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Preparation and evaluation of wound healing activity of phytosomes of crocetin from *Nyctanthes arbor-tristis* in rats

Manasi Varadkar, Chhaya Gadgoli [1, *]

Saraswathi Vidya Bhavan's College of Pharmacy, Kalyan Shil Road, Sankara Nagar, Sonarpada, Dombivli(E), 421203, Thane District, Maharashtra State, India

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ABSTRACT

Background and aim: Orange colored tubular calyx of the flowers of *Nyctanthes arbor-tristis* contains an apocarotenoid crocin as a major ingredient, which is originally detected to be major colouring principle of saffron stigma. Saffron stigma exhibits good wound healing activity owing to the presence of crocin. The present study is aimed at isolation of crocetin, from tubular calyx of N. arbortristis and improve stability through entrapping in vesicles followed by evaluation of wound healing activity of the topical formulation thereof.

Experimental procedure: Crocetin was isolated by treating ethanolic extract of tubular calyx of *N. arbor-tristis* with sodium bicarbonate followed by regeneration of crocetin using hydrochloric acid. The phytosomes were prepared by lipid film hydration technique. The gel containing phytosomes equivalent to crocetin 1% w/w, was then evaluated for wound healing activity through applications on incision and excision wounds inflicted in Wister albino rats.

Results: Stability of crocetin was found to be increased due to entrapment into phytosomes. The studies revealed that both types of wounds upon treatment with gel containing crocetin phytosomes, indicated good wound healing potential, as the epithelization period was significantly ($P < 0.001$)decreased as compared to the control group from 26 to 9 days, in excision wound model and significant ($P < 0.001$) increase in breaking strength of repaired skin, as compared to control from 328.8 to 857.0 gm in incision wound model.

Conclusion: Crocetin from tubular calyx of *Nyctanthes arbor-tristis* indicated to be potential wound healing phytoconstituent.

1. Introduction

Crocetin is an aglycon apocarotenoid dicarboxylic acid obtained from hydrolysis of Crocin, an apocarotenoid esterified with glucose units, responsible for coloration of stigma of flowers of Saffron that is Crocus sativus (Iridaceae).[1] Orange colored tubular calyx of flowers of Nyctanthes arbor-tristis (Oleaceae) is reported to contain crocin and it is considered as an alternative source for the same. The flowers of Nyctanthes arbor-tristis form an economical source of crocin, as these are readily available in India. The flowers blossom at night and wither next morning, also get perished in half an hour, thus get wasted.[2,3] Crocin present in saffron is reported to be an active ingredient, responsible for various pharmacological actions including wound healing,[4,5] antioxidant, anti-inflammatory properties along with expression of NF-kB related genes and glycosylation related genes (6). Crocin is reported to upregulate expression of collagen type 1 gene,[7] which reveals the potential of crocin in prevention of skin ageing. Saffron also forms one of the important ingredients of skin cosmetics (7). Crocin is a major constituent of tubular calyx of *N. arbor-tritsis* and being a carotenoid containing conjugated double bonds, it is highly unstable, The stability of crocin was improved[8] through preparation of biodegradable nano particles using Chitosan-alginate.

The aim of the study is to improve stability of crocin and crocetin by entrapping into phytosome. To achieve this aim, the objectives of

Abbreviations: NAT, *Nyctanthes arbor-tristis* extract.

* Corresponding authors.
E-mail addresses: E-mail addresses: E-mail address: chhaya.gadgoli@svbpharmacy.edu.in (C. Gadgoli).chhayahg@rediffmail.com (C. Gadgoli). chhayahgadgoli@gmail.com (C. Gadgoli).

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[1] Author to whom Correspondence should be addressed

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the studies involved utilization of tubular calyx of *N. arbor-tristis* as an alternative and economical source of crocin and crocetin. Crocin is a glycoside while crocetin is an aglycon. Due to presence of dicarboxylic groups on the molecule, it can be easily entrapped into phytosomes. This is followed by evaluation of stability and efficacy of gel formulations in healing of excision and incision wounds.

2. Materials and methods

2.1. Materials

All the chemicals used were of AR grade. Hydroxy proline was procured from Loba Chemicals, Mumbai, India. Crocin and crocetin were isolated from the tubular calyx of N. arbor-trisits. The solvents viz. ethanol, chloroform, were of AR grade while isopropyl alcohol, ethyl acetate, water utilized for HPTLC analysis were of HPLC grade.

2.2. Plant material

Fresh flowers of *N. arbor-tristis* were collected from the premises of the institute early in the morning and was authenticated at Agharkar Research Institute, Pune, Maharashtra, India. The voucher specimen (an- 0707,142) was deposited in the Institute (Fig.S1).

2.3. Extraction and isolation of crocetin (I)

The fresh flowers of *N. arbor-tristis* (about 750 gm) were cleaned by washing under running tap water and the orange-colored tubular calyx (about 500 gm) were manually separated from the petals and dried. After drying 67.3 gm of dried calyx was obtained. The dried tubular calyx (100 gm) was then macerated with about 350 ml ethanol in dark for about 6–8 h at room temperature. The orange red colour extract of tubular calyx of *N. arbor-tristis* (NAT Extract) obtained after filtration of the marc. The extract was evaporated in rotary flash evaporator and dried in vacuum oven at temperature not exceeding 50° c.

