ABSTRACT

This study evaluated the wound healing potential leaves of *Lawsonia alba* Lam by excision and incision wound model. Besides this activity we also studied the acute inflammation by carrageenan model and analgesic study by using tail immersion method in mice. The methanol extract and its active fraction contained glycosides, flavonoids, tannins, coumarins and quinines and were relatively stable when stored at room temperature for three months. Solvent free semi solid extract of *Lawsonia alba* was incorporated into an aqueous gel and applied (5%W/W) on excision and incision wounds in Swiss albino mice. Excision wound model results showed that all treated animal groups (Standard colloidal silver gel and 5% test extract gel) exhibited significant increase in the percentage of wound contraction as compared to control group from 5th to 32nd days. In incision wound model treated with ELA showed significant increase in tensile strength as compared to control. Both the MLA and ELA were found to possess anti-inflammatory and analgesic activities at 1/10th of its LD$_{50}$ dose of 100mg/kg and 50mg/kg i.p. respectively. MLA and ELA also enhanced peritoneal cell count and the number of macrophages in normal mice. It is plausible that the active fraction of MLA may be responsible for these activities of leaves of *Lawsonia alba* Lam.

KEYWORDS: Wound healing, *Lawsonia alba* Lam. gel, excision and incision model, peritoneal macrophage.
1. INTRODUCTION
Wound is commonly known as breaking in epithelial integrity of the skin altering the
structure and functions of underlying normal tissue, caused by contusion, haematoma,
lacerations or abrasions due to physical, chemical and microbial injury. Wound healing starts
from the time of injury and continue for varying periods of time, depending on the degree
of wounding. Wound healing is a normal biological process with numerous steps like
coagulation, inflammation, granulation tissue formation, matrix formation, and connective
tissue remodeling, collagenization and wound strength acquisition. The increasing risk of
infection in soft-tissue or wound is major problem worldwide. Large wounds often take
longer to heal than small wounds and, as such, are more likely to develop complications such
as infection, which may then slow healing. For chronic wounds, size may relate to the
severity of the underlying causative condition and the status of the surrounding skin, both of
which have the potential to delay healing. In addition, the persistent inflammatory process
associated with non-healing and long wound duration degrades the extracellular matrix and
vascular supply to the wound, resulting in poor cellular function and senescence – a loss of a
cell’s power to divide and grow. Lawsonia alba Lam. (LA) by Lam.-nom.-illeg. (syn. Lawsonia inermis Linn), a member of family ‘Lythraceae’ and commonly known as ‘henna’
and ‘mehndi’, is cultivated chiefly as a hedge and garden plant in semi-arid zones and
tropical areas of northern Africa, western and southern Asia, and northern Australasia. All
parts of LA plant are used in several ailments including antimicrobial, analgesic, anti-
fertility activity, anti-sickling activity, anti-diabetic, anti-inflammatory and hepato-
protective effects with anti-oxidant property. Lawsonia alba Lam (LA) has been
employed in folk medicine for wound care traditionally. In Ayurvedic an ointment prepared
from the leaves is used to cure wounds. Therefore, the present communication is an approach
to study the wound healing activity of methanolic extract and its various fraction of Lawsonia
alba Lam. wound healing activity along with the anti-inflammatory and analgesic effect.

2. MATERIALS AND METHODS
2.1 Chemicals and reagents
Petroleum ether, Chloroform, Methanol, Ethyl acetate, n-butanol, Folin Ciocalteau’s
reagent, Propylene glycol 400 Carbopol934, Methyl paraben, Propyl paraben, Isopropyl
alcohol, Ethanol, Formaldehyde (Merck), Quercetin, Carrageenan, Gallic acid (Gibbco USA),
Ibuprofen (Sigma), Aluminium chloride, Sodium carbonate, Potassium acetate (Lobachel),
Pentazocine (Glaxo Smith Kline), Mega gel (Aristo Pharma), Triethanolamine, Diethyl ether,
Xylene, Glycerol, Sodium salicylate, Hydrochloric acid, Sodium Bicarbonate, Magnesium sulphate (Ranchem), Phenol red (Lobachem) and other chemicals and reagents were of analytical grade and purchased from local firms.

