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Original Article

Cassia occidentalis Potentiates wound Healing Process in Type-2 Diabetic Rats

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ABSTRACT

Introduction: This work is focus on a cost-effective herbal hydrogel formulation comprising of extract, from the leaves of *Cassia occidentalis* L. (Caesalpiniaceae). The formulation is highly effective for delayed wound healing due to diabetes. **Background:** *Cassia occidentalis* (CO) is extensively used in folklore medicine system. It is a common weed scattered from foothills of Himalayas to West Bengal, South India, Burma and Srilanka. **Objectives:** The study aimed to explore the role of *Cassia occidentalis* (leaf) for wound healing potential in diabetic rats using the excision wound model. **Materials and Methods:** The characterization of the phytoconstituents and bioavailability profiles of methanolic extract of CO (MCO) were investigated using High-Performance Liquid Chromatography-Photodiode Array Detector analysis. Insulin resistance was induced in rats by feeding them a diet rich in fructose and hyperglycemia was induced by injecting streptozotocin (STZ) at a dose of 40 mg/kg body weight (bw), intraperitoneally (ip). After confirmation of diabetes fasting blood glucose level, the wound was induced. Excision wound healing activity was examined on Wistar rats. **Results:** The hydrogel of MCO (5 and 10%) reduced significantly wound size. Significant mean epithelialization period, higher hydroxyproline content and tensile strength were revealed at the concentrations of MCO hydrogel of 5 and 10% compared to the diabetic wound control group. **Conclusion:** These results provide promising baseline information for the wound healing potential of MCO in type-2 diabetes treatment.

Keywords: *Cassia occidentalis*, Hyperglycemia, Hydroxyproline, Hydrogel, Biochemical parameters.

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INTRODUCTION

Diabetes is projected to rise from 171 million to 366 million in 2030, affecting 4.4% of the population worldwide. Diabetes mellitus is a severe global health problem. Diabetes will be the seventh leading cause of death in 2030.[1] Uncontrolled higher glucose level (diabetes mellitus) may lead to mortality and morbidity due to their related complications such as macro and microvascular complications, cardiovascular diseases, neuropathy, retinopathy and foot ulcer.[2] Foot ulceration is a major complication of diabetes which is associated with sustained pain, bacterial infection and may lead to chronic impaired non-healing wound.[3] Diabetic patients are 15 to 20 times more likely to require amputation than those without diabetes worldwide. Impaired wound healing may lead to lower limb amputation.[4] Wound healing is a very orderly and highly controlled process characterized by four distinct but overlapping phases: hemostasis, inflammation, proliferation and remodelling. The repair process needs the coordination of various cells, growth factors and cytokines.[2] In individuals with diabetes mellitus, wounds remain in a chronic inflammatory state and fail to heal in a timely and orderly manner. The continual influx of inflammatory cells and sustained production of their inflammatory mediators cause imbalances in wound proteases and their inhibitors, preventing ECM synthesis and remodelling that is essential for normal wound healing.[5] *Cassia occidentalis* L called as «Kasmard» in Sanskrit, «Kasondi» in Hindi and «Coffee Senna» in English belongs to family Caesalpiniaceae. It is an ayurvedic plant with substantial medicinal importance. Leaves of *C. occidentalis* plant have ethnomedicinal relevance like a paste of leaves is externally applied on healing wounds, sores, itch, cutaneous diseases, bone fracture, fever, ringworm, skin diseases and throat infection.[6,7] Previous pharmacological investigations showed that *C. occidentalis* leaf extracts have antioxidant,[8] antidiabetic,[9] and antibacterial,[10] wound healing,[11] and anti-inflammatory[12] properties. The aim of the study is to evaluate the effect of methanolic extract of *Cassia occidentalis* for wound healing potential in diabetic rats using fructose-fed-STZ at the dose of 40 mg/kg bw administered intraperitoneally induced type-2 diabetes.

MATERIALS AND METHODS

Experimental animals

Healthy Wistar rats of either sex, 8-14 weeks (150-250 g) with no prior drug treatment were selected to carry out all the present *in vivo* studies. The animals were used after an acclimatization period of 10 days to the laboratory environment. They were housed in standard polypropylene cages and provided with food and water *ad libitum*. Before the selection of animals, they were screened for their normal glucose levels. The

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animals having fasting blood glucose level in the range of 80-120 ± 5 mg/dL were selected for the study.