For isolation of crocetin from the dried NAT extract, (10 gm) was suspended in mixture of 50 ml of distilled water and 10 ml of concentrated hydrochloric acid. The suspension was refluxed on water bath for 3 h and cooled. The reaction mixture was then partitioned into about 50 ml of diethyl ether. The diethyl ether layer was separated and treated with sufficient quantity of saturated aqueous solution of sodium bicarbonate. The sodium bicarbonate layer was separated and concentrated hydrochloric acid was added till the pH was acidic to litmus paper. The orange colored precipitate was obtained due to treatment with hydrochloric acid which was filtered and dissolved in diethyl ether. The solvent ether was evaporated at room temperature to get solids of crocetin (about 1 gm). For the complete study totally about 5 gm of crocetin was prepared from about 50 gm of ethanolic extract by above mentioned procedure.

2.3.1. Isolation of crocin

The NAT extract (1 gm) obtained by above procedure, was loaded on silica gel (60–120 mesh) column and it was eluted by isocratic mode with the mobile phase consisting of Ethyl acetate: Isopropanol: Water (65:25:10). The eluents were analyzed by Thin Layer Chromatography (TLC) on silica gel GF 254 and mobile phase (Ethyl acetate: Isopropanol: Water [65:25:10]). Eluents with identical TLC pattern indicating single spot corresponding to Crocin (Rf-0.22) were pooled together and concentrated under reduced pressure to get the desired compound (about 0.3 gm) The NAT extract was quantified for content of crocin by HPTLC method, using the isolated crocin as a marker compound. The isolated crocin was also utilized as a marker compound for analysis of crocin from the phytosomes and the formulation thereof.

2.4. Preparation of phytosomes of NAT extract and crocetin

The phytosomes were prepared by lipid film hydration method.[9] In brief, the NAT extract and crocetin individually in the quantity of 0.5 gm, along with 1.0 gm of phosphatidyl choline and 0.5 gm of cholesterol were dissolved in 10 ml of chloroform, followed by evaporation of the solution in rotary flash evaporator to get a thin film of lipid. The film was hydrated at 25 ± 2 °C by adding phosphate buffer saline (pH 7.4) and kept it for 1 h. The dispersion of phytosomes thus obtained was sonicated in ice bath for 30 min. The phytosomes were further stabilized by hydration for 8 h at ambient temperature and separated from free drug by centrifugation. The amounts of entrapped crocetin and NAT extract (equivalent to crocin) were determined HPTLC method.

2.5. Evaluation of phytosomes

The FT-IR spectra for crocetin, and phytosomes of crocetin were recorded to confirm the formation of phytosomes.

2.5.1. Determination of crocetin and crocin by HPTLC method

For construction of calibration curves for crocin and crocetin, these were individually dissolved in methanol and diluted to get effective concentrations in the range of 10–50 μg/ml. The amount of crocetin and crocin entrapped was determined by extracting individually 50 mg of phytosomes of crocetin and NAT extract respectively, in about 25 ml of methanol and shaken for 30 min and then made up the volume to 50 ml with methanol (Solution A1 and A2). The solutions A1 and A2 were filtered and the 5 ml of the filtrate from each solution was diluted to 10 ml (Solution B1 and B2). The solutions B1 and B2 were quantified for the content of crocetin and crocin respectively using HPTLC method.

10 μl of each concentration of the standard solutions of crocin, crocetin and the samples were applied to precoated silica gel 60 GF_{254} plates (thickness-0.2 mm) with the help of applicator Linomat V using Hamilton syringe. The plate was developed using Petroleum ether: Acetone (6:3) and Ethyl acetate: Isopropanol: Water (65:25:10) as mobile phases for crocetin and crocin respectively, and dried. The plates were then scanned using CAMAG Scanner 3 Version 1.14.28 and recorded the areas of crocin peak (Rf = 0.22) and crocetin peak (Rf = 0.24) from both the standard and the sample tracks. The concentration of crocetin and crocin in phytosomes were extrapolated from the standard curves of crocetin and crocin respectively.

2.5.2. Determination of phospholipid content

The phospholipid content of the phytosomes was determined using colorimetric method.[10] In brief 50 mg of phosphatidyl choline was dissolved in 50 ml of chloroform (1000 ppm). From this solution aliquot 10 ml was diluted to 100 ml using chloroform to get concentration of 100 ppm. From the solution of 100 ppm through serial dilutions various concentrations were prepared in the range of 10–100 ppm. The sample was prepared by sonicating 1000 mg of the phytosome with about 40 ml of chloroform for 15 min and made up the volume to 50 ml with chloroform. The suspension was filtered and 5 ml the aliquot was utilized for color development. The color was developed by mixing 5 ml of aliquot of each concentration of standard solutions and the sample with 2 ml thiocyanate reagent, using vortex mixer. The red colored lower layer was removed using Pasteur pipette and the absorbance was read at 488 nm (Elico-spectrophotometer (SL 159)) against the reagent blank. The concentration of phosphatidyl choline in phytosomes was extrapolated from the calibration curve.