2.2 Animal models

The experiments are carried out on Swiss albino mice (20-26gm) and Wister rats (150-180gm) weighing, bred in the ‘Animal House Unit’ at the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi (Jharkhand). The animals were housed under condition of 22±1ºC, 50±10% humidity and 12 hrs light and 12hrs dark cycle. During maintenance the animals were received food and water as required.\textsuperscript{[7]}

2.3 Collection and identification of plant specimen

\textit{Lawsonia alba} Lam leaves were collected from Ranchi, Jharkhand and identified by Dr. V. P. Prasad, Scientist - D, Central National Herbarium, Botanical Survey of India, Ministry of Environment and Forests, and Government of India at Howrah, West Bengal. The specimen authentication no is CNH/ Tech. II/2014/75/158.

2.4 Extraction, separation and preparation of \textit{Lawsonia alba} Lam. leaves extract

The leaves of \textit{Lawsonia alba} Lam were harvested during dry season and air dried, then grinded into powder and leaves powder (1kg) was defatted by using pet ether and soaked in chloroform to free chlorophyll for 3 days and then soaked in 6000ml of methanol (selected by Phytochemical screening\textsuperscript{[8]}) for 7 days at room temperature with occasional shaking. The mixture was then filtered by filter paper and the solvent was evaporated by rotary vacuum evaporator and then lyophilized for 4 hrs to produce methanol free extract. The sticky methanolic extract was obtained finally. It was kept in a container, sealed with parafilm and stored at 4° C in airtight container and was designated as MLA, methanolic extract of \textit{Lawsonia alba} Lam. leaves for desired experiments. 10gm MLA extract is subjected to further fractionation by separating funnel method using different solvents (200ml) like ethyl acetate (ELA), n-butanol (BLA) and water (WLA).\textsuperscript{[9-11]}

2.5 Estimation of total flavonoids content by Aluminium Chloride Colorimetric method

Quercetin (Q) was used to make the calibration curve.\textsuperscript{[2-4]} Quercetin (10mg) dissolved in 80% ethanol, diluted to 12.5, 25, 50, 100, 120µg/ml. To the diluted standard solutions (0.5ml), 1.5 ml of 95% ethanol, 0.1ml of 10% aluminium chloride 0.1ml of 1M potassium acetate and 2.8ml of distilled water were added separately. After incubation at room temperature for 30
min, the absorbance was taken at 415 nm. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank. Repeat the methodology with the ELA, active fraction of MLA for determination of flavonoid content as described above. Result was expressed as milligrams of quercetin equivalents (QE) per gm of dry extract.

2.6 Estimation of total phenolic content by the Folin-Ciocalteu method

0.5ml of ELA fraction having concentration of 2,4,6,8 and 10µg/ml was added to 1.5ml (1:10 v/v diluted with distilled water) Folin Ciocalteau’s reagent and allowed to stand at 22ºC for 5 min. Then 2 ml of sodium carbonate (Na₂CO₃, 7.5% w/v) was added and the mixture were allowed stand for another 90mins and kept in the dark with intermittent shaking. Then the absorbance of the blue color developed solution was measured at 725nm using spectrophotometer. Gallic acid (GA) was used for constructing the standard curve using the same process mentioned above and the total phenolic contents in the fraction was expressed as milligrams of Gallic acid equivalent (GAE) per gram of dry extract.[2-4]

2.7 Carrageenan-induced rat paw oedema method

In this method, rats are divided into three groups of three each. The animals are pretreated with drug and extracts in concentration of (50mg/kg and 100mg/kg, i.p). Carrageenan is injected into the sub plantar region of left hind paw of each rat. Swellings of carrageenan injected foot are measured at 15, 30, 60,120,180 min using plethysmometer. The right hind paw is injected with 0.1ml of vehicle. The animals receive the standard drug ibuprofen (100mg/kg, i.p).[12-15] The percent inhibition in increase of edema volume for each animal group is calculated by the following formula, Percentage inhibition of edema = \[\frac{(V_t - V_0)\text{ control} - (V_t - V_0)\text{ treated}}{(V_t - V_0)\text{ control}}\times 100\].

