were also treated with blank gel in the same manner as papain containing gels. For control, no treatment was given to cultured HaCat cells. After incubating for 24 h, MTT (having 0.5 mg mL^{-1} concentration) was mixed with cell culture and incubation was done for 2 h for reduction to formazan crystals. Towards the conclusion of the investigation, culture supernatant was separated and layer of cell with matrix was solubilized in 100 µL DMSO (dimethyl sulfoxide) and then was assessed in an Elisa plate reader (iMARK, Biorad, USA) at 540 nm and 660 nm. Darker is the color of the solution, greater will be the number of viable cells.

Selection of optimized batch

Selection of optimized batch from the prepared gel formulations was done based on the results of evaluated parameters like spreadability, extrudability, homogeneity, *in vitro* diffusion study and cell line study. The batch which exhibited good spreadability and optimum extrudabilty, was homogenous, gave good *in vitro* diffusion at the end of 12 h and had the best antiproliferative action was considered as the optimized batch.

Stability studies: Stability testing of gels is important for economy of the finished product. To estimate the formulation stability, accelerated stability study was carried out in accordance with ICH guidelines for a time period of 3 months. The stability study was conducted for the optimized formulation. The selected optimized formulation was subjected to 40 °C ± 2 °C /75 % RH ± 5 % RH temperature and humidity condition. Samples were removed at zero, first and third months and evaluated for alteration in color, odor, homogeneity, extrudability, speadability, viscosity and pH[15, 16].

RESULTS AND DISCUSSION

Formulation of gel

The gels were formulated by hydration method considering the time period from 6 to 24 h for the hydration of Carbopol 934, where 12 h time period was optimized for the hydration of Carbopol 934. Papain when directly added to the gel did not produce homogenous gels, hence papain were dissolved in propylene glycol and filtered through Whatman filter paper. Methyl paraben and propyl paraben were mixed with the filtered solution of propylene glycol. Varying concentrations of gelling agent (0.8, 1 and 1.5 %) were used during trials but 1 % was optimized. Papain (2.5, 3.5 and 4.5 %) was used for preparation of gel. Carbopol proved to be an excellent gelling agent as all the gels were clear and transparent. Propylene glycol was used as a permeation enhancer and solubilising agent. Triethanolamine was used to adjust pH to 6.8 for neutralization of Carbopol and also because papain shows good activity and stability below pH 7[17]. Also pH 6.8 has been found to be compatible with skin pH. Methyl and propyl paraben were employed as preservatives.

Evaluation of prepared gel

The composed gels were assessed for the following parameters (Table II).

Physical examination

Physical parameters like color and appearance were evaluated visually and all gels were found to be creamish.

pH: pH of all the papain gel formulations ranged from 6.8-6.9. This was highly desirable to minimize the possibility of irritation on application to skin[18]. Also, between pH 2.8 -7.0, papain shows good activity and stability.

Viscosity: Viscosity of the formulation influences drug release from the gel. If a gel has high viscosity, the release of drug from the formulation is low and if the same gel possesses less viscosity, the drug release is enhanced. Hence for the gel formulations optimum viscosity is required to get the optimum release of drug. Viscosity was found to range from 28252 ± 2.0- 44186± 2.5 mPas.

Extrudability: The extrudabiliy of the papain gel is important for its application. Extrudability of papain gel formulation was established to be good. Extrudability ranged from 0.126±0.018- 0.130±0.015 g.

Spreadibility: All formulations exhibited satisfactory spreadability when applied between glass plates. Consistency of these formulations was found to be smooth and acceptable on application. Spreadibility ranged from 3.0-3.2 cm.

Homogeneity: Formulations appeared to be homogenous with the absence of lumps.

***In vitro* diffusion study:** Diffusion studies were conducted with the help of Franz diffusion cell. At 12 h time point, the mean % cumulative drug release of formulations was found to be between 48.0 - 60.97 %, showing the release to be prolonged (Fig. 1).

Release kinetics: Information acquired from *in vitro* drug release studies of the papain gel formulations was fitted to different kinetic models like Zero order, First order, Higuchi and Korsemeyer-Peppas model, the outcome of which is presented in Table III and Fig. 1. The release kinetics of papain from the gel was found to follow kinetics of zero order for F1 and F3 batches and Higuchi kinetic model for F2 batch (3.5 %) based on the goodness of fit. The data of kinetic models can be seen from Table III. Following zero order release, release of papain from F1 and F2 batches is not dependent on the drug concentration in the gel. Drug release is more or less constant with respect to time. Higuchi kinetics model of F2 batch suggests that the drug release mechanism is diffusion dominated. The n value obtained for various batches can be used to determine release mechanism of drug. n of 0.145 for F1 shows the drug release to follow Fickian diffusion, n value of 0.800 for F3 exhibits non-Fickian transport and n= 0.9 for F2 elicits super case two transport[19].

