

CHAPTER 10: SUMMARY AND CONCLUSIONS

10.0 SUMMARY AND CONCLUSIONS

A wound can be defined as an injury or disruption to anatomical structure and function resulting from simple or severe break in structure of organ such as the skin and can extend to other tissues and structures such as subcutaneous tissue, muscles, tendons, nerves, vessels and even to the bone. Wound healing is a complex process involving several inter-related biological and molecular activities for achieving tissue regeneration. The main physiological events include coagulation, inflammation, removal of damaged matrix components, followed by cellular proliferation and migration, angiogenesis, matrix synthesis and deposition, re-epithelization, and remodeling. Wound healing is a global medical concern with several challenges including the increasing incidence of obesity and diabetes, an ageing population and the requirement for more effective dressings. The aim of the present research work was to prepare and evaluate Calendula flower extract (CFE) loaded hydrogel sheet and CFE loaded collagen film for effective treatment of wound.

10.1 PRE-FORMULATION STUDY

Calendula flower extract was prepared and characterized for physical appearance, pH, moisture content, DSC, FT-IR, total polyphenol content and total flavonoids content.

- pH and moisture content of CFE were found to be 6.5 ± 0.2 and $4.945 \pm 0.62\%$ respectively.
- It was confirmed by qualitative chemical analysis that the tannis, saponins, terpenoids, flavonoids and phenols were present in CFE.
- Total phenolic content of CFE was determined by colorimetric method using Folin Ciocalteu reagent and it was found to be 9.649 ± 0.6339 mg GAE/g.
- Total flavonid content of CFE was determined by colorimetric method and it was found to be 10.386 ± 0.275 mg QUE/g.
- The radical scavenging capacity of CFE was found to be 4.604 mg QUE/g extract using DPPH assay.

CHAPTER 10: SUMMARY AND CONCLUSIONS

10.2 FORMULATION DEVELOPMENT AND EVALUATION OF HYDROGEL SHEET CONTAINING CALENDULA FLOWER EXTRACT

PVA hydrogel sheet containing CFE was prepared by gamma irradiation technique and optimized by Box-Behnken desing (BBD) using DesignExpert® 7.0 (Stat-Ease Inc., MN). Optimized formulation was also evaluated for pH, FT-IR, DSC, total phenolic content, total flavonoid content, antioxidant activity and sterility.

- The prepared hydrogel sheet containing calendula flower extract was transparent, flexible, non sticky and golden yellow in color.
- % Gel fraction were reduced significantly with incorporation of CFE, decreased slightly with the increase of Solidifying agent and increased with irradiation dose.
- The swelling capacity decreased with increased in %GF because increased in crosslinking of polymer. Higher irradiation dose enhanced the crosslinking density and reduced the degree of swelling of hydrogel. The hardness of hydrogel sheet increased with irradiation dose because of chemical crosslinking in the PVA by gamma irradiation.
- Prepared hydrogel sheet was transparent, flexible and slightly yellowish in colour with 6.5 pH value.
- Optimized formulation of CFE loaded hydrogel sheet had 73% fluid absorptivity, 68 % gel fraction and 13 g hardness.
- Total polyphenols and flavonoids content were found to be 4 mg GAE/g extract and 4.2 mg QUE/g extract respectively.
- Radical scavenging capacity of it was found to be 0.6987 $\mu\text{mol QUE}/25 \text{ g}$ by DPPH assay. Developed hydrogel sheet was good barrier against microbes because there was no microbial growth was found on surface of hydrogel sheet in microbe penetration test.
- DSC study confirmed that the CFE and excipients were compatible.
- There was no heamolysis observed with formulation as well as CFE in *in-vitro* heamolysis study which proved its safety.

CHAPTER 10: SUMMARY AND CONCLUSIONS

10.3 FORMULATION DEVELOPMENT AND EVALUATION OF COLLAGEN FILM CONTAINING CALENDULA FLOWER EXTRACT

Collagen films were prepared by solvent evaporation method. Concentration of collagen, concentration of plasticizer, speed of homogenizer and drying method were optimized using OVAT method.

