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Research Article

Extraction, Phytochemical Screening and Wound Healing Activity of Herbal Formulation of *Saussurea lappa*

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Abstract: The aim of present research work was to develop emulgel and *In-situ* gels of methanolic extract of *Saussurea lappa* with the purpose to determine their wound healing and anti-bacterial properties. Phyto-chemical analysis of extract was also performed. Emulgel was prepared by using carbopol 940, Span 80, tween 80, polyethylene glycol (PEG 4000) and methyl paraben while in-situ gel was prepared by using polaxomer (P407) as thermo-sensitive and carbopol (934P) as pH sensitive polymers. All formulations were maintained at pH 6-7 and stored at 4 ◦C. Lyophilized extract was added in solution form to enhance the solubility as well as the stability. In-vitro release profile was performed by using Franz diffusion method and data was plotted in different pharmacokinetic models like first order, higuchi and hixon-crowell models. All formulations followed first order release mechanism. Hemolytic activity of extract was performed at concentration of 100µg/ml through heat induced hemolysis of erythrocyte membrane model system while anti-bacterial activity was determined by using agar well diffusion method. Acute toxicity assay of crude extract showed that 1000 mg/kg was safe dose with no toxic symptoms. Excision wounds were induced and wound healing potential of all formulations was determined. Results were compared and expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA) with $p<0.05$.

Keywords: *Saussurea lappa,* Wound healing, Emulgel, *In-situ gel,* Herbal formulation

1. INTRODUCTION

Natural products are very diverse and almost 35,000 - 70,000 plants species have been screened till date. Ethno pharmacological use of crude drugs provided a major clue in drug discovery. Data suggested that more than 50% of medicines used during last 30 years were of natural origin [1]. *Saussurea lappa*, commonly known as costus or kuth occur in South East Asia and Pakistan having 400 species with long and rich use in local traditional herbal products to treat internal heat or fever, menstruation, wound healing purposes, unbalanced blood circulation, unwanted bleeding, body pain and rheumatic arthritis [2]. *S. lappa* contains variety of phytochemicals like sesquiterpene lactones that have anti-inflammatory and wound healing potential. Gastric ulcers are inhibited by costunolide and Saussure amines while cyanopicrin is potent immunosuppressive agent [3]. Herbal formulations are becoming popular as they are considered safe due to their natural origin. Herbal medicines are used in variety of health ailments like liver problems, diabetes and heart problems etc.

Wound healing is a normal physiological process that involves hemostasis, inflammation, proliferation, and remodeling which highly programmed phases. All these phases are necessary for proper wound healing in a proper sequence and time frame [4]. Topical drug delivery system is becoming popular but conventional topical drug delivery systems like ointments and creams have a drawback of low bioavailability and poor retention [5]. Emulgels are the recent formulations in novel drug delivery systems that are combination of emulsion and gel. When applied topically they provide dual control release in the form emulsion

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as well as gel [6].

In-situ gelling systems involve use of polymers that have phase transition from solution to gel upon alterations in physico-chemical properties of drug [7]. In-situ gels effectively overcome the drawbacks of conventional topical dosage forms and drug is released in controlled manner because they have better stability and release profile upon gelation [8].

The purpose of present study was to formulate and evaluate anti-microbial and wound healing activity of herbal formulations of *S. lappa* for the cost effective treatment. Results were compared with standard formulations. The study was divided into three parts. First part the extraction and phytochemical evaluation of crude extract of *S. lappa*, Second part the designing of dosage forms i.e Emulgel and *In-situ* gels along with their post formulations studies to determine the *in-vitro* release profile, stability, sterility and other physico chemical properties and the third part acute toxicity study of crude extract in mice, wound healing activity of formulations in rats and anti-bacterial activity by agar well diffusion method.

2. MATERIALS AND METHODS

2.1 Plant collection and extraction

The roots of *S. lappa* were collected from the wild cultures growing in and around Swat in April, 2018. Roots were washed thoroughly with water and shade dried. Fine powder (750g) of dried roots was prepared by using pestle and mortar. Powder was macerated with 3 liters of methanol for 14 days and extract was dried by using rotary evaporator. Chemicals were purchased from Sigma Co., Aldrich, Merck of Germany and BDH Lab Supplies of England.

2.2 Thin Layer Chromatography

Analytical thin layer chromatography (TLC) was performed for the detection of sesquiterpene lactones. Analytical TLC plates (TLC Silica gel 60 F254 20x20 cm Merck KGaA, Darmstadt, Germany) were spotted with 5-10 µg of each extract and placed in glass chamber containing chloroform and methanol (9:1). After development all the plates were dried and placed in glass chamber containing Iodine. After complete sublimation of iodine, plates were examined.

2.3 Phytochemical Screening of Extract

The root extracts of *S. lappa* were analyzed for the presence of alkaloids, terpenoids, carbohydrates, proteins, Flavonoid, glycosides, phenolic compounds, saponins and tannins as described [9].