In vitro anti proliferative study on HaCat cell line

Antiproliferative activity of papain was evaluated for explaining the alleviation effect of papain gel on psoriasis. Therapy for psoriasis depends on prevention of hyperproliferation of keratinocytes as it is important in converting psoriatic lesion to normal epidermis.

MTT assay of papain 2.5 %, 3.5 %, 4.5 % and blank gels revealed 12.4 %, 3.9 % and 2.1 %. 97.7 % cells to be viable respectively with respect to control at 24 h (Fig. 2). This was determined by assaying the reduction of MTT to purple formazan crystals which were dissolved in DMSO to give a colored solution, where absorbance value was read on an Elisa plate recorded at 540 and 660 nm. The cell viability results clearly confirmed that papain has antiproliferative activity and can be used successfully for managing the psoriatic lesions. It was also ascertained from the results that on increasing the concentration of papain in gels, the antiproliferative activity increases, although difference between 3.5 % and 4.5 % gel antiproliferative activity was insignificant (at $p < 0.05$) when tested with one way ANOVA using Prism statistics software (GraphPad prism version 6.01).

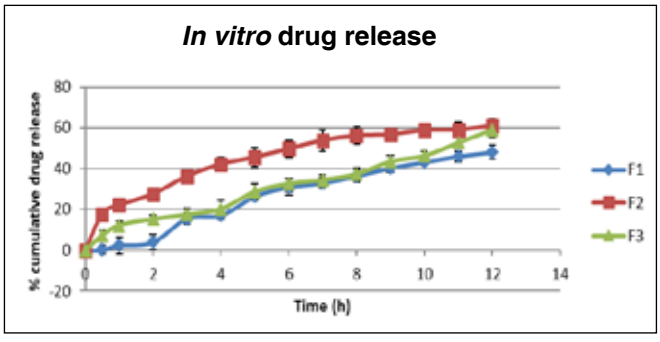

Fig. 1: Cumulative percentage drug release of papain gel in different formulations

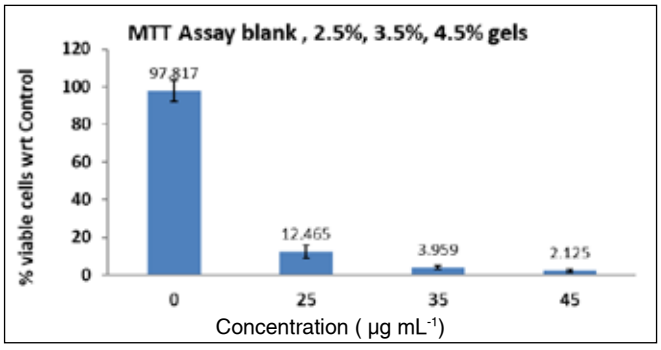

Fig. 2: Results of anti-proliferative activity of various gel formulations

Table II: Results of physicochemical evaluation of gels

Sr. No.	Test Parameter	F1	F2	F3
1.	Color	Creamish	Creamish	Creamish
2.	pH	6.98	6.82	6.82
3.	Viscosity (mPas)	28252± 2.0	34807± 2.0	44186± 2.5
4.	Homogeneity	Good	Good	Good
5.	Extrudability (g)	0.130± 0.018	0.130± 0.011	0.126± 0.015
6.	Spreadability (cm)	3.2 ± 0.1	3.0± 0.2	3.0± 0.3

Table III: Kinetic equation parameters for various formulations

Formulation Name	Zero order		First order		Higuchi		Korsemeyer-Peppas	
	R^2	K_0	R^2	K_1	R^2	K_h	R^2	K_p
2.5% Papain gel (F1)	0.972	4.358	0.793	0.122	0.904	16.36	0.944	1.582 n= 0.145
3.5% Papain gel (F2)	0.859	4.309	0.458	0.078	0.980	17.62	0.574	0.944 n= 0.900
4.5% Ppapain gel (F3)	0.985	4.370	0.691	0.095	0.945	16.35	0.698	0.87 n=0.800