Preparation of Plant Extract

The selection of plant was based on the traditional uses as in wound healing, cutaneous diseases and diabetes. The plant *Cassia occidentalis* leaves were collected from wild of Khargone District, Madhya Pradesh., India in September and authenticated by Prof. Zia Ul Hasan (Botanist and Head of Botany, Safia College of Science, Bhopal, Madhya Pradesh, India.) with voucher specimen (417/Bot/Safia/12). Dried and powdered plant leaves of *Cassia occidentalis* (50 g) were defatted firstly with petroleum ether at 40-60°C for 36 hr in soxhlet apparatus. Then, defatted material (exhausted material) was subjected to extraction with 500 mL of methanol in soxhlet apparatus for 36 hr, and the solvent was recovered to the obtained dry residue of extract. Methanolic extract was weighed and stored.

Preliminary Phytochemical Investigation

MCO was subjected to phytochemical investigation for the detection of the presence of various phytochemicals by using standard methods.[13]

Formulation of hydrogel of MCO

The dried MCO was taken for the preparation of hydrogels. The formulations were prepared by dispersing carbopol 934 (1 g) in distilled water (50 mL) with continuous stirring and kept overnight to get a smooth gel. Solutions of sodium metabisulphite (2 mL), methyl paraben and propyl paraben (5 mL) were appropriately mixed with the carbopol gel with continuous stirring. Then, the required amount of extract (5%) was added in the above mixture, and its volume was increased to 100 mL by adding distilled water. Triethanolamine was added dropwise to the formulation for adjusting the required skin pH (pH: 6.5-7.0) and obtaining necessary consistency.[14] Similarly, 10% of MCO hydrogel was prepared. Both preparations were packed in wide-mouthed plastic jars. All developed herbal formulations were tested for physical appearance and homogeneity by visual observation.

In vivo skin irritation study

The hydrogel was applied to the shaven skin of the rat. Skin irritation potential of 5% and 10% hydrogel of MCO were assessed by carrying out patch skin irritation test on albino rats (150-200 g). Rats were acclimatized for 7 days before the study. The fur from the dorsal surface of rats was removed with electronic hair remover without damaging the skin, 24 hr prior to the experiment. Animals were divided into three groups containing three rats in each group. Group I was kept as a control - applied hydrogel base topically only, Group II was shown as a treated group- applied topically 5% MCO hydrogel and Group III was observed as a treated group- applied topically 10% MCO hydrogel. The formulations were applied topically to approximately 1cm^2 area of the skin. The animals were then returned to their cages and were examined at 24, 48 and 72 hr after the application of the formulation. The sites were inspected for dermal reactions such as erythema and oedema. The mean erythemal and oedema scores were recorded based on the degree of severity:[15] no erythema/edema=0, slight erythema/edema=1, moderate erythema/edema=2, severe erythema/edema=3.

Diabetic wound healing activity assay

Diabetic wound healing activity of MCO against impaired wound healing process by diabetes was performed on the excision wound model in rats. The animals were divided into four groups, each group containing six animals. Group I was served as Normal control (only hydrogel base is applied topically), Group II was kept as negative control (10% Fructose fed orally for initial 2 weeks *ad libitum* and Streptozotocin was injected at a dose of 50 mg/kg bw, topically hydrogel base only applied), Group III and IV were kept as test groups (10% Fructose fed orally for initial 2 weeks *ad libitum* and Streptozotocin was injected intraperitoneally at dose of 50 mg/kg bw, and 5 and 10% hydrogel of MCO respectively). Wound induction was done on 2nd week of fructose feeding and served as the initial day (zero) of post wound day in all the groups excluding Group I. Control groups were supplied with standard drinking water *ad libitum*. In contrast, other groups were supplied with 10% fructose solution, respectively, *ad libitum* for the initial 2 weeks only, then provided with normal drinking water during the remaining period of the experiment. STZ was dissolved in a citrate buffer (pH 4.4) with a concentration of 15 mg/mL. All animals were fasted overnight, and each of the fructose-fed groups were injected intraperitoneally with single-dose STZ (50 mg/kg bw) whilst the animals in the control group were injected with vehicle buffer (citrate buffer pH 4.4) only. The blood sample was withdrawn by pricking vein from the tail at each week from starting of fructose feeding to 2nd week of fructose feeding. After STZ injection blood sample was collected on 0 day, 3rd day, 7th, 15th and 30th day. Blood glucose level was measured by digital Glucometer (Elegance Glucometer CT-X12, India). Confirmation of diabetes for wound healing activity was done on 7th day of STZ induction. Animals which showed fasting blood glucose level more than 150 mg/dL were considered as diabetic.[16]