2.5.3. Determination of cholesterol content

Cholesterol content in the phytosomes was determined by colorimetric method[11] . The standard solution of cholesterol was prepared by dissolving 400 mg of cholesterol R.S. in 50 ml isopropyl alcohol. The solution was appropriately diluted to get the concentrations of cholesterol in the range of 10–400 μg/ml.

The sample was prepared by sonicating 1000 mg of the phytosomes with about 10 ml isopropyl alcohol for 15 min. The suspension was then filtered and utilized for color development. The color was developed by mixing 5 ml of each concentration of standard solution and the sample with 3 ml of glacial acetic acid, 0.3 ml iron solution and 3 ml of concentrated sulphuric acid. The solutions were mixed thoroughly using vortex mixer and absorbance values were read at 560 nm (Elico-spectrophotometer (SL 159) against the reagent blank. The concentration of cholesterol in phytosomes was extrapolated from the standard curves.

2.6. Stability studies on the gel formulation

The formulation containing phytosomes of crocetin and NAT extract were subjected to accelerated stability studies as per ICH guidelines. These formulations were stored at 25 ± 5 °C with 60% relative humidity and 45 ± 5 °C with 75% relative humidity for duration of 12 weeks. The samples were withdrawn at interval of four weeks and were evaluated for the content of phosphatidyl choline, cholesterol, crocin and crocetin using the above described analytical methods.

2.7. Pharmacological evaluations

The pharmacological evaluations included evaluation of wound healing activity. The studies were carried out with the due approval of Institutional Animal Ethics Committee (SVBCP/IAEC/PG/11–12/40). All the studies were carried out on Wistar albino rats (150–200 gm) of either sex maintained in clean propylene cages, under standard environmental conditions of temperature 27 ± 2° c, 60% R_H and light –dark cycle of 12 h, fed with standard diet and water ad libitum.

2.7.1. Preparation of formulations

The gel base was prepared by dispersing 1 gm of Carbopol ® in 98 gm of distilled water followed by continuous stirring using magnetic stirrer till uniform milky colloidal solution was obtained. To this solution, glycerin (1 gm) was added and the pH was adjusted to 6–7 using triethanolamine to get gel consistency.[11] Two formulations were prepared viz. F1 containing phytosomes of NAT extract (equivalent to crocin 1% w/w) and F2 containing phytosomes of crocetin (equivalent to 1% w/w crocetin) were prepared by incorporating the phytosomes in the Carbopol gel base.

2.7.2. Acute dermal toxicity

Acute dermal toxicity of gel base and the formulations F1 and F2 was evaluated as per OECD guidelines no 402.[12] The animals were divided into three groups viz. Gel Base, F1 and F2. In brief, two patches of area about 1 cm^2 were marked on the shaven back of the animals. The gel base and formulations F1 and F2 were applied on the marked area. The entire trunk was wrapped with impervious material for 24 h. At the end of 24 h, the patches were removed and skin was observed for any visible changes such as erythema or edema. Observations were repeated after 72 h.

2.7.3. Evaluation of wound healing activity

In all the models of wound healing, the animals were grouped into four main groups viz. two control groups, standard and test with six animals in each group. The control groups were treated with the gel base alone and a gel base containing the blank phytosomes. The test groups involved treatment of wounds with the formulations F1 and F2, containing gels with phytosomes of NAT extract (equivalent to 1% W/W crocin), and crocetin phytosomes (equivalent to crocetin 1% w/w) respectively. The standard group was treated with the Betadene ® (Povidone Iodine 5% w/w, Win Care Pharmaceuticals). Evaluation of wound healing activity was carried out in acute wound healing models in rats viz. excision and incision wound healing models.

2.7.4. Excision wound model

Full thickness circular wounds (area approximately 500 mm^2) were inflicted on the shaved dorsal thoracic region under Ether anesthesia and the animals were housed individually in clean propylene cages. The wounding day was considered as day zero. The wounds were cleaned sterile water and the groups were treated with the respective gel formulations. Healing of the wounds were assessed by tracing area of wound on transparency sheets on day 4, 8, 12, 16 and thereafter on alternate days until healing was complete.[13]

2.7.5. Incision wound model

Two paravertebral incision wounds of 6 cm in length of full thickness of the skin were inflicted on either side of the shaved back of anaesthetized rats and the incisions were closed with interrupted sutures of 1 cm apart with thread (No.000) and curved needle (No.09). This wounding day was considered as day zero. The various groups were treated with the respective gel preparations for the period of 10 days. The sutures were removed on 8th post wounding day and breaking load of newly formed skin was measured on the 10th day by Tensiometer.[14,15] The weight in gm required to break open the healed skin is recorded as breaking load.

