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Original Research Article

Formulation of Wound Healing Transdermal Patch from Tubers Extract of *Momordica Cymbalaria* and its *In-vitro* Evaluation

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Abstract

Developing wound infection in type I or II diabetic people had risk complications like gangrene. Although there are enough antibiotic ointments or creams, still it needs physician dressing and continuous monitoring of wound. So, to make it simple and more effective, a combination of transdermal drug delivery route with herbal usage contribute in controlled release of medication with minimum undesirable side effects. And also help to treat wound infections by self-medication. This work is focused, to formulate wound healing transdermal patch for diabetics with *Momordica cymbalaria* tuber extract. A sequence of techniques was performed to analyse antioxidant content then Starch assay was performed *In vitro* to evaluate its healing efficacy, determine cytotoxicity using zebra fish embryos and artemia salina using hexane extract of tuber. Optimized combination is used to prepare Patch by solvent evaporation method then evaluated the patch by organoleptic and physico-chemical properties. The result proofs, the tuber extract had Strong Antioxidant activity whereas weak cytotoxicity showed in two concentration (25 and 50µg/ml) towards brine shrimp lethality assay and fish embryo toxicity. High migration rate found in 25µg/ml and 50µg/ml in scratch assay using Human Epidermal Keratinocytes. Evaluated the patch for its organoleptic and physico-chemical properties, it shows good result. As a final observation of the study, bio compounds present in tuber extract proved to enhance wound healing and could pave way as alternate to the synthetic wound healing patches owing to its ecofriendly, economical and herbal nature be natural replacement to hasten wound healing especially in diabetic patients.

Keywords: Diabetic wounds, Transdermal drug delivery system, *Momordica cymbalaria*, tuber extract, Antioxidant, Human Epidermal Keratinocytes, Artemia salina, Zebra fish embryo.

INTRODUCTION

Diabetes is the wide-ranging global health concern which is one of the 10 major causes of death. World health organization (WHO) states, in 2019 among 74% of global mortality 1.6 million death rate are due to diabetes (Pradeepa *et al*, 2021). Patients who having diabetes, their wounds were considered as a serious health concern and require attention. To maintain tissue homeostasis, Wound healing mechanism is mandatory to regain the lost tissue. To form a fresh healthy tissue in the wounded area is complicated mechanism and it involves the following process inflammation, angiogenesis, granulation tissue formation, re-epithelialization and ECM reconstruction. Cells include fibroblasts and other cells will migrate toward the wound area and helps in healing the wounded area. (Bolla *et al*, 2019).

Exploring the plant with their traditional properties and valuable medicinal uses reflects in discovering a newer drug with cost-efficient along with less side effects for treating various ailments. (Muhammad *et al*, 2013). Up-regulation of VEGF and TGF-β, activation of NF-KB activation of interleukin-8, increased expression of iNOS and alpha-1 type 1 collagen and anti-oxidant activity was some process that medicinal plant uses to heal the wound areas. (Firdous *et al*, 2018). *Momordica cymbalaria* found to best alternative for anti-diabetic when it comes to plant origin aspects. *Momordica cymbalaria* used for treating diabetics for a long period and also its pharmacological actions are examined and proved against anti-diabetic activity in animal models. Among all different type of solvents ethanol extract of this plant satisfied high phenolic and flavonoid (Elangovan *et al*, 2019).This forms a major element for choosing *Momordica*

cymbalaria as a potential wound healing agent for treating diabetic wounds.