Excision Wound Induction

Blood glucose levels were determined on 7th day after STZ injection, and only rats with blood glucose level concentration more than 150 mg/dL were used in the following study. The rats were anesthetized using ketamine and xylazine (50 and 5 mg/kg ip bw respectively). The dorsal area was depilated, and single full-thickness excision circular wound (1 cm diameter) was created on diabetic rats using sharp scissors and a scalpel. Wounds were left undressed to the open environment. In this model, wound contraction, epithelialization period, hydroxyproline estimation, histopathology was monitored.[17]

Wound contraction and Epithelialization period

The progressive changes in excision wound area were measured in cm by tracing the wound boundaries on a transparent paper on each 3 days interval until complete wound healing, after the wound creation. The wound areas in all groups were recorded on a graph paper. Wound contraction was expressed as a reduction in the percentage of the original wound size. The rate of wound contraction was determined using the following formula:

$$\text{Wound contraction (\%)} = \text{Healed area} / \text{Total wound area} \times 100$$

Epithelialization time refers to the number of days taken by the wounds to appear closed entirely with no moist granulation tissue, and the injury was covered with new epithelium.[18]

Tensile strength

Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists to breaking under pressure and may designate in part of fixed tissue. For that healed tissue is excised on 7th day of post wounding day by using anaesthesia and subjected to tensiometer. The instrument used for measurement is called tensiometer. Tensile strength was measured with the help of the tool made in the laboratory, which resembles a tensiometer.[19]

Hydroxyproline estimation

Hydroxyproline is an uncommon amino acid present in the collagen fibres of granulation tissues. The measurement of hydroxyproline can

be used for collagen turnover. For the determination of hydroxyproline content, the animals from each group were anaesthetized by using ketamine and xylazine. The wound tissues were excised on the 7th day of post wounding day and dried in a hot air oven at 60-70°C to constant weight, and the hydrolysate was neutralized to pH 7 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by the addition of 0.4 M perchloric acid and colour was developed with the help of Ehrlich reagent at 60°C. The absorbance was measured at 557 nm using a spectrophotometer. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline at the same time.[20]

Histopathological Studies

Histological analysis was performed after fixation with paraformaldehyde (4%) for 24 hr at room temperature. The specimens were embedded in paraffin and sectioned in a plane perpendicular to the incision. Sections 5 µm thick were mounted on polylysine-coated slides, dewaxed, rehydrated to distilled water, and stained with hematoxylin and eosin (H&E). Evaluations of all sections were performed by two experienced pathologists who were blinded to the previous treatment. Sections were semi-qualitatively assessed under a light microscope and observed for fibroblast proliferation, collagen formation, neovascularization, granulation tissue, and epithelialization.[21,22]

Statistical analysis

The values were expressed as mean ± SEM of six animals in each group. The data were statistically analyzed and regarded as significant if $p < 0.05$. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple parametric tests.

RESULTS

Extraction of plant material

The yield of *Cassia occidentalis* found to be 7.2 g (16%) of methanolic extract (Greenish black).

Phytochemical screening

Qualitative analysis was performed for petroleum ether, and methanolic extract of the *Cassia occidentalis* plant (leaf). Methanolic extract was found to contain alkaloids, glycosides, proteins, and amino acids, sterols, carbohydrates, phenolic compound, flavonoids, saponins, and tannins.

Skin irritation study

No sign of erythema and oedema was found up to 72 hr after application of the MCO hydrogel. This hydrogel was green and homogenous with excellent consistency.