2.7.6. Estimation of hydroxyproline and histopathological studies

For determination of hydroxyproline and histological examination, new set of animals were utilized and excision wounds were inflicted and treated as described in 2.7.3. On day 11, the granulation tissue was removed and the part of the tissue was utilized for histopathological studies. The granulation tissues collected on day 11 were dried in oven. The hydroxyproline content in the granulation tissues was determined by utilizing colorimetric method.[16] In brief, the granulation tissue was hydrolyzed by digesting with 6 N hydrochloric acid at 110 °C for 3 h, followed by reacting with Ehrlich's reagent (p- Dimethyl amino benzaldehyde in perchloric acid) to develop red color. The color was read at 558 nm (Elico-spectrophotometer (SL 159)) against the reagent blank. The content of hydroxyproline was extrapolated from the calibration curve for Hydroxyproline (R.S.).

For histopathological studies the granulation tissues were embedded in wax and sections were taken using microtome. The sections were stained with haematoxylin. The collagen was identified using Van Gieson stain.[17] These histopathological studies were carried out at Unique Bio-Diagnostics Enterprises (Veterinary Pathology Laboratory), Parel, Mumbai, India.

2.8. Statistical analysis

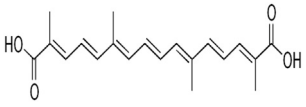
The data obtained from experiments is presented as mean ± standard error of mean (SEM). The animal experiments involved six animals in each group. The data was analyzed using one way ANOVA followed by Dunetts test for comparison. The values of $P < 0.05$ were considered as statistically significant.

3. Results and discussion

The mean content of crocin in NAT extract, as estimated by the HPTLC method was found to be 36.57% w/w. [Fig S2]. The molecule is an apocrotenoid which has highly conjugated double bonds and hence, there is a stability issue. It gets oxidized fast and prevents oxidation of other substances, hence it acts as an antioxidant. In the present study, the stability of the compound is increased by entrapping it into the vesicles of phosphatidyl choline and cholesterol, which are called as phytosomes.[18]

NAT extract containing crocin was hydrolyzed by treating with acid. This results in release of crocetin. The crocetin is then partitioned into non polar solvent like diethyl ether. Further purification of crocetin was carried out by preparation of sodium salt through reaction with sodium bicarbonate and then regeneration of crocetin by acidifying the solution containing sodium salt. The isolated crocetin was then utilized for further studies.

Preparation of phytosomes was optimized by varying the ratio phosphatidyl choline and cholesterol to get maximum entrapment. The optimum ratio of phosphatidyl choline and cholesterol was found to be 2:1, as it yielded maximum entrapment of NAT extract equivalent to crocin to be 34.4% w/w and crocetin (I) to be 71.4% w/w [Fig. S4] in phytosomes. The material balance for the extract crocetin phytosomes is presented in Table 1.



The FT-IR spectra of crocetin and its phytosomes indicate the presence 2852.26 cm^{-1} and 2930.32 cm^{-1} corresponding to phosphatidyl choline and cholesterol forming bond with crocetin as a part of entire phytosome apart from all the peaks of crocetin. (Fig. S3).

The gel preparation containing phytosomes of Crocetin (Equivalent to crocetin 1% w/w) was found to be stable in the accelerated stability studies as the mean content of crocetin in the gel preparation was found to be 93.13 and 90.38% w/w at the end of 12 th week, when stored at 25 °C/60 $\%R_H$ and 45 °C/75 $\%R_H$ respectively. In case of the gel containing NAT extract (equivalent to 1% crocin), the mean crocin content was observed to be 91.5 and 90.65% w/w at 25 °C/60 $\%R_H$ and 45 °C/75 $\%R_H$ respectively at the end of 12 th week. (Fig. 1). The contents of phosphatidyl choline and cholesterol were found to be in the range of 92–97% w/w after storage at 25 °C/60 $\%R_H$ and 45 °C/75 $\%R_H$ (Fig. 2).

Wound healing potential of gel preparations containing phytosomes NAT extract (equivalent to 1% w/w of crocin) and Crocetin (1% w/w) were evaluated in rats using excision and incision wound models.

There are three major overlapping phases of wound healing process. The first phase is homeostasis which involves formation of provisional wound matrix followed by inflammatory response. The first phase includes initiation of different clotting cascades, activation of thrombocytes, infiltration of leucocytes due to release of cytokines growth factors in turn leading to the activation of the inflammatory process. The phase lasts for about 2–5 days.[19–21] The untreated group wherein the wound was allowed to heal with the physiological process indicated about 20–21% contraction while the process was found to be accelerated in case of wound treated with F1 (equivalent to 1% Crocin) and F2 (equivalent to 1 %w/w crocetin), as the % contractions the wound area were significantly ($P < 0.001$) higher than the control group. Crocin is reported to have anti-inflammatory activity[6] which may be contributing to the acceleration of contraction in the first phase of the wound healing process. The standard group also indicated significant ($P < 0.001$) increase in the % contraction in wound area, as compared to the control through controlling infection as the compound povidone iodine is an antimicrobial agent and commonly used in clinical practice (Table 1).