Figure 1: Image of *Momordica cymbalaria*

Momordica cymbalaria is a perennial herbaceous climber which is called by the Tamil name Athalakkai (Umarani *et al*, 2015) and found in tropical regions of India and southeast Asia. *Momordica cymbalaria* has been used in ancient period. In India, it is abundantly present in some specific place such as Tamil Nadu, Madhya Pradesh, Karnataka, Maharashtra and Andhra Pradesh. It has major source of vitamin C, Beta carotene, calcium, iron, and fibres but this plant material are rarely cultivated because of lack in planting material (Rekha, 2015). Leaves of the plant are orbicular- reniform in outline and fruits are 20-25 mm long. The size and shape of the seeds are 4.6 mm long and oval with smooth surface. The nature of the flowers is unisex and had woody roots (Jha *et al*, 2017). It is a drought tolerant plant, grows with the height of 90-120 cm. Tuberous root of this plant in the soil will remain even after the plant dry or disappeared and will regrow again in the upcoming season (kiruba *et al*, 2020). Diabetes wound requires a lot of care to avoid further infection in the wounded area. Oral antibiotics and intravenous injection are in usage for treating mild and severe soft tissue injury in diabetic wound. These tablets have drawbacks such as side effects and particularly, old age people will suffer due to difficulty swallowing. To make the treatment easy with quick healing without any side effect, herbal drug with transdermal preparation is used as an option. This method is more practical as well as comfortable with faster systematic effects (Mita *et al*, 2018). Transdermal therapeutic systems defined as patches which helps in delivering an accurate amount of drug into the systematic circulation of particular area through skin (can *et al*, 2013).The use of this method was considered due to its potential like hepatic first-pass metabolism, continued delivery of drugs to maintain stable plasma profiles and increase therapeutic efficacy with reduction in systemic side effects (Sonawane *et al*, 2016). Due to the reason like effectively control blood sugar and minimize adverse effects related to oral delivery, for chronic disease like diabetes transdermal patch is a helpful delivery system (Zhou *et al*, 2021).

Wound healing medication considered as disappointing because of limited efficacy, low availability and various side effects. Many scientists delivered that medicinal plant extract and its compounds show improvement in wound healing mechanism (Sagástegui-Guarniz *et al*, 2021). Tuber extract of *Momordica cymbalaria* observed and declared to posse's antioxidant and hepatoprotective activities. GC-MS analysis of tuber extract showed existence of n- Hexadecanoic acid constituents proved that it will heal diabetic wound effectively (Manikandan et al, 2019). The current study was aimed to formulate transdermal patch loaded with tuber extract of *Momordica cymbalaria* from hexane solvent and its in-vitro evaluation.

EXPERIEMENTAL METHADOLOGY

Collection of Plant and Preparation of Extract

Garden-fresh tubers of *Momordica cymbalaria* were used in this study, which were collected from Peraiyur, Madurai district, Tamil Nadu.

Figure 2: Image of *Momordica cymbalaria* plant with tuber

Collected tubers were cleaned with running tap water and rinsed in distilled water. The tubers were cutted into fine pieces, shade dried for 4 days and grinded into fine powder by using mechanical method. About 10 g of *Momordica Cymbalaria* tuber power were extracted with 200 ml of Hexane solvent using Soxhlet apparatus (Dhanalakshmi *et al*, 019). Then the obtained extract is evaporated to obtain crude extract which are stored in vials for further experimental use.

In-vitro Antioxidant Assay

To determine Antioxidant or the free radical scavenging capacity of tuber extract are done by (DPPH) 1, 1-Diphenyl-2-Picryl Hydrazyl assay.

Prepare DPPH stock solution (0.1mM) using Methanol. Different concentration of standard (Gallic acid) and tuber extract of *Momordica Cymbalaria*

(sample) were prepared by serial dilutions then make the final volume to 1ml with methanol. In 96 well plates, add 100 µl of 0.1mM DPPH solution to 100 µl of samples and 100 µl of standards which are prepared with different concentration. 200µl of DPPH solution is added as control for antioxidant assay. The reaction mixture was mixed then kept to react for about 30 minutes at Room Temperature in Dark Environment. Absorbance value for the samples was read at 517 nm with UV spectrophotometer. Inhibition of radical scavenging activity percentage is calculated by **Radical Scavenging activity (%) = $A_0 - A_1 / A_0 \times 100$**

Whereas, A_0 - Absorbance of DPPH radicals with methanol

A_1- Absorbance of DPPH radicals with sample extract or standard

Calculate the IC_{50} Values of extract and standard using Microsoft Excel. Conduct the experiment in triplicate and note the average value of the data (Muniandy *et al*, 2018).