Table IV: Results of stability study of F2 batch

Formulation code	Time duration	Physical appearance	pH	Viscosity (mpas)	Extrudability (g)	Spreadability (cm)	Homogenity
F2	0 Month	Creamish	6.8	34807±2.5	0.130±0.015	2.5±0.3	Clear gel with no lumps
	1 Month	Creamish	6.7	33958±2.5	0.125±0.015	2.6 ±0.3	Clear gel with no lumps
	3 Month	Creamish	6.7	33883±2.5	0.129±0.015	2.9±0.3	Clear gel with no lumps

3.5 % w/w gel was considered as an optimum formulation for managing psoriasis, as no significant advantage was derived in terms of antiproliferative activity on elevating the concentration of papain to 4.5 % in the gel. It can also be derived from the results that the reduction in proliferation of cells is due to the presence of papain and not because of the other ingredients used in formulation of gels as there is a very large difference in antiproliferative activity of blank gel and papain containing gel.

Selection and optimization of gel formulation

After analysis of all formulations for evaluation parameters like pH, spreadabilty, extrudability, *in vitro* diffusion study and cell line study, the formulation batch F2 (3.5 % papain) was found to show good results. The pH of F2 batch was determined to ensure that the gel can be put to use without the possibility of irritation to the skin. pH was established to be 6.82 ± 0.5, which was close to the neutral pH, suggesting safe application of F2 batch. The batch F2 had optimum viscosity (34807± 2.5 mpas). Spreadability (3.0± 0.3) and extrudability (0.130± 0.015) results of F2 batch show optimum spreadability and extrudability. This batch also shows highest cumulative *in vitro* drug release (60.97 %) during *in vitro* diffusion investigation. The results of cell line study also gave good result with percentage of viable cells being only 3.9 % with respect to control at the end of the study.

Stability study of optimized formulation

Stability of a pharmaceutical active in a drug form under specific environmental conditions is crucial as it decides the shelf life of formulation modification in the tangible features like color, odor and texture show the stability or instability of drug. Papain has been reported

to be stable below a temperature of 60 °C. It shows pH dependent solution stability and has been shown to retain its activity between pH 2.5 and 7.5. The outcomes of stability studies are indicated in Table IV. The optimized F2 batch of gel was exposed to a temperature of 40 °C ± 2 °C /75 % RH ± 5 % RH for 3 months[20, 21]. It was analyzed for visual appearance, pH, viscosity, spreadability, extrudability, homogeneity at 0, 1 and 3 months' time points. Evaluation parameters of the formulation F2 and physical alterations exhibited during stability studies are shown in Table IV.

Drug excipients compatibility by FTIR and DSC

FTIR: The FTIR spectra of papain (Fig. 3A) showed characteristic peaks around 3245.41 cm^{-1}, due to stretching of H of the secondary amide bond. The peaks at 1637 and 1551 cm^{-1} were due to –CONH amides I and II, [22, 23] respectively. The peak at 2980 cm^{-1} is due to $–CH_2–$ asymmetric stretching[23] while peaks at 1148, 1077 and 520 cm^{-1} can be due to sulphide and disulphide –CS stretching (Fig. 3B). The FTIR spectra of papain gel explicitly show the retention of these peaks of papain pure