The second phase of wound healing is proliferative phase and it starts from about day 3 and lasts till day 10, from the day of wounding.[22,23] In this phase, the process of healing is through covering of wound surface, formation of granulation tissue and

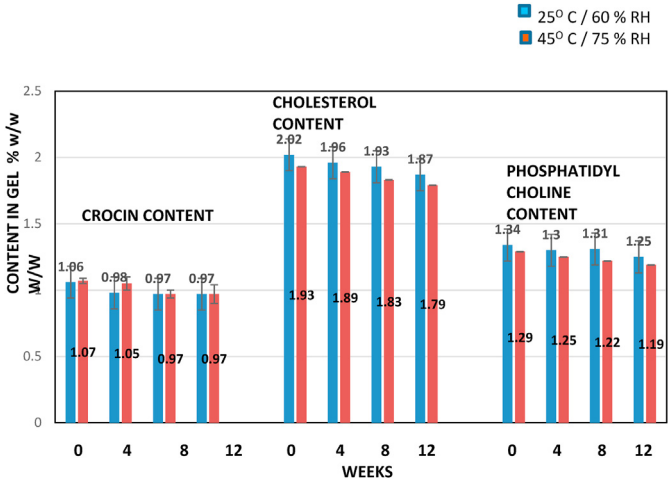


Fig. 1. Stability studies on gel formulation containing phytosomes of *Nyctanthes arbor-trisits* extract (Equivalent to 1% w/w of crocin).

Table 1
Effect of topical application of gels containing phytosomes of Crocetin and NAT Extract on wound contraction of excision wounds inflicted in albino rats.

Groups	% Mean Wound contraction ± SEM								Epithelization period (days) Mean ± SEM	Hydroxyproline content (µg/500 mg) Mean ± SEM
	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 20		
CT	20.84 ± 2.20	28.50 ± 1.50	40.89 ± 1.31	51.02 ± 1.52	57.84 ± 1.45	70.3 ± 1.80	83.51 ± 0.60	86.40± 0.10	26 ± 1.06	9.19± 0.56
CT1	22.50 ± 2.13	30.05 ± 1.45	48.91 ± 3.02	60.12 ± 2.13	75.63 ± 1.56	81.90 ± 1.43	88.95 ± 1.21	92.31± 0.15	21 ± 1.06	8.74± 0.6
STD	51.83 ± 1.10	61.69 ± 1.15	89.84 ± 2.10	93.59 ± 0.21	100***	100***	100***	100***	12 ± 1.06***	20.35± 0.56*
F1	32.09 ± 1.00	43.36 ± 1.38	54.48 ± 1.20	62.05 ± 1.31	79.63 ± 0.95	86.04 ± 0.73	98.03 ± 0.52	100***	19 ± 1.06***	16.36± 0.51*
F2	52.77 ± 2.31	65.81 ± 2.20	92.13 ± 0.16	99.13 ± 0.16	100***	100***	100***	100***	9 ± 0.73***	27.25± 0.54*

Number of animals in each group n = 6, CT: Control without any treatment; CT1: Control treated with gel base containing blank phytosomes, STD: Betadiene ointment containing 5% Povidone Iodine; F1: Gel formulation containing phytosomes of NAT (*Nyctanthes arbor-tristis*)Extract (Equivalent to 1% w/w crocin), F2: Gel formulation containing phytosomes of Crocetin (equivalent to 1% w/w of crocetin). ***P < 0.001, **P < 0.01, *P < 0.05 when compared with control group.

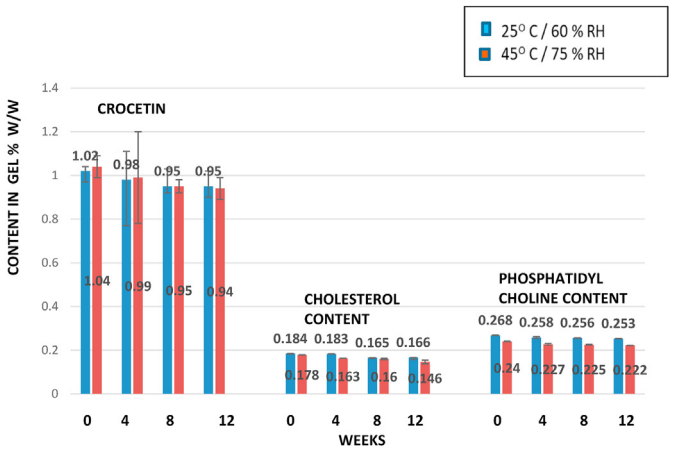


Fig. 2. Stability studies on gel formulation containing phytosomes of Crocetin (Equivalent to 1% w/w of crocetin).

restoring of vascular network. In case of Control group the physiological healing process indicated about 50–51% contraction of the wound area, while the treatment with the standard preparation viz. Betadiene ® and wounds treated with the F2 (crocetin 1% w/w), indicated more than 90% of contraction of wound area, and the treatment with F1 (Equivalent to 1% crocin) indicated about 60% reduction in the wound area on day 10 from the infliction of the wound (Fig. 3).