Brine Shrimp Lethality Bioassay

Artemia salina egg capsule were purchased from nearby aquarium and dried cysts were dissolved in the artemia collecting tank in which 50g of rock salt dissolved in 1 liter of distilled water. On the one side of the tank, one capsule contain quality egg were added and were allowed to hatch in dark environment. Then the mature nauplii were collected at another end to serve as test organism. Constant oxygen and light are provided throughout the hatching period at room temperature. After 48 hours, nauplii were used for evaluation of tuber extract. In an Eppendorf tube, stock solution is prepared by taking 10 mg of hexane extract of tubers and dissolves it in 1ml of artificial sea water. From the stock solution prepare 25µg/ml, 50µg/ml, 100µg/ml, 250 µg/ml concentration of sample solution using artificial sea water.

In a 96 well plate, add 200µl of sample suspension with different concentration in each appropriate well of microtiter plate. To the each well, add 10 nauplii per wells using Pasteur pipette and 10 nauplii with 200 µl of prepared sea water is taken as control. Incubate them for 24 hours at room temperature. After 24 hours count the number of surviving nauplii in each well using stereoscopic microscope. Conduct the experiment in triplicate for each concentration. Compare the number of survivors in test and control wells to analyze lethality rate (Rajabi *et al*, 2015). Use Abbott's formula to calculate the percentage.

Percentage of Lethality = {(Test-Control) / Control} × 100

Cytotoxicity Assay on Zebrafish Embryo

Zebra fish embryos were purchased from the zebra fish aquarium. To perform the assay, 10 healthy zebra fish embryos are shifted to each well in 24-well plate containing different concentration of sample with 1 ml of embryo water (which are prepared by the formulation: 60 mg of sea salt/ liter of ultrapure water) and incubate for 72 hrs . The test was performed in duplicate. At every end of 24, 48 and 72 hours observe the mortality rate of the zebra fish embryo and photograph the different stage using light contrast microscope.

***In vitro* Wound Healing Activity**

Take 60-mm culture dish and coat with ECM substrates and incubate the dish overnight for 2 hours at 37ºC without shaking. Then remove unbound ECM substrate and coat with 3 ml of bovine serum albumin at 37ºC for 1 hour. Wash it with PBS and add media into the dish before plating the cells. Resuspend the subconfluent growing cells by washing with PBS twice in culture dish. Add trypsin and mix the cell with serum containing medium. Disperse the cell equally by pipetting the solution and rock the dish gently. Using hemocytometer, cell counts of aliquot from cell suspension are determined. Add cells to 60mm culture dish to form confluent monolayer. Incubate them at 37ºC for 6 hours to adhere to substrate and develop into monolayer.

***In vitro* Scratch Assay**

Scrape monolayer of the cell horizontally using p200 pipette tip and remove the debris by washing with PBS. Wash the cell again with 1ml of growth medium and replace it with 5 ml of medium. Create a reference mark on the culture plate by using a razor blade or with fine tipped marker. Then the cells were treated with extract at 25µg/ml and 50µg/ml. cells without treatment are considered as control whereas positive control is cell treated with EGF (2ng/ml). Place the culture dish on the phase contrast microscope and record the first image of scratch at 0 hour. Keep the culture dish in CO2 incubator, observe them at the interval of 24h and 48 hr using the microscope and record the second and third set of Image. Migration rate of the cells are analysed by using the software "ImageJ" (Liang *et al*, 2007).

Formulation of Herbal Transdermal Patch

Formulation of wound healing transdermal patch with tubers extract of *Momordica Cymbalaria* is prepared by solvent evaporation techniques using bangles. It is performed by wrapping the aluminium foil around the bottom of the bangles in which the polymeric solution is poured. The polymeric solutions are prepared by dissolving required amount of polymer in distilled water and the solutions were keep in boiling water bath to get a clear polymeric solution. Then plant extract (tuber extract of *Momordica cymbalaria*, leaves extract of *senna auriculata*), few drops of clove oil and

DMSO were added into the polymeric solution. Mix them well using stirrer and tween 80 was added. The prepared solution was poured into the aluminum foil and keeps it for dry at room temperature. After 24 hours, the patch was peeled out from the foil and stores them for further evaluation needs in desiccator.