2.4 Hemolytic Activity of Crude Extract

The blood was collected from healthy human volunteer and centrifuged at 3000 rpm for 10 min which was then washed three times with equal volume of normal saline. The volume of blood was measured and re-constituted as 10% v/v suspension with normal saline. The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 µg/ml) and 1 ml of 10% RBCs suspension. Aspirin was used as a standard drug and normal saline was used as control. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The percentage inhibition of hemolysis was calculated as:

$$\text{Percentage inhibition} = (Abs_{control} - Abs_{sample}) \times 100 / Abs_{control} \quad [10]$$

2.5 Animal Studies

Male Balb-C mice (20-30 gm) and Sprague-Dawley rats (180-220 gm) of local breed were housed in at the Animal House of Riphah Institute of Pharmaceutical Sciences, Islamabad, in plastic cages (47x34x18) cm^3 at 23-25 ^{0}C. All the animals were maintained under standard husbandry environment with food and water *ad libitum*. Experiments were performed and compiled following rules of institute of Laboratory Animals Resources, Commission on Life Science University, National Research Council (1996) and approved by Research Ethical Committee of Riphah Institute of Pharmaceutical Sciences (Ref. No: REC/RIPS/2017/00).

2.6 Acute Toxicity Study

Twelve Male Balb-C mice (20-30g) were divided into two groups (n=6). Experimental group was

orally treated with 1000 mg/Kg dose of methanolic extract dissolved in normal saline while control group was administered with normal saline (10ml/kg). Mice were strictly monitored for 24 hours with free access to food and water *ad libitum* to observe any lethal effects [11].

2.7 Formulation of Extract into Emulgel

For topical application 0.5%, 1%, 2%, 3% ,4% and 5% of emulgel of crude extract was formulated (F1 to F6) as described by [12]. First of all, 30g of carbopol 940 was dissolved in 1000ml of distilled water which have pH 6.2 and pH was adjusted with few drops of NaOH upto 7.1. Mixture was left for overnight. Oil phase was prepared by mixing 1.8ml of span 80 and 10ml liquid paraffin while aqueous phase was prepared by mixing 2ml of tween 80 with 10ml of distilled water separately for each formulation. 0.5g, 1g, 2g, 3g, 4g and 5g of extract was taken and mixed with 10ml PEG, 0.12g methyl paraben and 0.1ml of eucalyptus oil for fragrance. Aqueous phase was added in oil phase followed by mixing extract phase. Stable emulsions were formulated and further formulated into emulgel by mixing with gel made with carbopol 940 (100g for each formulation).

2.8 Formulation of Extract into *In situ* Gel

2.8.1 Lyophilization of Extract

Lyophilization of extract was done for its better reconstitution. 14g of extract was dissolved in 20ml distilled water and was frozen at -40 ^{0}C for 10 hours. Primary drying was done by increasing temperature to -10 ^{0}C at 0.6 ^{0}C/min for 5 hours and further up to 20^{0}C at 0.25 ^{0}C/min with pressure range of 1000 to 5 pascals [13].

2.8.2 Preparation of Gel

15% solution of polaxomer P (407) was prepared and refrigerated for 24 hours. 2% Carbopol 940 was dissolved in phosphate buffer and left for 24 hours to become hydrated. 15% of hydroxy propyl methyl cellulose (HPMC) and poly vinyl alcohol (PVA) were also dissolved in phosphate buffer separately. All the polymers were then mixed and gel was formulated with 3%, 4% and 5% of lyophilized extract. Benzyl alcohol was used as preservative [14].

2.9 Characterization of Formulations

2.9.1 Appearance

Formulations were visually examined for color, texture and grittiness [15].

2.9.2 Viscosity

Viscosity was determined by brookfield viscometer with spindle 61 at various speeds and results were recorded as centipoises (cp).

2.9.3 pH

pH was determined by using pH meter as described by Sultana et al., 2016 [12].

2.9.4 Spreadability

350 mg of each formulation was placed over a glass slide and second slide was dropped from distance of 5cm over the top of first slide. Diameter of the circle was determined [15].

2.9.5 Swelling Index

1g of each formulation was taken on porous aluminum foil and placed in beaker containing 10ml 0.1N NaOH. Samples were removed and dried at various time intervals. After drying, samples were reweighed and % swelling index was calculated by [16].

$$\text{Swelling Index (SW) \%} = [(Wt - Wo) / Wo] \times 100$$

2.9.6 Centrifugal Test

5g of formulations were centrifuged at 3750 rpm for few minutes at room temperature [17].

2.9.7 Accelerated Stability Test

Formulations were stored at 40 ^{0}C and physical parameters like pH and viscosity were determined at every week for the period of 4 weeks [17].

2.10 *In vitro* Permeation Study

Emulgel with 5% extract and *in situ* gel with 5% extract of *S.lappa* were used for *in vitro* permeation studies. Donor compartment of Franz diffusion cell was filled with formulation while the recipient compartment was filled with phosphate buffer (pH 6.0). 0.22 micrometer pore size dialysis membrane was used to separate both compartments. Outer jacket of the cell was filled with water and maintained at 37 ^{0}C with magnetic stirring at 50

rpm. Samples were taken at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hours and UV absorbance was determined at 332nm. For UV analysis, phosphate buffer was used as blank. Sample was prepared after withdrawn from the cell and dilution with 10ml phosphate buffer. 150**µl sample was** collected from the solution and again diluted with 10ml phosphate buffer and UV absorbance was determined [18]. Percentage absorbance was determined by:

UV absorbance %= [(absorbance of sample / absorbance of standard) x 100]

After the UV analysis, results were fitted into various models to determine release rate pattern of formulations.