2.8 Tail immersion method in mice

The animals are numbered, weighed and divided into four groups as standard, control and test with four animals in each. Mice are held in position in a suitable restrainer with the tail extending out. The tail (up to 2cm) is then dipped in beaker of water at 55°C. The time in seconds taken to withdraw the tail clearly out of the water is taken as the reaction time. The mice are administered the test drug (50mg/kg, i.p). Pentazocine (10mg/kg body weight, i.p) is used as standard. The reaction time in control and treated animals is recorded at 0, 30, 60 and 90 min after the treatment and compare the values with each others.[13]
2.9 Formulation of LA topical gel containing ELA extracts (5% w/w)

Mixture A: 500mg of carbopol 934 dispersed in 30 ml of distilled water with continuous stirring and kept for overnight.

Mixture B: Methyl paraben (0.5%) dissolved in hot water and Propyl Paraben (0.2%) in Propylene Glycol.

Required quantity of Ethyl acetate fraction of methanolic extract of LA leaves is mixed in distilled water with continuous stirring and this mixture is added to the mixture A with continuous stirring. Mixture A and Mixture B are mixed properly. Finally volume is made up to 50ml and Triethanolamine is added drop wise to the formulation for adjustment of required skin pH (6.8-7) and to obtain gel at required consistency. Same method is followed for the preparation of control sample without adding any leaves extract. Evaluation of topical gel formulation is done by physical evaluation, measuring pH, spreadibility test, homogeneity, skin irritation test etc.\(^{[16-17]}\)

2.10 In-vivo Wound Healing Studies

2.10.1 Excision wound model

Group the animals as untreated (negative control) mice, wound treated topically with 5% LA gel (test), wound treated topically with Mega gel (positive control). The mice were anaesthetized by open mask method using anesthetic ether. Mice were depilated on the dorsal back, Ethanol (70%) was used as antiseptic for the shaved region before making the wound, and an excision wound is made by removing a 7mm × 7mm full thickness piece of the skin from a predetermined shaved area on the back of each animal. The wound was left undressed to the open environment and no local or systemic anti-microbial agents are used. The mice are distributed in groups and each mouse is placed in separate cage. Throughout the study period, the excision wounds were cleaned with normal saline every morning prior to medication. In each animal group the wound inflicted to animals treated with Mega gel (positive control), 5% LA gel (test), normal saline (negative control), respectively. The size of wounds was traced at 0\(^{th}\), 4\(^{th}\), 8\(^{th}\), 12\(^{th}\), 16\(^{th}\), 20\(^{th}\), 24\(^{th}\), 28\(^{th}\) and 32\(^{nd}\) days and the evaluated surface area was then employed to calculate the percentage of wound contraction. This model was used to monitor rate of wound contraction.\(^{[9,18]}\)

Determination of wound contraction was done by using formula, Percentage of wound contraction= \((\text{wound area on } 0^{th} \text{ day} - \text{wound area on } n^{th} \text{ day}) / \text{wound area on } 0^{th} \text{ day} \times 100\)
2.10.2 Incision wound model

Animals in each group are anaesthetized and one ‘Para vertebral long incision’ is made through the skin and cutaneous at a distance of 1cm on each mouse with depilated back. Full aseptic measures are not taken and no local or systemic antimicrobial agents are used. After the incision is made, the parted skin kept together and stitched with surgical thread and curved needle. The wound is left undressed. Test LA gel, standard and control drug are topically applied once daily for 11th day; when wounds are cured thoroughly the sutures are removed on the 11th day and tensile strength is measured. This model is used to measure the tensile strength, resisting the breaking under tension.\(^9\)[18]

Measurement of tensile strength was done by using formula, Percentage Tensile Strength standard or LA gel = (Standard or LA gel TS - Control TS)/ control TS × 100.