Confirmation of diabetes and wound induction

All the animals showed the elevated blood glucose level (fasting) more than 150 mg/dL on 7th day of diabetes induction. In all the animals of group II, III and IV, glucose level was found to be more than 150 mg/dL on the 7th day of STZ administration. On that day, the excision wound was induced and evaluated for various parameters.

Wound contraction and epithelialization

In the comparison of diabetic wound rats, 5% MCO hydrogel and 10% MCO hydrogel treated rats showed a significant reduction ($p < 0.01$) in wound contraction time (Table 1, Figure 1). In the case of Group IV, 100% wound contraction was achieved on 24th day while that in the case of Group III, 100% wound contraction was achieved on 26th day. In the case of diabetic wound rats, the process of wound healing was delayed, and 100% wound contraction was observed on 30th day. In case of Group IV (10% MCO hydrogel) period of epithelialization was observed on 21st day and in comparison, of Group II diabetic wound group significant ($p < 0.01$) reduction in the meantime of epithelialization which is enlisted in Table 2. However, 5% MCO hydrogel treatment did not show a significant change in epithelialization time in comparison with diabetic wound rats.

Table 1: Wound contraction (%) in treated diabetic rats with MCO at 5 and 10% concentrations.

Post wounding day	Group I	Group II	Group III	Group IV
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2nd	50.41 ± 7.85	24.14 ± 1.86	34.25 ± 1.22	36.53 ± 1.11
4th	61.10 ± 6.35	37.86 ± 1.77	45.26 ± 2.62	45.13 ± 0.98
6th	72.09 ± 6.22	51.25 ± 1.22	52.05 ± 1.61	53.91 ± 0.93
8th	86.82 ± 1.86	57.3 ± 1.59	57.81 ± 1.63	60.35 ± 1.88
10th	92.69 ± 0.91	63.36 ± 1.71	62.66 ± 3.01	67.73 ± 1.85
12th	94.56 ± 0.73	70.11 ± 1.03	67.06 ± 1.13	73.21 ± 1.31
14th	97.25 ± 0.69	74.50 ± 2.56	71.80 ± 1.11	79.04 ±1.15
16th	98.39 ± 0.69	77.45 ± 1.71	78.1 ± 1.31	84.91 ± 1.25**
18th	99.60 ± 0.16	82.20 ± 2.19	83.75 ± 1.21	89.71 ± 1.23**
20th	100.00 ± 0.00	86.70 ± 2.12	88.33 ± 1.14	93.23 ± 0.64**
22nd	-	90.20 ± 1.31	93.85 ± 1.19*	98.78 ± 0.52**
24th	-	94.05 ± 1.03	99.03 ± 0.24**	100 ± 0.00**
26th	-	96.73 ± 1.11	100.00 ± 0.00**	-
28th	-	98.70 ± 0.99	-	-
30th	-	100.00 ± 0.00	-	-

-: Total recovery, Group I- Normal control (vehicle treated only hydrogel base), Group II- Diabetic control (vehicle treated only hydrogel base), Group III- Treatment by 5% hydrogel of MCO, Group IV- Treatment by 10% hydrogel of MCO, values are expressed as mean ± SEM, $n = 6$, *, **indicate a significant difference at $p < 0.05$ and $p < 0.01$ respectively, compared to the diabetic control group (Dunnett's test).

Hydroxyproline and tensile strength

Hydroxyproline was found significant ($p < 0.01$) higher in comparison to diabetic wound group in the treatment group. Significant reduction of hydroxyproline was found in diabetic wound group in comparison to the normal control group (Table 3). Tensile strength of treated rats significantly improved in comparison with diabetic wound rats (Table 3).

Histopathological study

The histopathological studies of the tissue of excision wound area treated with standard, control and extract ointments (5 and 10%) are shown in Figure 2. There was a marked infiltration of the inflammatory cells, increased blood vessel formation and enhanced proliferation of cells as a result of treatment with MCO. There was full thickness re-epithelialization, in which epidermis was thin and well organized, comparable to the normal adjacent skin which was not involved in the wound generation and healing process. The granular layer was well-formed and one cell in thickness. In all, complete epithelialization, vascularization and hair follicles formation were observed in treated rats.