Table 1: Optimization of wound healing transdermal patch composition

Ingredients	Formulation Code		
	TP1	TP2	TP3
Hexane extract of *M. cymbalaria* tuber(mg)	25	25	25
Hexane extract of *senna auriculata* leaves(mg)	10	10	10
Clove oil	2 drops	2 drops	2 drops
Pectin (mg)	100	150	300
DMSO (ml)	2.5	2.5	2.5
Tween 80 (ml)	0.5	0.5	0.5
Distilled water (ml)	6	6	6

Evaluation of Herbal Drug Loaded Patch

1. Organoleptic Characteristics

The physical appearance of the patch was analysed by using naked eye for its appearance, colour, clarity, flexibility and smoothness (Tripathi *et al*, 2017).

2. Physico-Chemical Evaluation

➢ **Thickness of Patch**

The thickness of prepared herbal drug loaded patch was measured by taking measurement at different point of patch by using Vernier calliper. Take 3 patches from same formulation and calculate the Average mean value that determines the thickness of the patch (Reddy *et al*, 2021).

➢ **Weight Uniformity**

Take 10 randomly prepared patches from same batch and dry them at 60ºC for 4 hours. Then the patch is weighed in digital balance and calculates the average weight of the patch (Reddy *et al*, 2021).

➢ **Determination of Surface P^H**

The p^H of the patch is determined by exposing the patch to swell with 1 ml of distilled water for 2 hours at room temperature. Keep the P^H electrode at the surface of patch to note the p^H value and allow it to equilibrate for 1 minute (Reddy *et al*, 2021).

➢ **Folding Endurance**

Evaluation of folding endurance involves determining the folding capacity of the patch. A specific area of strip is cutted and repeatedly folded at the same place till it broke. The value of folding endurance of the patch is measured by counting the number of folds in film at same place without breaking (Das *et al*, 2021).

➢ **Moisture Content**

Prepared patch to be weighed initially and kept in desiccator containing calcium chloride for 24 hours at room temperature. After 24 hours, the patch was taken out and reweighed as final weight. The percentage of moisture content are determined by using the formula,

Moisture content (%) = [Initial weight – Final weight / Final weight] × 100

➢ **Moisture Uptake**

Prepared patches to be weighed initially and kept in desiccator containing Aluminium chloride for 24 hours at Room temperature. Then take out the patch and reweighed as final weight. The percentage of moisture uptake is determined by the formula (Reddy *et al*, 2021).

Moisture uptake (%) = [final weight- initial weight / Initial weight] × 100

➢ **Drug Content**

Dissolve the prepared patch of size 2×2 cm in 100 ml of distilled water using magnetic stirrer for 5 hours to estimate amount of drug present in a patch. Filter the solution using filter paper and analyse the drug content with proper dilution using spectrophotometer at 382 nm.

RESULTS

Antioxidant Activity of Hexane Extract of *Momordica cymbalaria* Tubers

The plant extract was allowed to react with violet colored DPPH solution which was reduced to yellow color product, diphenyl picryl hydrazine. The IC50 value of tubers extract of *Momordica cymbalaria* mentioned in the Table 2. Therefore, the result indicates that crude extract of *Momordica cymbalaria* tuber has high radical scavenging activity and graphically represented in the Figure 3.