2.11 Pharmacokinetic Models

Drug diffusion and polymer chain relaxation are two parameters that determine drug release at a particular time. Pharmacokinetic models were developed and data obtained from *in vitro* release was fitted into each model. Various mathematical models were used to correlate drug permeation profile with drug release kinetics [19].

2.11.1 First order model

It establishes relationship between drug release verses time. Integration and rearrangement of equation is as follows:

$\log C_t = \log C_{0-K1t/2.303}$

K_1= first order rate equation expressed in time-1 or per hour,

C_0= initial concentration of the drug,

C_t=remaining percent of drug at time t

2.11.2 Higuchi Model

It is most prominent pharmacokinetic model that involves drug dissolution and diffusion which depends on drug concentration. Simplified form of Higuchi equation is as follows:

$Q = K_H \times t^{1/2}$

Q = Cumulative amount of drug released at time t

K_H = Higuchi release rate constant

$t^{1/2}$= square root of time

2.11.3 Hixson-Crowell Model

It mainly describes the release of drug from the system that involves change in surface area and diameter of drug particles. Simplified relationship between drug release and time is as follows:

$W_0^{1/3} - W_t^{1/3} = K_{Hc}\, t$

$W_0^{1/3}$ = cube root of initial amount of drug present in the matrix.

$W_t^{1/3}$= cube root of remaining amount of drug in matrix at time t.

KHc = release rate constant.

t= time

2.12 Wound Healing Study

36 male albino rats were divided into nine groups (n=4). Animals were closely monitored and infectious rats were excluded from the study. During wound healing study, no other topical or systemic treatment was given. Group I was untreated group while group II was treated with crude methanolic extract and group III received pyodine gel treatment. Group IV, V and VI were treated with 3%, 4% and 5% herbal emulgel. Group VII, VIII and IX were administered with 3%, 4% and 5% herbal *in-situ* gel. All the animals were given free access to food and water ad libitum. All the doses were administered topically. For wound healing study, dorsal skin of each rat was depilated and marked on the back of the rat by a standard ring. Rats were then anesthetized with ketamine (25 mg/kg intraperitoneally and excision wound of 380 mm^2 was induced). Full thickness of the marked skin was cut carefully. Wound was cleaned and kept open. 0.5g of each formulation was applied once daily from the day 1 to day 20 of wounding. 200mg/10ml extract was also applied topically. Wound size was measured after every 4 days for 21 days [20].

% wound contraction = [(initial wound size - specific day wound size) / initial wound size] x 100

2.12.1 In-vitro Antibacterial Activity

Rats were anesthetized with chloroform and excision wound was induced with sterile needles. Sterile cotton was placed over the wounds and exudate was collected. Exudate was cultured by using LB broth media [21]. After 48 hours, bacteria were streaked on LB agar plates. After growth of bacteria they were again streaked on separate agar plate for biochemical analysis like gram staining, coagulase test and catalase test. Zone of inhibitions of formulations and extract were determined by using agar well diffusion method [22].

2.12.2 Statistical Analysis

All values were expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA).

3. RESULTS

TLC of extracts was performed by using cholorform and methanol (9:1). Plates were analyzed by using iodine. 1g of iodine was placed in closed chamber along with plate. Sublimation of iodine left spots on the plates that appeared brown with sespuiterpene lactones. Ethyl acetate and choloform extracts showed yellow spots of flavanoids. Hexane showed no significant spots, while methanolic extract showed brown spots that were our desired sesquiterpene lactones and other terpenoids which have significant *in vivo* activities.

3.1. Phytochemical Screening

Preliminary phytochemical analysis of crude extract was shown to contain alkaloids, glycosides, flavanoids, terpenoids, saponins, tannis, phenols and carbohydrates but no proteins were detected in the extract. Results are shown in table 1.

3.2. Hemolytic activityof crude extract

Methanolic extract of *Saussurea lappa* was shown to be effective in inhibiting heat induced hemolysis at concentration of 100 µg/ml. *S. lappa* efficiently protect the membrane against hemolysis induced by heat . Percentage inhibition of *S. lappa* was 42.8% while aspirin was 51% at 100 µg/ml. Results were reported in the form of graph as shown in figure 1.

3.3. Acute Toxicity Studies

Twelve male Balb-C mice weighed between (20-25g) were divided into groups (n=6) with free access to food and water *ad libitum.* Control group was given normal saline (10mg/kg) while experimental group was given crude extract (1000mg/kg) orally. Weight over fixed dose approach was followed. Extract was dissolved in normal saline. Each mouse weighed between (20-25g). All mice were monitored strictly for 24 hours and symptoms of toxicity were observed. After 24 hours, no toxic symptoms were observed in any of the mice [11].