The results presented in Table 1 indicate that the highest content of hydroxyproline (27.25 μg/500 mg) was obtained in wounds treated with the F2. The control groups indicated hydroxyproline levels in the range of 8–9 μg/500 mg. The formation of granulation tissue was revealed through the content of hydroxyproline in the granulation tissue formed on the wound on day 10. Hydroxyproline is an amino acid acts as a precursor for synthesis of collagen. Collagen synthesis forms an essential aspect of strength of repaired skin.[24] The content of hydroxyproline in the group of animals treated with standard and F1 were also found to be significantly ($P < 0.001$) higher than the control group.

In case of incision wound model, the progress of healing of the wounds was monitored by determined by estimating breaking load of the healed skin/repaired skin on day 11 later infliction of the wounds. It is weight in grams required to break open the repaired skin after removing the sutures. The mean breaking loads of the wounds treated with F2 and F1 were found to be about 2.5 and 2 times higher than the control group (328.8 gm), indicating healing of the skin (Fig. 4). Incision wound model simulates surgical wounds and clinical increased intra-peritoneal pressure which contributes to dehiscence. The common parameter utilized for assessment of healing of these types of wounds is measurement of breaking strength of the healed skin.[25] It is the weight in gm required to tear the skin. The higher the weight required to tear the skin indicates higher tensile strength and acceleration of healing.

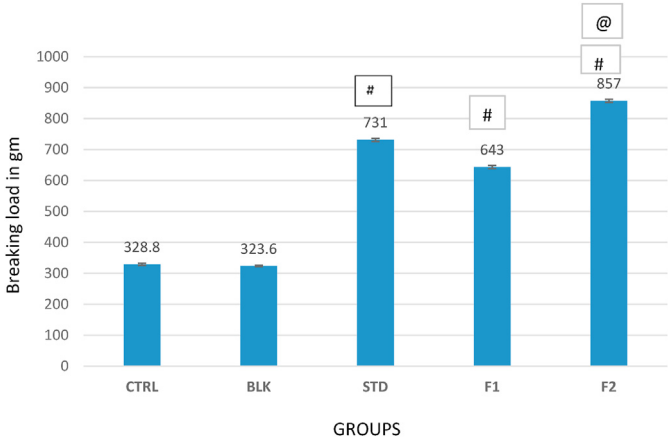


Fig. 4. Effect of treatment of incision wounds with F1, F2 and Betadine on breaking strength of healing skin of rats.
#P < 0.001 When compared with CTRL and BLK; @ P < 0.1 When compared with F1. Groups: CTRL: Control (treated with gel base), BLK: Blank (treated with gel containing blank phytosomes), STD: standard (treated with Betadine® containing 5% Povidone Iodine), F1: Gel formulation containing phytosomes of *Nyctanthes arbor-tristis* extract equivalent to crocin 1% w/w, F2: Gel formulation containing phytosomes of crocetin equivalent to crocetin 1% w/w.

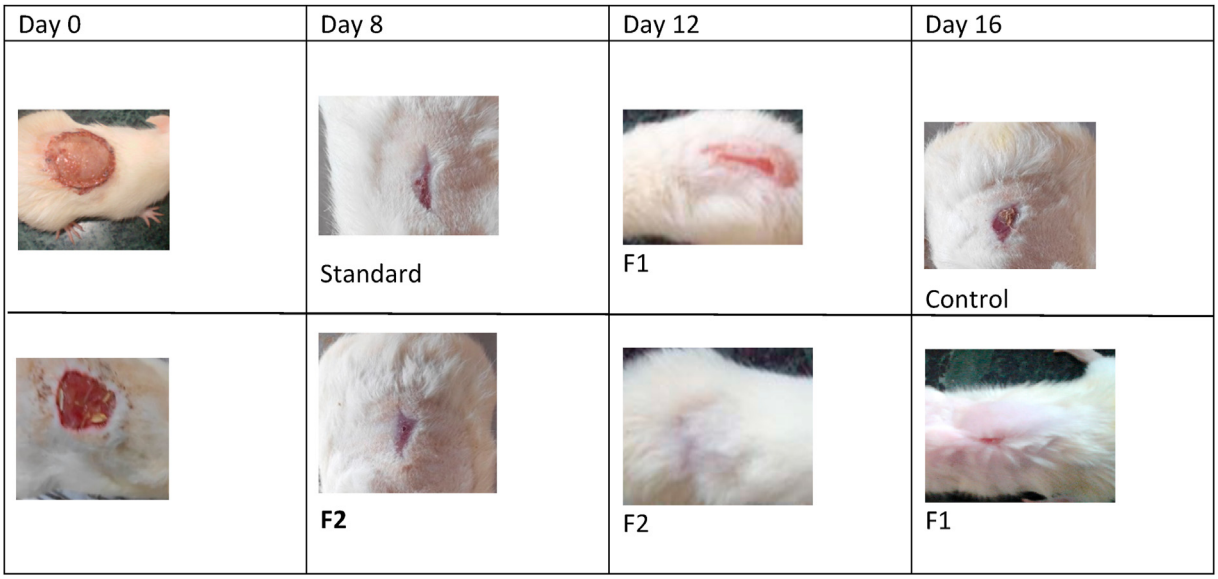


Fig. 3. Effect of topical applications of F1, F2 and Betadine on contraction of excision wound in rats.
Control group treated with gel containing blank phytosomes, Standard group treated with Betadine containing 5% w/w Povidone Iodine, F1 Group treated with gel containing extract phytosomes (equivalent to 1% crocin w/w), F2 Group treated with Gel containing isolated phytosomes containing 1% w/w crocetin.