DISCUSSION

Diabetic foot ulcers (DFUs) are rapidly growing complication of diabetes, and it is delayed the wound healing process. Over half of diabetic patients who develop a single ulcer will subsequently develop another ulcer of which the majority will become chronic non-healing ulcers.[23]

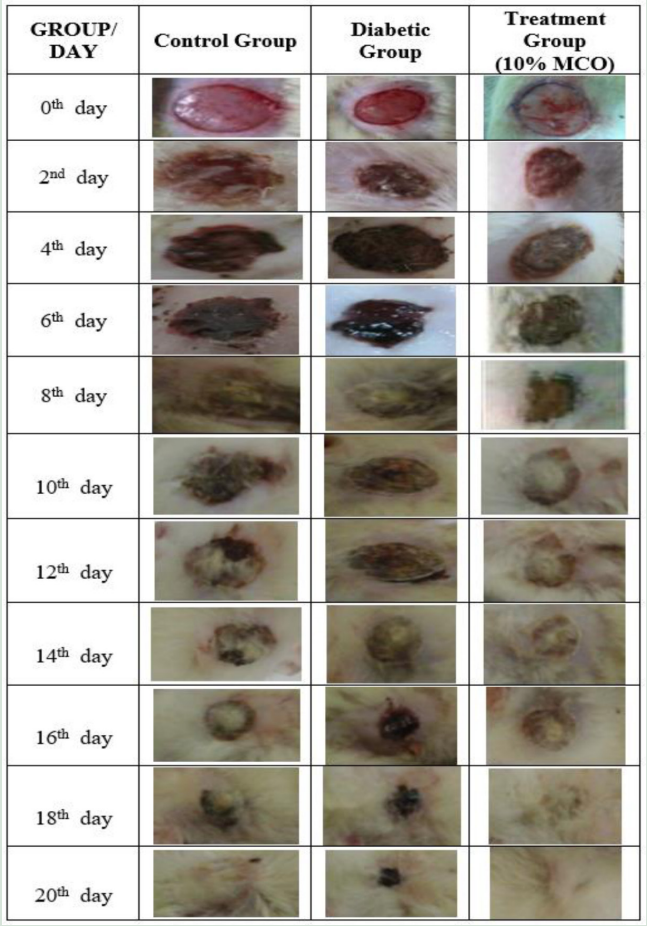

Figure 1: Effect of 10% MCO hydrogel on the rate of wound contraction model in excision wound.

Table 3: Hydroxyproline estimation and tensile strength observed in treated diabetic rats on 9th day of post wound with MCO at 5 and 10% concentrations.

Groups	Hydroxyproline (mg/g tissue) on 9th day of post wound	Tensile strength (g) on 9th day of post wound
Group I-Normal control (vehicle treated only hydrogel base)	55.5 ± 2.02	23.33 ± 0.71
Group II-Diabetic control (vehicle treated only hydrogel base)	23.88 ± 1.01	11.50 ± 0.429
Group III- Treatment by 5% hydrogel of MCO	41.5 ± 0.62**	18.83 ± 0.94**
Group IV- Treatment by 10% hydrogel of MCO	48.33 ± 0.95**	21.00 ± 0.57**

Values are expressed as mean ± SEM, $n = 6$, **indicates a significant difference at $p < 0.01$ respectively, compared to the diabetic control group (Dunnett's test).

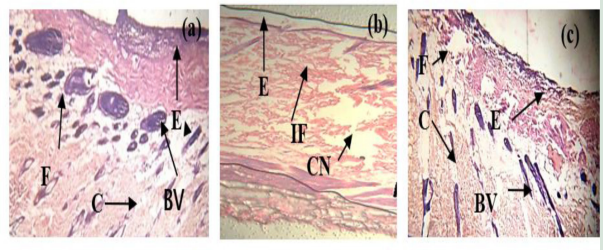

Figure 2: Photomicrographs showing histological changes in skin tissue on day 17; (I) Control group (II) Diabetic group (III) Treated group (10% MCO) (100 X); E: Epidermis; BV: Blood vessels; C: Collagen fibres; IC: Inflammatory cells; CN: Cellular necrosis.

Table 2: Period of epithelialization observed in treated diabetic rats with MCO at 5 and 10% concentrations.