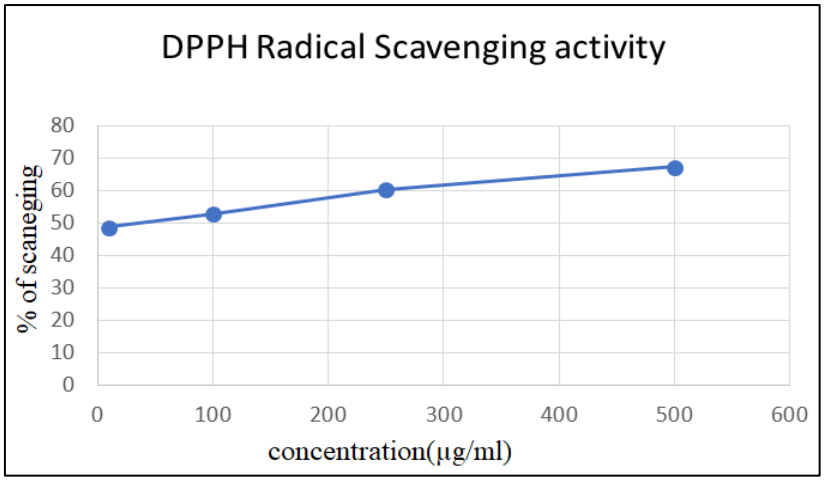

Figure 3: DPPH radical scavenging activity of tuber extract

Table 2: percentage of inhibition and IC 50 value for anti-oxidant activity

Plant name	Concentration	OD value	% of Inhibition	IC50 value
Momordica Cymbalaria tuber extract *(Hexane)*	10	0.174	48.6	69.51
	100	0.160	52.8	
	250	0.135	60.1	
	500	0.111	67.2	

Cytotoxicity of Tuber Extract Using Brine Shrimp Lethality Assay

Four different concentration of tuber extract of *Momordica cymbalaria* were exposed to this assay. The graph was plotted for calculating LC50 value of the tuber extract and represented in Table 3.

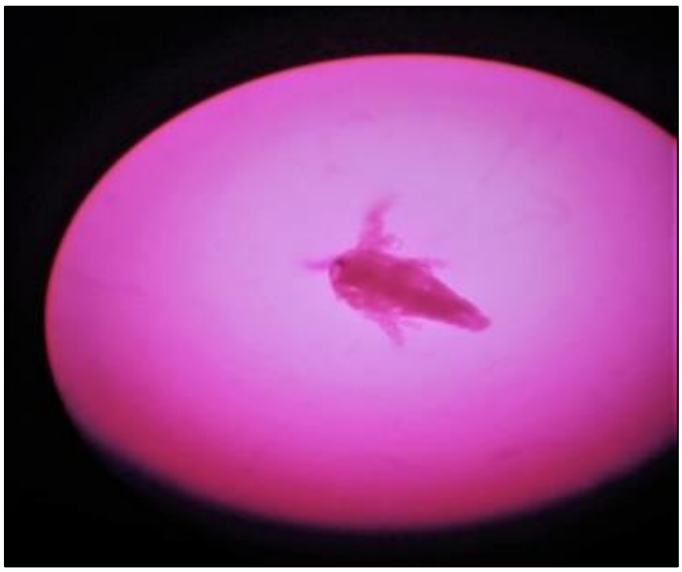

Figure 4: Image of Artemia salina which is exposed in crude plant extract and viewed in light microscope at 10x magnification after 24 hours of incubation

Table 3: Lethality percentage and LC50 value for tuber extract of *Momordica cymbalaria(Artemia salina)*

Plant name	Concentration	No. of. survival	No. of. death	% of Lethality	LC50
Momordica cymbalaria Tuber extract	25	7	3	12.5	133.34
	50	7	3	12.5	
	100	5	5	37.5	
	250	3	7	62.5	

Cytotoxicity Testing On Zebrafish Embryo

Zebra fish embryo was analysed with different concentration of *Momordica cymbalaria* tuber extract for its developmental toxicity. The survivability rate of the fish was regularly monitored by using microscope. The percentage of lethality is mentioned in the T able 4 and microscopic image of the survived zebra fish are showed in Figure 5.

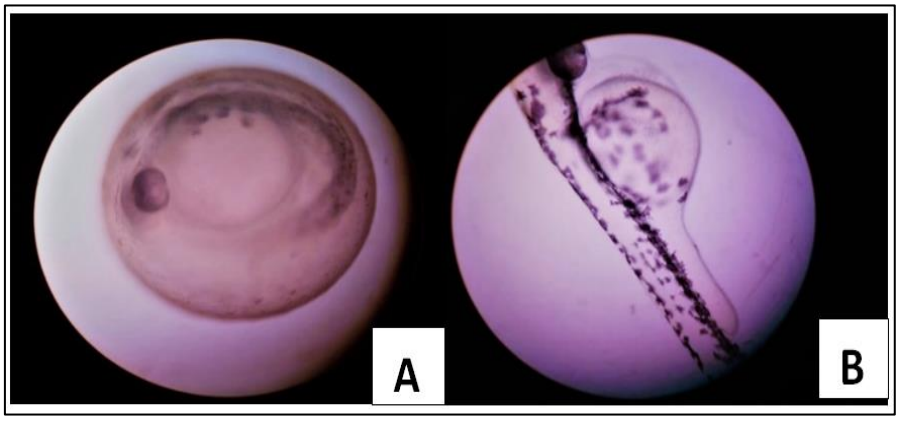

Figure 5.a: Zebrafish embryo at 0 hour before exposing into plant sample under microscope at 10x b) image of zebrafish after incubating in plant sample for 24 hours at 10x magnification