2.11 Histopathological Examination

After deep ether anesthesia, the cross-sectional full-thickness skin specimens from each group were collected at the 10th day of the experiment to evaluate for the histopathological examinations. Tissues were fixed in neutral buffered formation (10% formaldehyde in Phosphate buffered saline) overnight. After fixation, the tissues were placed in 70% isopropyl alcohol for 2 hr and then in each ascending strength (80%, 90%, 100% isopropyl alcohol) for 1 hr each. The amount of alcohol used should be 15 times of the size of the tissue. After that, xylene was added to check for the appearance of milkyness. If milkyness appeared then repeat the dehydration procedure. The dehydrated tissue was impregnated in paraffin wax (m.p.56°C) for a period of 1 hr at 58-60°C. Molten parafin poured into L-block along with the tissues and allowed it to become hard. The tissue was sectioned into very thin (2-8 or 5-10 micrometer) sections using a microtome. The tissue Mounted on the slides with Mayer’s albumin solution (a mixture of equal parts of egg white and glycerin, beaten and filtered with the addition of 1% sodium salicylate) and incubated in warm oven for 2hr at 60°C. Slides containing paraffin sections were placed on a slide holder. Slides were deparaffinized with Xylene for 30 min and the excess xylene blotted. The tissue was rehydrate sucessively with 100%, 90%, 80% isopropyl alcohol for 2-3 min. each and put into water for 1-2 min. and then kept in tap water for 1-2 min. The slides containing tissue sections dipped into 1N HCL followed by Scott’s water (Sodium Bicarbonate 3.5g, Magnesium sulphate 20gm, distilled water1L) for 1min each. The tissue was immersed in Eosin stain for 30 sec. The tissue was dipped into 70% alcohol and then into 90% alcohol and pure alcohol subsequently and left
for 2mins each.Finally one drop of gum (DPX) was poured onto the slide and section was covered with cover slip.[14,22]

2.12 Peritoneal macrophage and total peritoneal cell count
In the total peritoneal cell study male or female albino Swiss mice weighing 23-26 gm were used for experiments. They were grouped and select as test and control group as each containing 4 mice. After that test group was treated with MLA (100µg/kg) and ELA (50µg/kg). After 6hrs euthanized the mice individually, sprayed it with 70% ethanol and mounted it on the Styrofoam block on its back. 1 ml of ice cold PBS was injected into the peritoneal cavity using a 27g needle. The needle was pushed slowly in the peritoneum being careful not to puncture any organs. After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. Immediately the peritoneal fluid was suck out. If visible blood contamination was detected then the contaminated sample should be discarded. Then one drop of the fluid was taken in neubauer's chamber and covered with cover slip to count the total peritoneal cells. After that, to differentiate the macrophages from peritoneal cells stained with phenol red. Lastly peritoneal macrophage cells were counted. The methodology was repeated for 12, 24, 48 hrs.[19]

2.13 Statistically Analysis
The data is analyzed statistically using one way ANOVA followed by Dunnet’s t-test. Values are expressed as mean *P<0.05, **P<0.01, ***<0.001 When compared to control. The data are expressed as mean ± SEM. “P” value less than 0.05 imply significant.

3. RESULTS
3.1 Preliminary Phytochemical Screening
Methanolic leaves extract of Lawsonia alba Lam. showed of better presence of phytochemical constituents like flavonoids, tannin, coumarin, carbohydrate, quinone etc than other extracts. Researchers also prove that ethyl acetate fraction of methanolic extract shows more potent than the other fractions such as n-butanol, aqueous fractions.
Table 1. Phytochemical screening of leaves extract of *Lawsonia alba* Lam.

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Methanol</th>
<th>Aqueous</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
<th>Pet ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Napthoquinone</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coumarin</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroid</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinone</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Where amount present indicated by ‘+++’ = Highly present, ‘++’ = Moderately present, ‘+’ = Slightly present, ‘-’ = absent.*

3.2 Estimation of total flavonoids content of ELA fraction of MLA extract by Aluminium chloride colorimetric method

Estimation of total flavonoids content reported that, the EA fraction showed the flavonoids content was 76.82 mg/gm.

3.3 Estimation of total phenol content of ELA fraction of MLA extract by the Folin-Ciocalteu method

Estimation of total phenol content reported that, the EA fraction showed the phenol content was 107.88 mg/gm.
3.4 Carrageenan-induced rat paw oedema method

In carrageenan-induced rat paw oedema model methanolic extract (MLA) showed significant decrease in paw oedema volume and increase in percentage inhibition of paw oedema volume at 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th} hrs and ethyl acetate fraction (ELA) 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} hours almost same as standard (Ibuprofen 100mg/kg, i.p) when compared to control. This investigation proved that ELA fraction was more potent than MLA extract.