Groups	Epithelialization period (Mean time in days)
Group I- Normal control (vehicle treated only hydrogel base)	17.83 ± 0.47
Group II- Diabetic control (vehicle treated only hydrogel base)	26.00 ± 0.40
Group III- Treatment by 5% hydrogel of MCO	24.16 ± 0.58
Group IV- Treatment by 10% hydrogel of MCO	21.66 ± 0.66**

Values are expressed as mean ± SEM, $n = 6$, **indicates a significant difference at $p < 0.01$ respectively, compared to the diabetic control group (Dunnett's test).

Diabetic patients are 15 to 20 times more likely to require amputation than those without the disease. The prevalence of DFU ranges from 4 to 10% in hospitalized patients. The risk of developing a foot ulcer in diabetic patients could be as high as 25% in their lifetime.[24] Prolonged and poorly controlled diabetes irreparably damages bodily tissues. Nerve damage in the lower limbs results in diabetic neuropathy, whereby the patient's somatosensory and autonomic functions are diminished or completely lost. The subsequent loss of protective sensation, impaired gait control, bone deformities (e.g., Charcot's foot), callus formation, and/or inhibited sweat response result in excessive shear and pressure that damages the diabetic foot.[25]

It was reported that an incision skin wound was healed by the growth of granulation tissue and re-epithelialization.[26] *In-vivo* topical application of MCO significantly accelerates the healing process and provides the strength to collagen tissue. The phytochemical screening of MCO revealed the presence of various components, which would promote individually or synergistically its wound healing activity. For instance, flavonoids and tannins present in this extract could induce wound healing through several cellular mechanisms, like scavenging of free radicals and reactive oxygen species, promoting contraction and increasing fibroblasts. Previous studies on *Cassia occidentalis*, revealed strong antioxidant activity against the superoxide radical, nitric oxide radical and hydroxyl radical,[8] and bactericidal activity against *Bacillus subtillis*.[10] Therefore, the ability to scavenging free radicals and exert a bactericidal effect on the surface of the skin. It is known to play an important role in the treatment of diabetic wounds at the proliferative stage. Reactive oxygen species can induce severe tissue damage and even lead to neoplastic transformations decreasing the healing process by damage in cellular membranes, DNA, proteins and lipids.[26]

CONCLUSION

This study confirms the promising wound healing activity of methanolic extract of *Cassia occidentalis* in Type-2 diabetes. Phenolic compounds

as flavonoids were found in the MEOH fraction of *Cassia occidentalis* which could be responsible for the protective properties as discussed. Further studies should be carried out to characterize these compounds and emphasized on their exact mechanism of action.

Authors' Contributions

RSP designed the study. FAT participated in the design of the study. ASM and SK carried out the experiments. ALMK wrote and reviewed the manuscript. All authors took part in analyzing and interpreting data. All authors approved the final version.

Ethical Approval

The animal study was performed in Department of pharmacology, Faculty of Pharmacy, VNS Group of Institutions, Bhopal (M.P.) with due permission from the Institutional Ethical Committee (CPCSEA protocol no. PH/IAEC/VNS/2K12/006).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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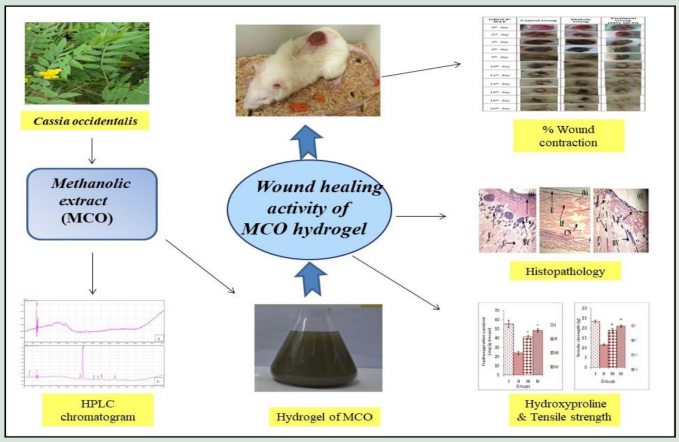
GRAPHICAL ABSTRACT



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