Table 4: **Lethality concentration of tuber extract of *Momordica cymbalaria(zebra fish embryo)***

Plant name	Concentration	No. of survival	No. of Death	% of Lethality
Momordica cymbalaria Tuber extract	25	9	1	10
	50	9	1	10
	100	6	4	40
	250	2	8	80
	Control	10	0	0

***In vitro* Wound Healing Assay**

To determine the wound healing potential of tuber extract of *M. cym*balaria, scratch assay was performed in Human Epidermal Keratinocytes with the concentration 25µg/ml and 50µg/ml of tuber extract against the control and EGF (2ng/ml). Migration rate of wound healing represented in Figure 6 and Table 5.

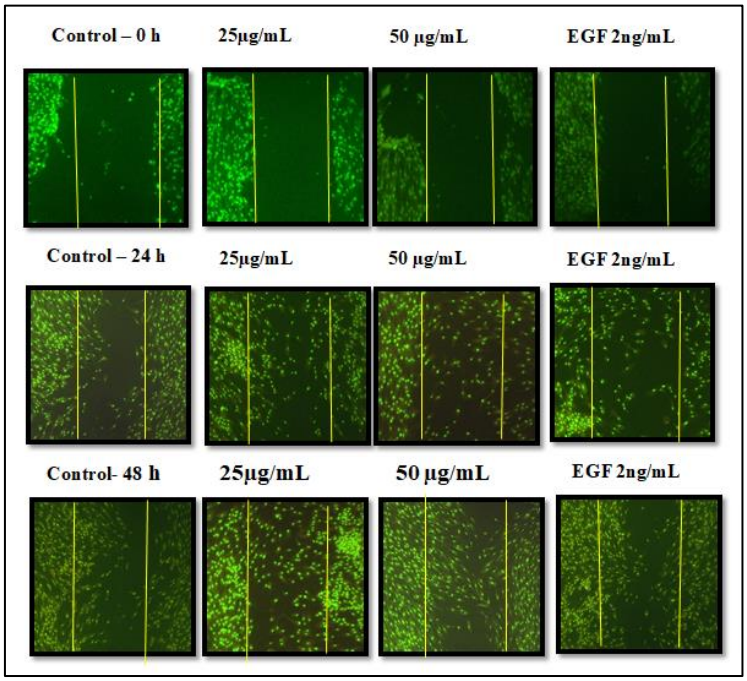

Figure 6: Analysis of cell migration by *In vitro* scratch assay

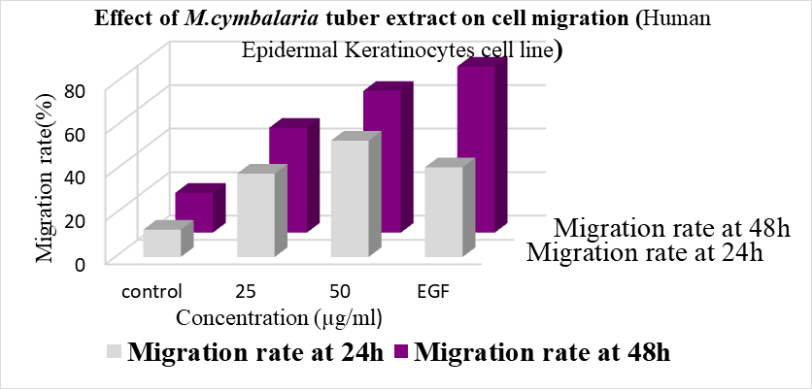

Figure: 7 Effect of *M. cymbalaria* tuber extract on cell migration rate

Table 5: Percentage of Migration rate in Human Epidermal Keratinocytes using tuber extract of *M. cymbalaria*

Sample	Concentration (µg/ml)	Migration rate (%)	
		24 hours	48 hours
Control	-	12.50	18.30
Tuber extract	25	38.28	48.18
Tuber extract	50	53.47	65.07
EGF	2	41.21	76.27

Formulation of herbal wound healing transdermal patch

The optimized patch composition is mentioned in Table 6 and the image of the patch is showed in Figure 8.