Table 1. Phytochemical analysis of *S.lappa* extract

No	Phytochemical	Indication	Results
1.	Alkaloids	Reddish brown pecipitates	+
2.	Glycosides	Brown color	+
3.	Saponins	Stable froth for 10 minutes	++
4.	Phenols and tannins	Blue green color	++
5.	Proteins	No change	_
6.	Carbohydrates	Brick red color	+
7.	Flavanoids	Intense yellow color	++
8.	Terpenoids	Gray color	++

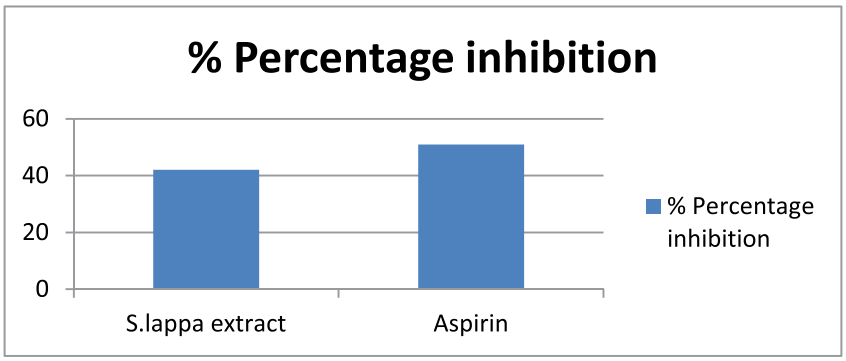

Fig. 1. Hemolytic activity of *S.lappa* extract

Table 2. Formula for emulgel preparation

No	Ingredient	Quantity/ 37g	
1.	Crude extract	**F1**	0.5g
		F2	1g
		F3	2g
		F4	3g
		F5	4g
		F6	5g
2.	Carbopol	3g	
3.	Span 80	1.8ml	
4.	Paraffin oil	10ml	
5.	Tween 80	2ml	
6.	Distilled water	10ml	
7.	Methyl paraben	0.1g	
8.	PEG 4000	10ml	
9.	Eucalyptus oil	0.1ml	

3.4. Formulation of Extract into Emulgel

Six formulations were prepared F1 to F6. Each formulation contained variable amount of extract ranging from 0.5% to 5% but amount of emulsion and other ingredients were not changed significantly [12] . Emulgel was formulated as shown in table2.

3.5. Formulation of Extract into *In situ* Gel

14g extract was dissolved in 20ml distilled water and lyophilized for 24 hours. 2% carbopol 940, 15% hydroxy propyl methyl cellulose (HPMC), 15% of polaxomer P (407) and 15% poly vinyl alcohol (PVA) solutions were prepared in phosphate buffer and left for 24 hours. After 24 hours, all the polymers were mixed and 3%, 4% and 5% (G1 to G3) *in situ* gels were formulated as shown in table 3. [14].

Table 3. Formula for in situ gel preparation

No	Ingredient	Quantity/ 37g	
1.	Crude Lyophillized extract	**G1**	3g
		G2	4g
		G3	5g
2	Carbopol	5g	
3	HPMC	5g	
4	PVA	5g	
5	Polaxomer P (407)	5g	

3.6. Characterization of Emulgels

Formulations F1 to F6 were characterized for appearance, viscosity, pH, spreadability, swelling index, centrifugal test and accelerated stability test. Results are shown in table 4.

3.7. Characterization of *in situ* Gels

Formulations G1 to G3 were characterized for appearance, viscosity, pH, spreadability and results

Table 4. Characterization of emulgel

Formulation	Color	Grittiness	pH	Viscosity (cps)	Centrifugation (Phase separation)	Swelling Index (%)	Spreadability (cm/sec)
F1	Cream	No	6.83	15678	No	56.2	18
F2	Pale yellow-brown	No	6.92	15750	No	69.3	20
F3	cream	No	6.35	15890	No	88.5	16
F4	Pale yellow-brown	No	6.79	15960	No	85.4	16
F5	Pale yellow-brown	No	7.12	16123	No	89.6	18
F6	Pale yellow-brown	No	7.33	16190	No	97.6	20

Table 5. Characterization of emulgel

Formulation	Color	Grittiness	pH	Viscosity (cps)	Spreadability (cm/sec)
G1	Brown	No	6.73	16650	17
G2	Brown	No	6.62	16825	19
G3	Brown	No	6.89	16960	16

are shown in table 5.

3.7.1. In vitro Permeation Study

In vitro permeation study was carried out by using franz diffusion cell of diamter 20 mm. The recipient compartment was filled with phosphate buffer surrounded by water jacket to maintain temperature at 37^0C and stirred at 50 rpm. A 0.22 µm pore size dialysis membrane was used to separate the donor and recipient compartment. Donor compartments were fillled with 5% emulgels and 5% *in-situ* gels. 150 µL of samples were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hours, diluted with phosphate buffer and absorbance was checked at 332 nm using phosphate buffer as blank. After each collection, the compartment was filled with equal volume of phosphate buffer [18]. Cumulative release of formulations over time was determined by fitting the data into various models.

3.8. Models

Various mathematical models were used to correlate drug permeation profile with drug release kinetics [19]. The results showed that formulation released drug constantly over the period of time and better realease rate was observed with *in-situ* gels as compared to emulgels.

3.8.1. First Order Model

It establishes relationship between drug release verses time. For both emulgel and *in-situ* gel formulations log cumulative percentage release was plotted against time as shown in figure 2.