- Prepared film was 2-3 mm thin, transparent, and flexible and slight yellowish in color. It had about 15.5 ± 1 g/g fluid absorption capacity and 4 hr pepsin digestion time.
- It had $10.8 \pm 1\%$ moisture content.
- pH of the prepared film was in between 3 to 4.
- Its folding endurance was more than 250 times.
- Total polyphenols and flavonoids content were found to be 5.35 mg GAE/g extract and 5.14 mg QUE/g extract respectively.
- Free radical scavenging capacity of film was found to be 0.8987 $\mu\text{mol QUE/g}$.
- DSC studies confirmed absence of chemical reaction between collagen and CFE.
- FT-IR study confirmed hydrogen bonding in collagen film loaded with CFE.
- There was less than 1% hemolysis observed with formulation in *in-vitro* hemolysis study which proves its safety.
- Collagen of sterilized prepared film was in native form which was confirmed by AFM study and gel electrophoresis study.

10.4 IN-VITRO DRUG RELEASE STUDY

In-vitro release study of total polyphenolic compound from CFE loaded hydrogel sheet was carried out using total immersion method and release kinetics were determined.

- There was initial fast release of polyphenolic compound (about 50%) during 1st hr and then sustained release from CFE-hydrogel sheet.
- CFE-loaded hydrogel sheet followed Korsmeyer Peppas release kinetics as Korsmeyer equation ($R^2 = 0.940$) showed better fit than Higuchi ($R^2 = 0.839$),

CHAPTER 10: SUMMARY AND CONCLUSIONS

first order ($R^2 = 0.745$), Hixon-Crowell cube root ($R^2 = 0.671$) and zero order equations ($R^2 = 0.671$). This type of drug release is controlled by combination of polymer swelling, polymer erosion and diffusion through the hydrated matrix (Diffusion and chain relaxation).

10.5 CELL LINE STUDY

10.5.1 MTT ASSAY: MTT study was conducted using Swiss 3T3 fibroblasts cell line.

- The toxicity profile comparison between growth media, CFE, placebo formulation and formulations containing CFE **by** MTT assay showed that the formulations containing CFE (collagen film and hydrogel sheet) were non-toxic in nature.
- The CFE, CFE loaded collagen film and Hydrogel sheet loaded with CFE significantly stimulated the cell proliferation as number of cells markedly increased after the treatment with formulations compared to placebo and growth media sample.

10.5.2 SCRATCH ASSAY: *In-vitro* wound healing efficacies of developed formulations were evaluated using scratch assay.

- Wells treated with CFE-hydrogel sheet and CFE-collagen film had more population of fibroblast cells in the scratched area compared to non-treated wells and placebo formulations treated wells which confirmed the usefulness of developed formulations for wound healing.
- *In-vitro* scratch assay showed that the developed formulations promoted cell migration and proliferation better than placebo in artificial wound.

CHAPTER 10: SUMMARY AND CONCLUSIONS

10.6 IN-VIVO STUDY

The experimental protocol for the study was evaluated and approved by the Institutional Animal Ethics Committee, Faculty of Pharmacy, The MSU of Baroda, Gujarat, India.

- Two animal models were used for study: Burn Wound model on Wistar rats and cutaneous excision wound healing model on SD rats.
- Wound area was observed and measured using proper scale during 21 days.
- After 21 days post injury, the rats of all groups were anesthetized and under sterile conditions, a skin incision was made to get skin sample (about 3cm x 3cm) for histopathological and biochemical studies.