Table 6: Optimized formulation (TP2) for wound healing Transdermal patch

S.NO	Ingredients	Amount
1	Hexane extract of *M. cymbalaria* tuber(mg)	25
2	Hexane extract of *senna auriculata* leaves(mg)	10
3	Clove oil	drops
4	Pectin (mg)	150
5	DMSO (ml)	2.5
6	Tween 80 (ml)	0.5
7	Distilled water (ml)	6

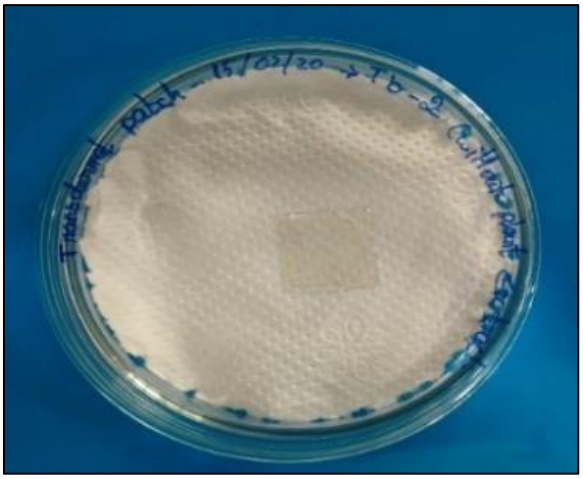

Figure 8: Prepared transdermal patch

Evaluation of Herbal Wound Healing Transdermal Patch

The physical appearance of the patch was analysed by using naked eye for its appearance, colour, clarity, flexibility and smoothness and image are attached in Figure 9. Obtained result was tabulated in Table 7.

The result for physico-chemical properties of the prepared patch were tabulated in the Table 8.

Figur 9: Result for organoleptic characteristics

Table 7: Evaluation of patch for organoleptic characteristics

S. No	Physical Appearance	Result
1	Appearance	Jellified preparation
2	Color	Golden Brown
3	Clarity	Translucent
4	Flexibility	Yes
5	Smoothness	Good

Table 8: Evaluation of patch for physico chemical parameters

S. No	Parameters	Result
1	P^H	7±0.2
2	Thickness(mm)	0.4
3	Weight uniformity	0.13
4	Folding endurance	111
5	Moisture content	63.96
6	Moisture uptake	62
7	Percentage of Elongation	50
8	Drug content	77.83

DISCUSSION

In this finding, Momordica cymbalaria are explored to treat diabetic wounds through transdermal delivery route. To begin the process, Antioxidant assay were performed to analyse the sample. The plant extract was allowed to react with violet colored DPPH solution which was reduced to yellow color product, diphenyl picryl hydrazine. Conversion rate indicates the presence of antioxidant potential in the plant sample and the reduction reaction in plant sample occurs in the concentration dependent manner. The plant extract was reduced into yellow color and activity were analysed using UV spectrophotometer at 517 nm. DPPH radical scavenging activity for Tuber extract of *Momordica cymbalaria* showed high inhibitory concentration. The IC_{50} value of *M.cymbalaria* tuber extract was found to be 69.51µg/ml which is compared against the standard, gallic acid. The amount of decolourization takes place in the plant sample indicates the percentage of scavenging activity and is observed with UV spectrophotometer. As a result, *M.cymbalaria* has strong antioxidant activity. Here, the preliminary cytotoxicity of the plant extract was evaluated for further toxicity check by using brine shrimp lethality assay and fish embryo toxicity assay on Zebrafish to set the optimum concentration usage for living cells. Brine shrimp lethality assay was recommended by Michael *et al.*, Brine shrimp lethality assay were used as preliminary assessment of cytotoxic content in the plant extract. Artemia salina is very convenient test organism for studying toxicity. Four different concentration of tuber extract of *Momordica cymbalaria* were exposed to this assay and two concentration (25 and 50µg/ml) showed a week cytotoxicity whereas 100µg/ml exhibit moderate and 250µg/ml showed high toxicity towards the brine shrimp larvae (nauplii). Zebra fish embryo was analysed with different concentration of *Momordica cymbalaria* tuber extract for its developmental toxicity. The survivability rate of the fish was regularly monitored by using microscope. It is largely used as a toxicology model organism for in vivo assays because it is available at low cost, easy to maintain and are easy to check toxicity studies. A successful hatching was occurred at 3 concentration (25, 50, 100µg/ml) which indicates that the crude extract is not toxic. 25µg/ml concentration is suggested to optimize the patch which indicates that the consumption of these medicinal plants doesn't cause toxic to cells.