3.8.2. Higuchi Model

It is most prominent pharmacokinetic model that involves drug dissolution and diffusion which depends on drug concentration. For both emulgel and *in-situ* gel formulations, cumulative percentage

Fig. 2. First order release pattern of emulgel and *in situ* gel containing 5% *S.lappa* extract

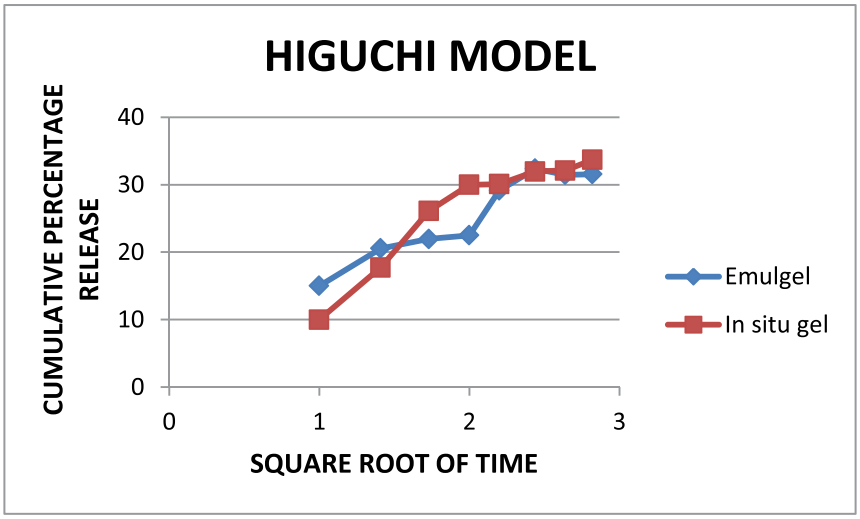

Fig. 3. Higuchi model for release pattern of emulgel and *in stu* gel containing 5% *S.lappa* extract

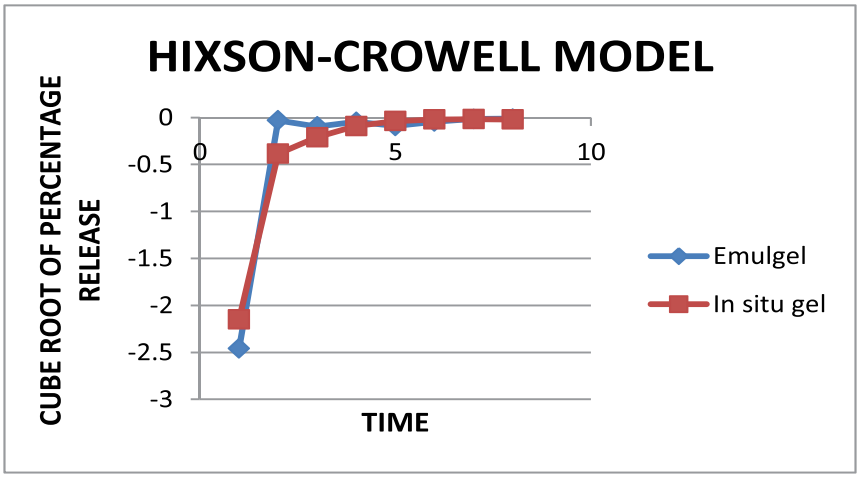

Fig. 4. Hixson-Crowell model for release pattern of emulgel and *in situ* gel containing 5% *S.lappa* extract

release was plotted against square root of time as shown in figure 3.

3.8.3. Hixson-crowell Model

It mainly describes the release of drug from the system that involves change in surface area and diameter of drug particles. For both emulgel and *in-situ* gel formulations, cube root of percentage released was plotted against time as shown in figure 4.

3.9. Wound Healing Activity

15 mm wounds were induced in 36 male albino rats divided into 9 groups (n=4). Group I was untreated group while group II was treated with crude methanolic extract and group III received pyodine gel treatment. Group IV, V and VI were treated with 3%, 4% and 5% herbal emulgel. Group VII, VIII and IX were administered with 3%, 4% and 5% herbal *in-situ* gel. All the animals were given free access to food and water ad libitum. Percentage wound contraction was detrmined by:

% wound contraction= [(initial wound size-specific day wound size) / initial wound size] x 100

All treatments were given for 20 days and wound sizes were determined after every 4 days. All treatments showed significant reduction in wound size as compared to control group. *In-situ* formulations showed faster and better wound healing potential as compared to emulgel formulations which were shown better than marketed formulation and extract. *In-situ* formulations also showed better permeation as compared to others. *In-situ* gel (G3) containing 5% extract of *S. lappa* was better than 3% (G1) and 4% (G2) formulation. 5% (F6) emulgel was also found effective but not as much better than *in-situ*. It was also shown that as the concentration of extracts in formulations increased wound healing activity was also increased. Percentage wound contraction was increased. All values were expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA). Results are shown in table 6. Percentage wound contraction for all formulations is shown in figure 5 while for optimized formulation G3 and F6 these are shown in figure 6. Figures 7 through 9 highlight the wound healing images of rats treated with (a) herbal emulgels of *S. lappa* extract, (b) herbal *in situ* gels of *S. lappa* extract, and (c) pyodine gel, *S. lappa* extract and a control