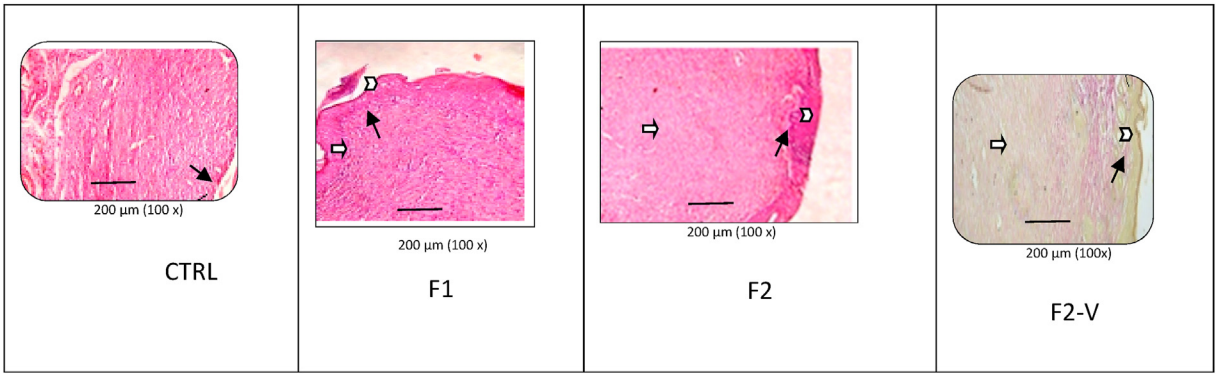


Fig. 5. Histopathological studies on granulation tissue collected on day 11, obtained through treatment of excision wounds with F1, F2 and Blank phytosomes. CTRL control group treated with gel containing blank phytosomes, F1 Group treated with gel containing extract phytosomes (equivalent to 1% crocin w/w), F2 Group treated with Gel containing isolated phytosomes containing 1% crocetin, F2–V Group treated with gel containing phytosomes of crocetin (equivalent to 1% w/w) stained with Van Gieson stain. (↘) Epithelization (⇨) Inflammatory cells (ɒ) collagen.

Highest weight of 857 gms was required to break own the wound in case of wounds treated with F2, indicating good healing and improvement in the tensile strength of the wound. The property of Crocin to inhibit collagenase enzyme and upregulation of Collagen type 1 enzyme support the production of collagen and in turn improving the strength of the repaired skin.

During the proliferative phase oxidative stress of vascular cells is increased.[26] Treatment of the wounds with F1 and the F2 led to significant increase in the rate of wound closure in the proliferative phase which may be due to reduction in the oxidative stress in the wound as the formulations contain carotenoids and these are good antioxidants.

The last phase is remodeling phase involving changes in the extracellular matrix and synthesis of collagen I by replacing collagen III, produced in the proliferative phase. This process can last for about one year. Period of epithelization is the number of days required for fall of eschar from the wound indicating complete closure of wound.[27] The lesser the number of days required as compared to the control group indicating the physiological healing, the better is the process of wound healing. Wounds treated with the F2 indicated least number of days for epithelization, as compared to the wounds in control group (26 days), revealing promotion of wound healing process.

The results presented in Fig. 5 indicated that wounds treated with F2, the granulation tissues contained comparatively few inflammatory cells, and greater collagen, fibroblast and proliferating blood capillaries compared with control group. The higher collagen content of wounds as assessed by the Haematoxylin and Eosin stain was better highlighted upon staining with Van Gieson.[28] Staining with Van Geison stain indicates staining of newly deposited collagen fibers in blue/green color. In addition, the alignment of the collagen fibers was also much better in wounds treated with the F1 and F2, also these were compact and parallel to the surface of the skin. Treatment with the F1 and F2, it is also observed to stimulate and enhance the lay down of collagen fibers and new blood vessels in granulation tissue as compared to placebo-treated control group. Collagen content was found increasing in wounds treated with formulations in the following order: CT < F1 < STD < F2. The molecule crocin is reported to have anticollagenasae activity and the molecule up regulates the expression of Collagen type 1 gene.[6].

4. Conclusion

The stability of crocin an apocarotenoid in orange colored tubular calyx could be enhanced by entrapping in phytosomes and the aglycon crocetin indicated good entrapment efficiency. The studies revealed that crocetin an apocarotenoid isolated from NAT extract and the NAT extract have potential in promotion of wound healing process. Crocetin has better potential as compared to the NAT extract.