To optimize the dose, M. cymbalaria tuber extract was treated with Human Epidermal Keratinocytes cell line to identify the potential activity in healing the diabetic wounds. Wound closure is takes place by the mechanisms, proliferation and migration of cells in the wounded area. So, migration rate of cell indicates the presence of bio active compounds that possess wound healing. Two concentrations of plant extract (25 and 50µg/ml)were treated HPK cell lines for 24 hr and 48 hr, 50 µg/ml concentration showed positive result with highest migration rate 65.07% upto 48 hours compared to 25 µg/ml concentration and control. Three different compositions of transdermal patches were prepared by solvent casting technique with tuber extract of *M.cymbalaria* to optimize the patch. Among three, one composition (TP2) showed thin, smooth and good elasticity, this composition was used for formulating the herbal wound healing transdermal patch. The patch was prepared with the natural polymer, pectin by dissolving in distilled water. Pectin helps in controlled release of drug compared to synthetic polymers. To that add plant extracts, *M.cymbalaria tuber* extract to help in wound healing process, *Senna auriculata* which helps to avoid microbial contamination in the wounded area and clove oil was added with 2 drops which increase the stability of the patch. DMSO added in the formulation that helps in drug permeation and tween 80 were used in transdermal patch to avoid dryness and moisturize patch. The prepared patch was evaluated for organoleptic characteristic features and some physico-chemical properties like thickness, weight uniformity, drug content, moisture uptake, moisture content, elongation percentage and folding endurance which are important parameters for basic transdermal patch. The observed organoleptic characters of the prepared patch were Golden brown in color with jellified preparation, translucent in nature with good smoothness and flexibility. Whereas for physico-chemical parameters, the thickness of the patch is 0.4mm with 0.13 mg weight and ph. is 7. Moisture uptake and moisture content of the patch will be 62 and 63.96% and also folding endurance will be 111 and percentage of elongation is 50%. The drug content of the patch is found to be 77.83µg/ml.

CONCLUSION

As a final observation, it is concluded that *Momordica cymbalaria* tuber had potent pharmacological activity and showed potent antioxidant activity against gallic acid. *Momordica cymbalaria* had high hypoglycaemic and anti- diabetic activity that helps in treating the diabetic wounds. Wound healing potential of tuber extract of Momordica cymbalaria exhibits greater response in wound healing and further studies like GC-MS can be performed to analyse the presence of particular wound healing associated phytocompounds like n-Hexa decanoic acid. Using of analytical methods, the phytochemical can be isolated, purified and characterize the structure of isolated compound using NMR spectroscopy. The prepared patch showed reliable physical appearance and extra study like diffusion studies and animal studies can be carried out to assess the release of drug content in the prepared patch. Also, instead of using plant extract, the phytocompounds which is responsible for wound healing can also use to incorporate in the transdermal patch which helps to treat the diabetic wound in effective manner with lesser side-effects. This finding leads up to develop an alternative eco-friendly , economical, herbal drug for wound healing from natural source which could substitute the commercial synthetic drug-based patches provided .The preclinical studies in mice models are performed as a future scope of this work .

Declaration of Competing Interest

All the authors declare that they have no conflict of interest

Ethical statement

In this study there is no animal has been used.

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