![Standard curve of gallic acid](image)

**Fig.2: Total Phenol content: Standard curve of Gallic acid**

Table 2. Percentage inhibition of paw oedema volume of rat in carrageenan induced anti-inflammatory model

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Paw volume in ml at specific time intervals(% inhibition of paw oedema)</th>
<th>1hr</th>
<th>2hrs</th>
<th>3hrs</th>
<th>4hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.532±0.075</td>
<td>0.678±0.084</td>
<td>0.627±0.093</td>
<td>0.621±0.132</td>
</tr>
<tr>
<td>Ibuprofen 100mg/kg</td>
<td></td>
<td>0.172±0.4*(67.66%)</td>
<td>0.18±0.46**(73.45%)</td>
<td>0.1±0.32**(84.05%)</td>
<td>0.07±0.19***(88.72%)</td>
</tr>
<tr>
<td>MLA 100mg/kg</td>
<td></td>
<td>0.267±0.5%(49.81%)</td>
<td>0.25±0.1*(63.12%)</td>
<td>0.2±0.086**(68.1%)</td>
<td>0.1±0.07**(83.89%)</td>
</tr>
<tr>
<td>ELA 50mg/kg</td>
<td></td>
<td>0.239±0.346*(55.07%)</td>
<td>0.22±0.093**(67.55%)</td>
<td>0.18±0.075**(71.29%)</td>
<td>0.09±0.063**(85.50%)</td>
</tr>
</tbody>
</table>

*Values are expressed as mean *P<0.05, **P<0.01, ***<0.001 When compared to control. One way ANOVA followed by Dunnet’s t-test. Data are mean ± S.E.M, n= 4 per group

3.5 Tail immersion method in mice

Test drug (ELA) and standard drug (Pentazocine) showed significant increase in response (tail withdrawing time) at 15mins, 30mins, 45mins respectively when compared to control. So, test drug has central analgesic activity.
Table 3. Tail withdrawing activity of mice in tail immersion method

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Tail withdrawing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15mins</td>
</tr>
<tr>
<td>Control</td>
<td>1.33±0.00</td>
</tr>
<tr>
<td>Pentazocine 10mg/kg</td>
<td>3.86±0.32*</td>
</tr>
<tr>
<td>ELA 50mg/kg</td>
<td>3.53±0.29*</td>
</tr>
</tbody>
</table>

Values are expressed as mean *P<0.05, **P<0.01, ***P<0.001 When compared to control. One way ANOVA followed by Dunnet’s t-test. (Data are mean ± S.E.M, n= 4 per group)

3.6 Formulations of LA topical gel containing ELA extract (5%w/w)

LA hydro-gel incorporating 5% test (ELA) fraction showing the better dosage form of application by investigating the spreadibility, pH and appearance. The remedial gel was stored at ambient temperature.

Acute skin irritation test: In the skin irritation study, the tested gel did not show any type of irritation and there was no evidence of inflammation.

Table 4. Physical evaluation of all formulation at the time of gel formulation

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Color</th>
<th>Appearance</th>
<th>Spreadibility (g.cm/sec)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Mega gel</td>
<td>Colorless</td>
<td>Clear and transparent</td>
<td>19.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Control (Hydro-gel)</td>
<td>White</td>
<td>Clear and transparent</td>
<td>18.9</td>
<td>6.7</td>
</tr>
<tr>
<td>LA topical gel 5% w/w</td>
<td>Dark brown</td>
<td>Clear and transparent</td>
<td>20.3</td>
<td>7.2</td>
</tr>
</tbody>
</table>

3.7 In-vivo Wound Healing Studies

3.7.1 Excision wound model: Excision wound model results showed that all treated animal groups (Standard colloidal silver gel and 5% test extract gel) exhibited significant increase in the percentage of wound contraction as compared to control group from 5th to 32nd days gradually but no significant increase in the percentage of wound contraction on 1st day. However, it was seen that the significant healing of wound occurred in standard drug as well as 5%w/w test extract gel as compared to control group. Also, wound contraction on different days shown by histogram and photographical representation proved the significant healing of wound created by excision wound on skin of mice.
Table 5. Percentage wound contraction of mice in excision wound model