10.6.1 BURN WOUND MODEL FOR CFE LOADED HYDROGEL SHEET

- CFE loaded hydrogel sheet was evaluated using burn wound model on wistar rats for wound healing efficacy. 24 male wistar rats were randomly allocated to following 4 groups having 6 rats in each group: Group I, Group II, Group III and Group IV. Deep partial thickness burn was inflicted on the back side of the rat by placing the heated and moistened aluminum template at right angles a perpendicular to the dorsum of the rat. All wounds in group II and III were treated with placebo hydrogel sheet and CFE loaded hydrogel sheet respectively for 21 days. Wound from group IV were treated with marketed product for 21 days. Wound from group I were not given any treatment, they kept as a control group. All wound were observed and % wound contraction was noted.
- CFE loaded hydrogel sheet showed a significant burn healing progression on the day 4, 7, 14 and 21 compared to untreated group. The healing percent of lesion area ranged from 4.72% to 25.41% in the period from 2 to 21 days in the control groups (Group I), 6.21% to 72.63% , with Placebo hydrogel sheet, 7.69% to 87.01% with CFE loaded hydrogel sheet and 4.86% to 84.0% with marketed product.

CHAPTER 10: SUMMARY AND CONCLUSIONS

10.6.2 CUTANEOUS EXCISION WOUND HEALING MODEL ON SD RATS FOR CFE-COLLAGEN FILM

- CFE-collagen films were evaluated using cutaneous excision wound model on Sprague-Dawley (SD) rats for wound healing efficacy. 24 male SD rats were randomly allocated to following 4 groups having 6 rats in each group: Group I, Group II, Group III and Group IV. A skin incision was made in a square shape 2 cm x 2 cm on the back side of the rat using surgical blade and skin was removed. Wounds in group II and III were treated with placebo collagen film and CFE-collagen film respectively for 21 days. Wound from group IV were treated with marketed product for 21 days. Wound from group I were not given any treatment, they kept as a control group. All wound were observed and % wound contraction was noted. After 21 days, rats were euthanized and skin sample were removed for hisopathological and biochemical study.
- The rate of wound contraction was significantly high in CFE loaded collagen film treated wounds ($92.18 \pm 1.2\%$) when compared to control group ($56.25 \pm 4.1\%$), placebo treated group ($62.5 \pm 5.1\%$), Nemigen® treated group ($68.75 \pm 6.1\%$) on day 21.
- Developed formulations were significantly enhanced wound healing activity in by increasing cellular proliferation, formation of granulation tissue, neovascularization, synthesis of collagen epithelization and early histological maturation in rat.
- There was significant increase in the collagen amount in the treated group compared to non treated and placebo group. This indicates a positive role of the extract on the cutaneous wounds healing process. The triterpene faradiol palmitic ester stimulated the proliferation and migration of fibroblasts which stimulates the fibroplasias.

CHAPTER 10: SUMMARY AND CONCLUSIONS

10.7 Stability study

The developed formulations were evaluated for stability under ambient temperature (for real time stability study, 25°C±2° C, 60% RH±5% RH) and accelerated condition (40°C±2° C, 75% RH±5% RH) for 6 months as per ICH guidelines. Samples were withdrawn after 1, 2, 3 and 6 month for evaluating different physicochemical parameters.

- CFE loaded hydrogel sheet were evaluated for physical appearance, pH, %GF, %hardness, % water absorption, total polyphenolic content, total flavonoid content and sterility.
- CFE loaded collagen film were evaluated for physical appearance, %fluid absorption capacity, folding endurance, pepsin digestion time, %LOD, total flavonoids content, total polyphenolic content and sterility.
- The samples did not show any significant changes in any stability indicating parameters during stability study from the initial value. Hence both the developed formulations (CFE-hydrogel sheet and CFE-collagen film) were found to be stable for a period of 6 month.

10.8 CONCLUSION

Stable calendula flower extract loaded hydrogel sheet and collagen films were successfully developed and evaluated. The methodologies utilized in the present work have produced potentially useful stable formulations which were able to improve the wound healing process. *In-vivo* study indicated that calendula flower extract containing formulation showed significantly better wound healing activities compared to placebo formulations. We concluded that *Calendula officinalis* extract containing biodegradable film and hydrogel sheet stimulated wound healing process and increased the cell proliferation better than placebo formulation due to enhanced bio-activity of hydrogel sheet and biodegradable film by *Calendula officinalis* flower extract.