Declaration of competing interest

The authors declare that there is no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2021.10.002.

References

1. Alonso GL, Salinas MR, Garijo J, Sanchez-Fernandez M. Composition of crocins and picrocrocin from Spanish saffron (Crocus sativus L.). *J Food Qual*. 2001;24:219–233.
2. Bhuskat P, Amrite O, Patel N, Gadgoli C. Nyctanthes arbor-tristis -A substitute for saffron color. *Indian Drugs*. 2007;44(8):640–642.
3. Shelke S, Gadgoli C. Crocetin from the tubular calyx of Nyctanthes arbor-tristis. *Nat Prod Res*. 2010;24(17):1610–1615.
4. Bukhari SI, Manzoor M, Dhar MK. A comprehensive review of the pharmacological potential of Crocus sativus and its bioactive apocarotenoids. *Biomed Pharmacother*. 2018;98:733–745.
5. Deldar N, Monsefi M, Salmanpour M, Ostovar M, Heydari M. Wound healing potential of crocin and safranal, main Saffron (Crocus sativus L.). *the active constituents in excision wound model in rats*. 2020;10. https://doi.org/10.31661/gmj.v10i0.1900.
6. Crocin. A natural molecule with potentially beneficial effects against skin ageing. *Int J Cosmet Sci*. 2018;40(4):388–400. https://doi.org/10.1111/ics.12472. Epub 2018 Aug 9.
7. Naik AA, Gadgoli CH, Naik AB. Evaluation of gene expression and in vitro enzyme study for antiaging effect of crocin and lutein. *J. Natural Prod*. 2019;9:1–10.
8. Somayeh RQ, Seyed AS, Maryam H, Sohrab M, Seyed HR. Improvement of crocin stability by biodegradable nanoparticles of chitosan-alginate. *Int J Biol Macromol*. 2015;79:423–432. https://doi.org/10.1016/j.ijbiomac.2015.04.041. Epub 2015 Apr 28.
9. Maryanaa W, Rachmawatib H, Mudhakir D. formation of phytosome containing silymarin using thin layer-hydration technique aimed for oral delivery. *Mater. Today Proceed*. 2016;3:855–866.
10. Charles J, Stewart M, Crawford N. An improved method for the determination of free and total cholesterol using the ferric chloride reaction. *Clin Chim Acta*. 1958;3:357–367.
11. Charles J, Stewart M. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem*. 1980;104(1):10–14.
12. Chaudhary H, Rohilla A, Rathee P, Kumar V. Optimization and formulation design of carbopol loaded piroxicam gel using novel penetration enhancers. *Int J Biol Macromol*. 2013;55:246–253.
13. Morton JJP, Malone MH. Evaluation of vulnerary activity by an open wound procedure in rats. *Archives in Pharmacodynamics and therapeutics*. 1972;196(1):

117–126.
14. Ehrlich HP, Hunt TK. The effect of cortisone and anabolic steroids on the tensile strength of healing wounds. *Ann Surg*. 1968;57:117–121.
15. Kokane DD, More RY, Kale MB, Nehete MN, Mehendale PC, Gadgoli CH. Evaluation of wound healing activity of root of Mimosa pudica. *J Ethnopharmacol*. 2009;124:311–315.
16. Bergman I, Loxley R. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal Chem*. 1963;35(12): 1961–1965.
17. Robertson I, Hodge PR. Histopathology of healing abrasions. *Forensic Sci*. 1972;l:17–25.
18. Pawar HA, Bhangale BD. Phytosome as a novel biomedicine: a micro-encapsulated drug delivery system. *J Bioanal Biomed*. 2015;7(1):6–12.
19. Leibovich SJ, Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol*. 1975;78(1):71–100.
20. Epstein HF. Cutaneous wound healing. *N Engl J Med*. 1999;341:738–746.
21. Strodtbeck F. Physiology of wound healing. *Nborn Infant Nurs Rev*. 2001;1: 43–52.
22. Gurtner GC, Sabine W, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature*. 2008;453:314–321.
23. KohTJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expet Rev Mol Med*. 2011;13:1–23.
24. Kaplan B, Gönül B, Dinçer SF, Dinçer NK, Babül A. Relationships between tensile strength, ascorbic acid, hydroxyproline, and zinc levels of rabbit full-thickness incision wound healing. *Surg Today*. 2004;34:747–751.
25. Bradley MP, Pickett MC, Lawrence LPA, et al. Tensile strength vs healing time for wounds closed under tension. *Arch Otolaryngol Head Neck Surg*. 1996;122: 565–568.
26. Wound healing and indigenous drugs: role as antioxidants: a review. *Res Rev J Medicine Health Sci*. 2013;2:5–16.
27. Hosgood G. Stages of wound healing and their clinical relevance. *Veternary Clinic of Small Animals*. 2006;36:667–685.
28. Hblund B, Clemmensen I, Junker P, Lyon H. Fibronectin in experimental granulation tissue. *Acta Pathol Microbiol Scand Suppl A*. 1982;90:159–165.