<table>
<thead>
<tr>
<th>Days</th>
<th>Control (Normal saline)</th>
<th>Standard (Mega Gel)</th>
<th>LA topical gel (5% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.945 ± 0.656</td>
<td>2.0559 ± 0.5982</td>
<td>1.178 ± 0.174</td>
</tr>
<tr>
<td>4</td>
<td>14.0232 ± 2.348</td>
<td>24.0905 ± 4.3217*</td>
<td>21.7797 ± 4.112*</td>
</tr>
<tr>
<td>8</td>
<td>37.8712 ± 4.103</td>
<td>44.0638 ± 6.5142**</td>
<td>41.0876 ± 5.896**</td>
</tr>
<tr>
<td>12</td>
<td>52.6601 ± 5.365</td>
<td>69.5129 ± 7.7788**</td>
<td>65.7654 ± 6.546**</td>
</tr>
<tr>
<td>16</td>
<td>70.0158 ± 6.136</td>
<td>92.6442 ± 3.0855***</td>
<td>85.4602 ± 4.554***</td>
</tr>
<tr>
<td>20</td>
<td>82.5320±6.874</td>
<td>99.8577±1.0457***</td>
<td>91.5675±1.076***</td>
</tr>
<tr>
<td>24</td>
<td>91.6708±4.324</td>
<td>Healed</td>
<td>98.7490±0.987***</td>
</tr>
<tr>
<td>28</td>
<td>98.0128±1.340</td>
<td>Healed</td>
<td>Healed</td>
</tr>
<tr>
<td>32</td>
<td>Healed</td>
<td>Healed</td>
<td>Healed</td>
</tr>
</tbody>
</table>

Values are expressed as mean *P<0.05, **P<0.01, ***<0.001 When compared to control. One way ANOVA followed by Dunnet’s t-test. (Data are mean ± S.E.M, n= 8 per group).

Fig. 3: Photographical representation of wound contraction and comparison of wounds healing on different days in standard and test treated group animal compared to control group.

3.7.2 Incision wound model

In incision wound model, the incision wounds treated with test (5% w/w ELA fraction gel) and standard (colloidal silver gel) showed significant tensile strength 423.33 ± 22.546 gm (P < 0.05) and 481.2 ± 18.089 gm (P < 0.01), respectively compared to control group; 240 ± 8.40 gm. So, above observation for tensile strength showed that the incision wounds treated with 5% w/w test extract gel showed significant increase in tensile strength as compared to control.

Table 6. Measurement of tensile strength of mice in incision model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tensile strength(gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Normal saline)</td>
<td>240 ± 8.40</td>
</tr>
<tr>
<td>Standard (Mega Gel)</td>
<td>481.2 ± 18.089**</td>
</tr>
<tr>
<td>LA topical gel (5% w/w)</td>
<td>423.33 ± 22.546*</td>
</tr>
</tbody>
</table>
*P<0.05, **P<0.01, when compared to control. One way ANOVA followed by Dunnnet’s t-test. (Data are mean ± S.E.M, n=6 per group)

Fig. 4: Photographical representation of incision wound model treated with mice

3.8 Histopathological study

Hematoxylin and eosin stained sections of granulation tissue showed more aggregation of mononuclear cell (M), poor migration of fibroblast cells (F), less formation of new blood vessel (BV) and less collagen (C) deposition which is not good, indicates incomplete healing of wound in 10\textsuperscript{th} post wounding day in control group of incision and excision wound. But in Standard drug treated group of incision and excision wound model less aggregation of mononuclear cell (M), increased migration of fibroblast cells (F), formation of new blood vessel (BV) and increased collagen (C) deposition indicates complete healing of wound in 10\textsuperscript{th} post wounding day. Comparatively, in 5% fraction of test LA gel treated group of incision and excision wound showed less aggregation of mononuclear cell (M), enhanced migration of fibroblast cells (F), formation of new blood vessels (BV) and enhanced collagen (C) deposition indicates nearly complete healing of wound in 10\textsuperscript{th} post wounding day.
Fig. 5: Hematoxylin and eosin stained sections of granulation tissue showed aggregation of mononuclear cell (M), migration of fibroblast cells (F), formation of new blood vessel (BV) and collagen (C) deposition which, indicates status of wound healing in 10th post wounding day in control, standard, test group of excision and incision wound model

3.9 Peritoneal macrophage and total peritoneal cell count

In the Peritoneal Macrophage study by *in-vitro* assay there was significant increase in the peritoneal macrophage count with a time and concentration dependent manner compared to that of the untreated control group upon treating group with MLA (100μg) and ELA (50μg) for 6, 12, 24, 48 hours in peritoneal cavity of mice (Fig.6 and 7). So, there is possibility of immune modulator effect of MLA and ELA fractions of LA leaves.
Fig. 6: The histogram showed gradually increase in total peritoneal cell count treated with MLA and ELA fraction compared to control in a concentration and time dependent manner. Data are mean ± S.E.M.