Table 6. Wound healing activity assessment of herbal formulations of S.lappa

Formulation	Percentage wound contraction				
	Day 4	Day 8	Day 12	Day 16	Day 20
3% Emulgel	18 ± 0.829	42 ± 0.43	60 ± 0.707	85 ± 1.47	95 ± 0.43
4% Emulgel	22 ± 0.829	38 ± 0.829	63 ± 0.866	88 ± 1.08	98 ± 0.433
5%/ Emulgel	25 ± 0.829	47 ± 0.707	72 ± 0.829	93 ± 0.707	98 ± 0.433
3% In situ gel	27 ± 0.5	51 ± 0.829	73 ± 0.829	94 ± 0.707	100 ± 0
4% In situ gel	25 ± 0.43	53 ± 0.707	74 ± 0.707	95 ± 0.43	100 ± 0
5% In situ gel	30 ± 0.5	50 ± 0.54	87 ± 0.707	100 ± 0	100 ± 0
Pyodine Gel	13 ± 0.707	30 ± 0.5	57 ± 1.11	75 ± 0.829	91 ± 0.829
Extract	18 ± 0.82	40 ± 0.707	60 ± 0.707	81 ± 0.707	91 ± 0.43
Control group	12 ± 0.43	22 ± 0.43	37 ± 0.5	62 ± 1.08	82 ± 0.43