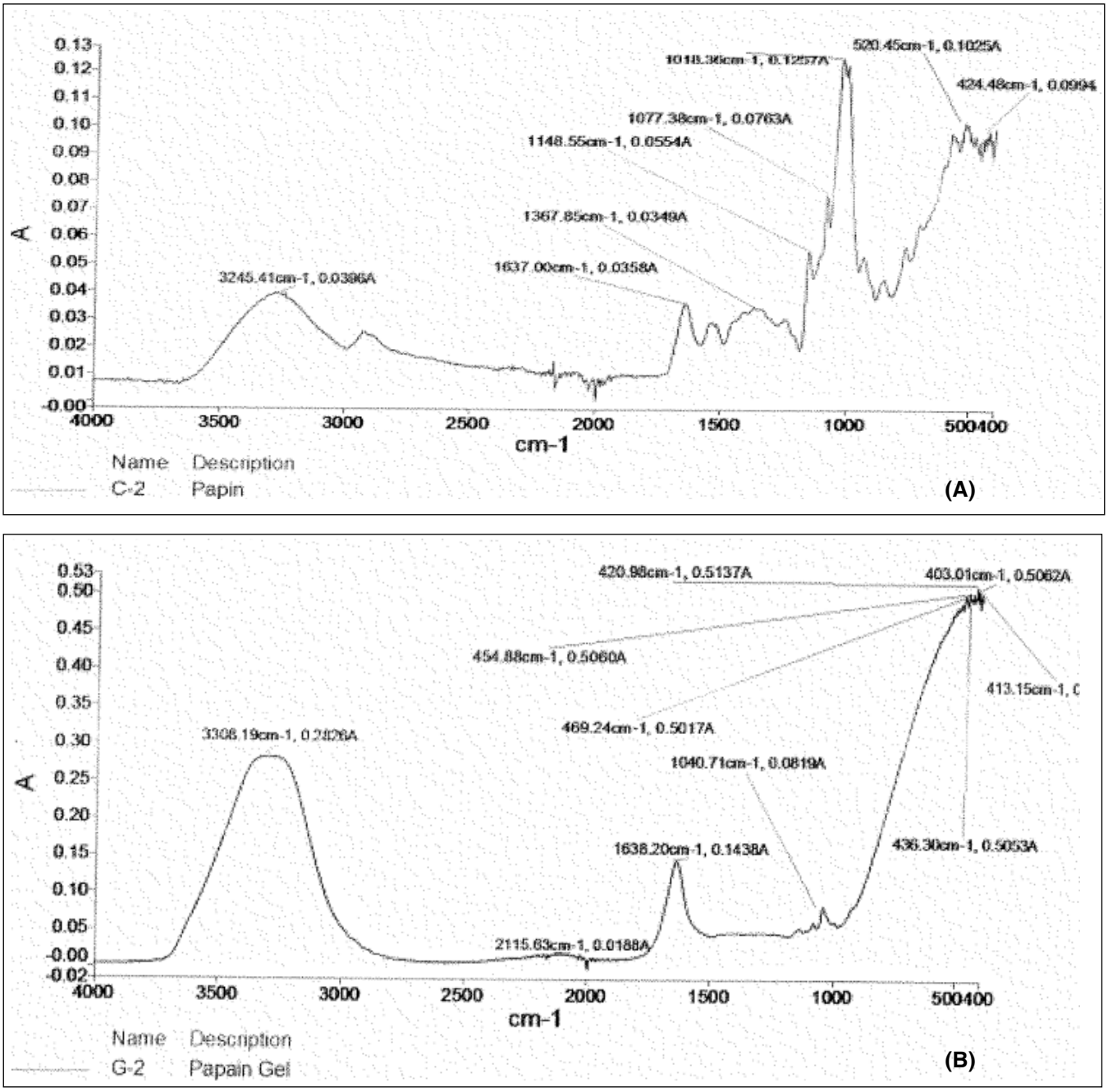

Fig. 3: FTIR spectra of (A) Papain (B) Papain gel

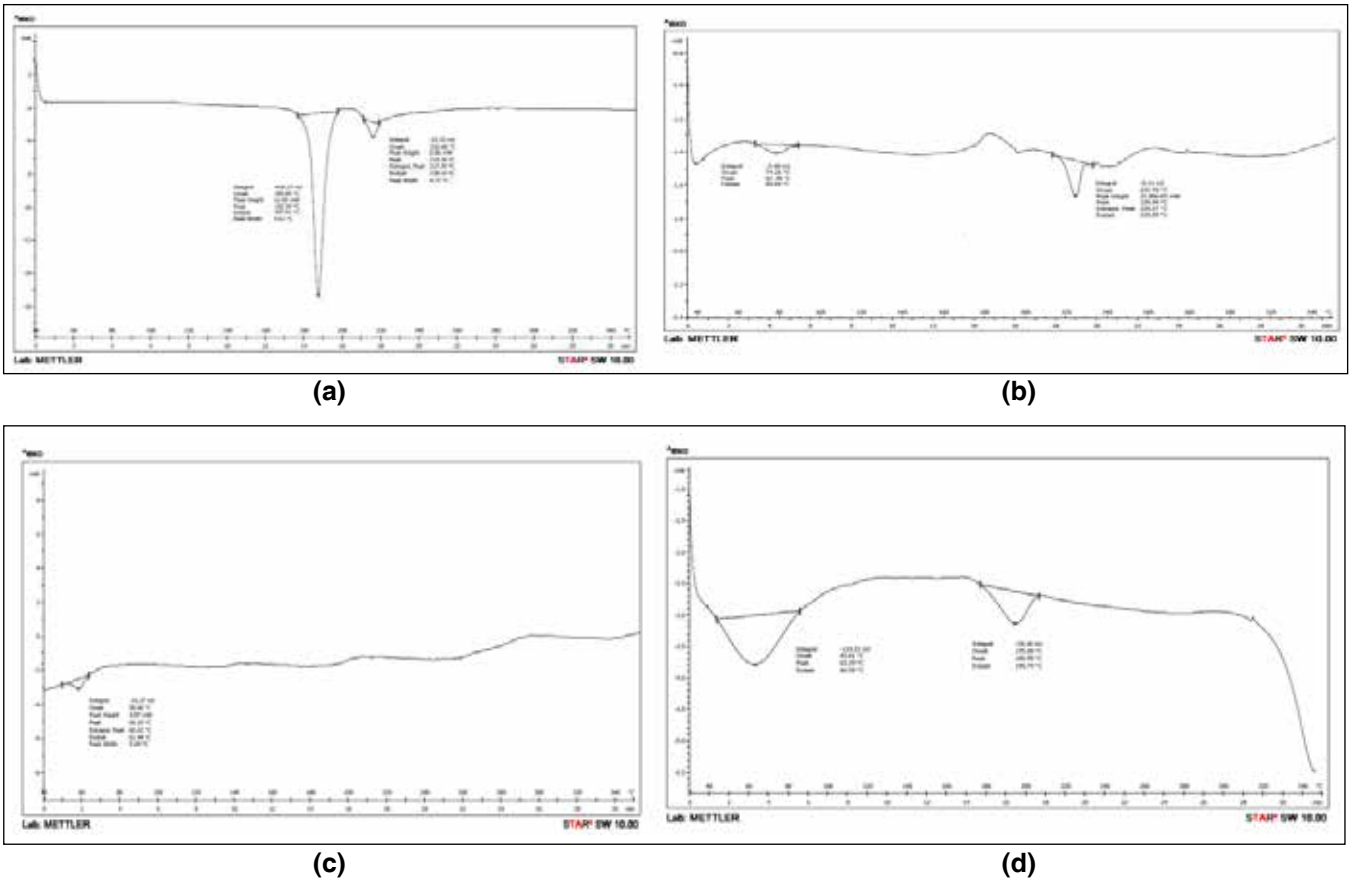

Fig. 4: DSC of a) Papain powder b) Carbopol c) Blank gel d) Papain gel

drug, thus manifesting absence of interaction between drug (papain) and selected polymers.

DSC: The differential scanning calorimetry thermogram of the papain is given in Fig. 4. DSC is an important technique used for characterization of the drug and for checking its compatibility with the excipients. Fig. 4a shows the DSC thermogram of papain powder with a pointed endothermic peak at 192.39 °C, which represents the point at which the drug melts. The sharp pointed peak exhibits the drug to be in the crystalline state (Fig. 4a). The DSC thermogram of Carbopol has a rounded endothermic peak at 82.98 °C, which is its melting point (Fig. 4b). The DSC thermogram of blank gel with a round endothermic peak at 60.10 °C, shows the excipients mixture to have reduced melting point in comparison to major excipient, Carbopol. This may be because of presence of some liquid excipients (Fig. 4c). The DSC graph of papain gel (Fig. 4d) has a rounded endothermic peak at 63.39 °C and 189.5 °C. There is slight shifting in endothermic peak of papain and Carbopol, may be because of presence of excipients as impurities. Rounded peak indicates that papain has acquired a less crystalline, disordered form. Rounded peak at 63.39 °C is the endothermic peak of Carbopol with slight shifting due to mixing with some liquid excipients.

CONCLUSION

It can be inferred from this study that papain gel can be safely and effectively used as a topical therapy for alleviating and managing the symptoms of psoriasis. It can be used as an economical and natural alternative to synthetic drug formulations. The *in vitro* anti-proliferative activity assessment carried out on HaCat cell line confirmed its efficacy in managing the psoriatic lesion which is the preeminent feature of psoriasis. The stability of the formulations, established at 40 °C ± 2°C /75 % RH ± 5 % RH ascertained that the gel formulation does not require any special storage conditions and can be easily stored and used at normal, ambient conditions of temperature and humidity. The *in vitro* diffusion study proved controlled release of papain from gel over a period of 12 h, ascertaining its action over a long period of time on single application. However, the findings need to be probed further through *in vivo* study on animal model as there are significant differences between *in vitro* cell lines and animal physiology.

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