Values are mean ± SEM (n=4) P<0.05

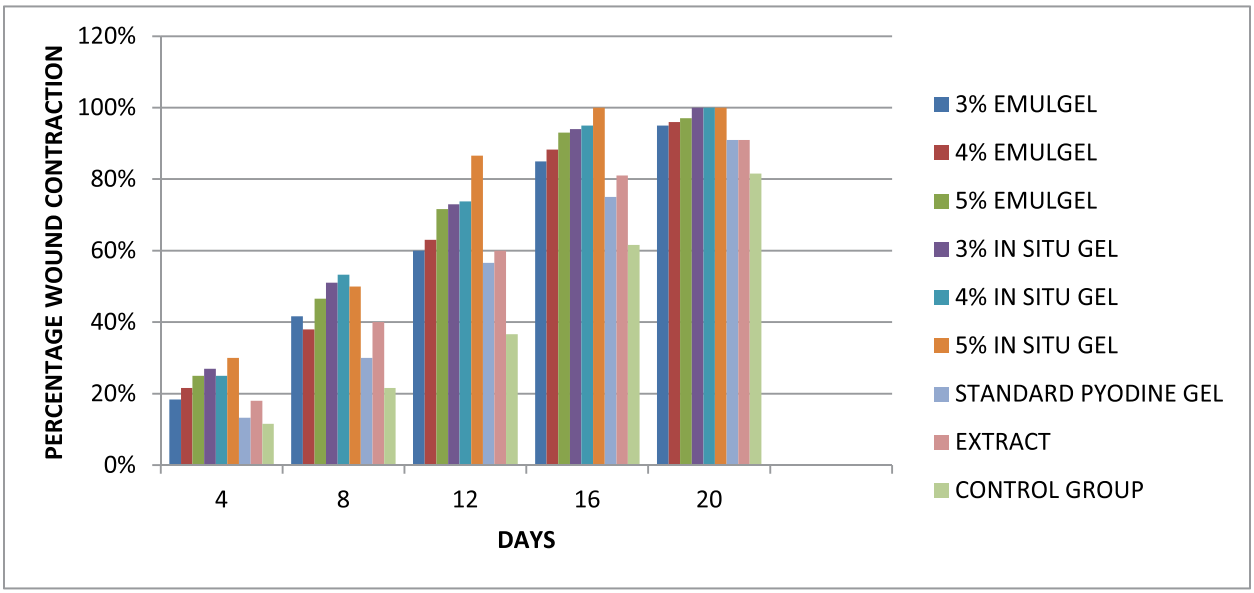

Fig. 5. Wound healing activity assessment for all formulations

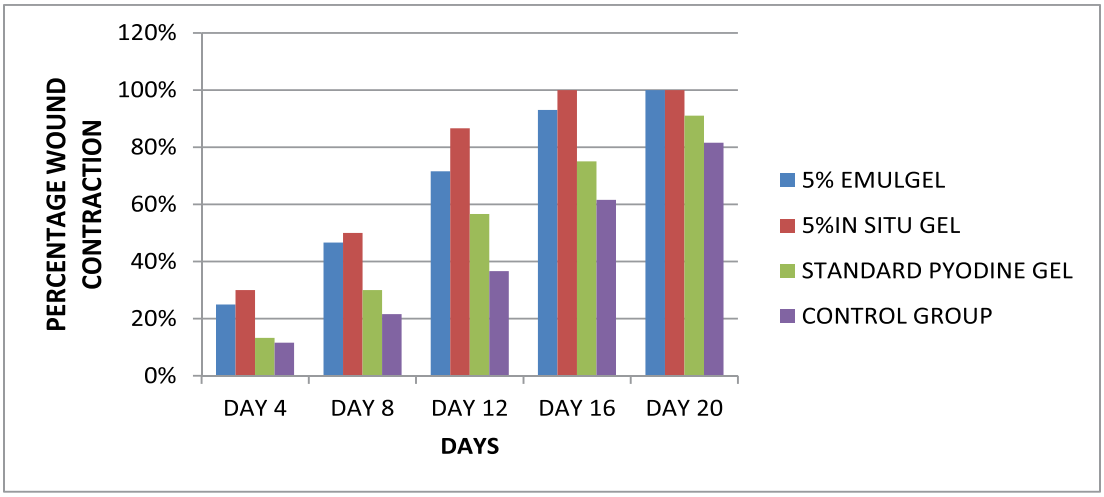

Fig. 6. Wound healing activity assessment of optimized G3 and F6 formulation

Fig. 7. Wound healing images of rats treated with herbal emulgels of *S. lappa* extract

Fig. 8. Wound healing images of rats treated with herbal *in situ* gels of *S. lappa* extract

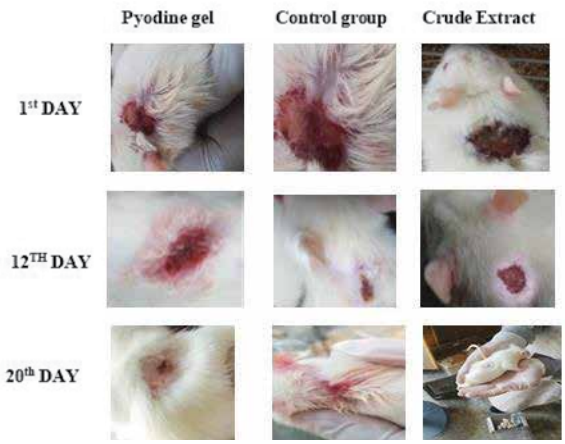

Fig. 9. Wound healing images of rats treated with pyodine gel, *S.lappa* extract and a control group

group, respectively.

3.9.1. In-vitro Antibacterial Activity

Rats were anesthetized with chloroform and excision wound was induced with sterile needles. Sterile cotton was placed over the wounds and exudate was collected. Exudate was cultured by using LB broth media [21]. After 24-48 hours bacteria were streaked on LB agar plates. After growth of bacteria they were again streaked on separate agar plate for biochemical analysis like gram staining, coagulase test and catalase test. Identification tests for bacteria are shown in table 7. *Staphylococcus aureus* was detected in pus culture.After identification *in vitro* anti-bacterial activity of formulations F3, F4, F5, G1, G2, G3, and crude extract was performed by using agar well diffusion method. Sulfazalazine was used as standardand different zones of inhibitons were determined which is reported in table 8. Results showed that sulfasalazine was having zone of inhibiton 31.3 mm, while extract was having 32.2 mm as compared to formulations and 33.1 mm in case of 5% formulation.

Table 7. Biochemical tests for wounded bacterial culture for identification of bacteria

Biochemical test	Observation
Coagulase test with plasma	Positive
Catalase test with hydrogen peroxide	Positive
Gram staining	Gram positive
Shape	*Cocci*
Type of microorganism	*Staphylococcus aureus*

Table 8. Zones of inhibitions of different formulations of *S. lappa* against standard

Formulation	Zone of inhibition (mm)
3% Emulgel	22.3 ± 4.5
4% Emulgel	19.6 ±5.85
5%/ Emulgel	25 ±3.15
3% In situ gel	*22.5 ±4.4*
4% In situ gel	*25 ±3.15*
5% In situ gel	*33.3 ±1.65*
Sulfasalazine cream	*31.3 ±0.55*
Extract	*32.2 ±0.55*

Values are mean ± SEM P<0.05

3.11. Statistical Analysis

All values were expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA). The P value was found to be < 0.05 which showed that results were statistically significant.

4. DISCUSSION

Saussurea lappa contains multiple chemical constituents that are responsible for anti-bacterial and wound healing actvities. Phytochemical screening was carried out to evaluate the presence of these particular chemical constituents. *S. lappa* is shown to contain alkaloids, glycosides, flavonoids,

terpenoids, carbohydrates, saponins and taninns but no proteins have been detected [9]. In crude form, it is difficult to estimate which chemical is responsible for these particualr activities. So for, better estimation of these multiple phytochemicals subsequent fractionation of crude extract is better option.

In previous study it was found that 6 g extract of *S. lappa* when dissolved in 100 mL of water and applied topically to the rats significantly reduced the wound size [23]. In the light of this previous study we have tried to develop two topical dosage forms i.e. emulgels and *in-situ* gels in our present study. For further pharmacological studies acute toxicity studies also give estimation of dose that is considered to be safe. In mice, 1000 mg/kg dose of crude extract was administered and mice were strictly monitored for 24 hours to check if any death occurred. After 24 hours it was observed that no death or any toxic symptom occurred in any mice. These results revealed that no physiological or behavioral differences between any control group and treated group were observed [11]. So, the present study is a postive indication of lower toxicity profile of *S. lappa.*

Bioactivity of crude extract was assessed by *in-vitro* anti-hemolytic activity of crude extract at concentration of 100µg/ml through heat induced hemolysis of erythrocyte membrane model system. Percentage inhibition of *S. lappa* was 42.8% while aspirin was 51% at 100 µg/ml [10]. Although, *S. lappa* has little anti-inflammatory activity as comapred to aspirin but it can be a better option to use it as anti-inflammatroy agent as long term use of aspirin has a major side effect of gastric ulcer. Therefore, further work should be carried out to compare its effectiveness with other NSAIDS like declofenac sodium and naproxen etc. that can be beneficial for patients suffering from arithritis.

Conventional topical dosage forms like ointments and creams have low bioavailability and poor retention. After careful selection of drug carrier we have been able to design both formulations that have better bioavailability and improved retention [24]. Emulgels have better controlled release as well as increase drug loading capacity. When appliead topically, they also provide dual control release in the form of gel and emulsion [6]. Six formulations F1 to F6 were prepared with carbapol 940 which was found to be compatible with ingredients. Carbapol 940 is a cross-linked polyacrylate water soluble biodegradable polymer that provide controlled release and increased stability to our formulations. All the formualtions contained same amount of excepients and polymer but varying amount of crude extract. *pH* of all formulations was ranging from 6.3 - 7.3 that was also similar to physiologic *pH* of skin. All formualtions were found to be stable after stability testing of four weeks.

Keeping in view above mentioned properties of emulgels and crude extract another effort was carried out in present study to make thermo senisitive *in-situ* gel forming biodegradable topical system using carbopol 940, HPMC K 15 and polaxomer P407 that have better *in-vitro* release properties than emulgels. Polaxomer shows gelation at 37 degrees celsius and it also inhibit the effect of efflux pumps that cause drug to stay into the cells for a longer period of time [25]. *In-situ* gelling systems involves use of polymers that have phase transition from sol to gel upon alterations in physico-chemical properties of drug [7]. While designing emulgels and in situ gels factors like *pH* and viscosity should be kept in mind because these parameters should define the release pattern of formulations. Increasing temperature from 25 °C to 37 °C did not cause any significant increase in viscosity. However, too much increase in viscosity than required in case of *in-situ* gels will lead to delay release of formulation [26].

In the present investigations, *in-vitro* drug release study was performed by using Franz diffusion cell that is being used over centuries for diffuion study of semisolid preparations like gels and ointments etc. Formualtions F6 of emulgel and G3 of *in-situ* gel were used to determine drug release pattern. Dialysis membrane of 0.22 micrometre pore size was used for the determination of *in-vitro* release pattern and results showed that both formualtions followed first order release over the period of 8 hours. But in situ gel was having better and improved release pattern than emulgel [18].

Lyophillization of crude extract was done for *in -situ* gels formulation to minimize the moisture content and better stability of final products [13]. Lyophillization also helps in easy reconstitution of

freeze dried product with minimum contamination.

Wound healing activity was assessed by applying each formulation locally against excision model of rats. All treatments were given for 20 days and wound sizes were determined after every 4 days. Results showed that there was significant reduction in wound size in experimental groups as compared to control group. *In situ* gel (G3) with 5% crude extract of *S.lappa* showed excellent healing in 14 days as comapred to standard pyodine gel [20]. So, further work should be carried out on this formulation for more efficient wound healing because *S. lappa* extract has shown to accelerate wound healing. Although extact mechanism of wound healing is still unknown as crude extract contains multiple chemical constituents so fractionation of crude extract is necessary. Results also revealed that as the concentration of crude extract increases from 0.5% to 5% in all formulations, the wound healing activity also increases in similar manner.

In-vitro anti-bacterial activity was also performed by inducing pus in rats. Pus culture was prepared and bacteria were streaked on LB agar plates [21]. Biochemical analysis like gram staining, coagulase test and catalase test were performed for identification of bacteria. After identification *in vitro* anti-bacterial activity of formulations F4, F5, F6, G1, G2, G3 and extract agaisnt standard sulfazalazine was performed. Different zones of inhibitions were measured. Results showed that sulfasalazine was having zone of inhibiton 31.3 mm. While extract was having 32.2 mm as compared to formulations and 33.1 mm in case of 5% formulation. So, in future, it may be beneficial to develop cost effective and resistance free herbal formulation to prevent wound infections that is leading cause of illness in majority of world population. Morever, localized topical herbal formulaion will have less side effect and minimum chances of bacterial resistance as comapred to the systemic ones.

Extraction is the most important step in biological evaluation of medicinal plants, so it should be performed under controlled temperature. Choice of extraction method is very important as high temperature may lead to deterioration of heat sensitive constituents. Choice of solvent is also a critical step. Non polar solvent like n-hexane and ethyl acetate should be used for extraction of lipophillic compounds while solvent like methanol, ethanol and chloroform should be used for extraction of hydriphillic compounds [27].

5. CONCLUSION

The study reveals that *Saussurea lappa* has effective wound healing, anti-inflammatory and anti-microbial properties which are proved through various physical and biochemical parameters. Formulation of extract into *in-situ* gels enhances the wound healing potential of *S. lappa* because it provides controlled drug release pattern and greater stability to the extract. Further studies are required to confirm the main constituents responsible for wound healing properties.

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