Fig. 7: The histogram shows gradually increase in peritoneal macrophage count treated with MLA and ELA fraction compared to control in a concentration and time dependent manner. Data are mean ± S.E.M.

3. DISCUSSION

The wound healing is a complicated biological process in cellular and molecular level for regeneration of the damaged tissue. Wound healing, complex sequences of events involve four phases, coagulation which prevents blood loss; inflammation and debridement of wound; epithelial repair including proliferation, mobilization, migration and differentiation; tissue remodeling and collagen deposition. In inflammation stage the platelets, thrombin, fibrin and other coagulation factors aggregated sub-endothelially to form haemostatic plug. In case of proliferative phase there is a rapid generations of collagen fiber and it deposited upon the wounded area. The neo-angiogenesis in proliferative phase also enhance the wound healing process by proper blood circulation around the wounded area. In final stage of wound healing there is a rapid differentiation of fibroblast cells to mayo-fibroblasts that causes wound...
Inflammation is an immune-vascular response involving to eliminate the cause of cell injury, clear out necrotic cells and tissues damaged from the original insult. Although acute inflammation acts as protective mechanism in wound healing but chronic inflammation can lead to major problem like cancer. It is condensed that reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. It is also evident that the antioxidant supplementation helps in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. Preliminary phytochemical screening which was carried using chemicals for the identification of various Phytoconstituents showed the significant presence of tannins, coumarins carbohydrates, flavonoids, quinone in methanolic extract (Table 1). Flavonoids and other phenol compounds of leaves origin have been reported as scavengers and inhibitors of lipid per-oxidation. Higher the flavonoids and phenol contents show stronger the wound healing activity. From the results of total flavonoids content (Fig.1) and total phenol content (Fig.2) of ELA fraction it was found that ELA fraction has stronger wound healing activity.

We know when injury occurs and inflammation create then inflammation markers histamine release at 1st hour, serotonin at 2nd hour, bradykinin at 3rd hour and prostaglandin release at 4th hour and working for body defense mechanism. Practical experience has shown that once a plant extract is found to possess anti-inflammatory activity, it is better to test whether it also possesses wound healing activity because it is now pretty well established that inflammation and wound healing go hand in hand. So, the anti inflammatory activity is useful for wound healing activity. Treated with MLA and ELA of Lawsonia alba leaves (Table 2) showed significant reduction in paw oedema volume as compared to control which possesses anti-inflammatory activity of the extract. When wound occurs, it is accompanied by pain, so the analgesic activity is supportive to wound healing activity. From the results of analgesic study by tail immersion method on mice, it was found that the methanolic extract and the ELA fraction of MLA extract of Lawsonia alba leaves (Table 3) showed significant reduction in central analgesic property as compared to control.

Natural remedies are more acceptable in the belief that they are safer with fewer side effects than the synthetic ones. Herbal formulations have growing demand in the world market. The herbal gel was dark brown in color and translucent in appearance and gave smooth feel on application, pH was found 6.7 to 7.2, spreadibility was also measured and found to be with less variation, the values of spreadibility indicate that the gel is easily spreadable by small
amount of shear, spreadibility of formulated gels (5%) was 20.3g cm/sec and showed good homogeneity with absence of lumps, physical evaluation was done at time of gel formulation. The topical gel thus formulated was non-irritant upon application on to the skin (Table 4).

In general, it may be suggested that due to the different experimental approaches and models used, investigations have sometimes yielded different results. Hence, the use of a standardized and reproducible model is inevitable to obtain objective information of the wound healing process as well as to better understand the pathological process and to improve medical technologies. Hence two different in vivo models (Excision wound model and Incision wound model) had been chosen in our study to assess the effect of herbal gel of most active fraction on wound healing. The data from excision model and photographical representation revealed that 5% gel of active fraction showed significant wound healing promoting activity by increasing rate of wound contraction as compared to control animals (Table 5 and Fig.3). In incision model the braking strength of the wounds after topical application of herbal 5% gel has been ensured. The breaking strength is the ability of healing wound which is measured experimentally by the amount of force required to disrupt it. In this study it was observed that topical application of 5% gel has shown significant increase in the breaking strength (Table 6 and Fig.4).

Histopathological evaluation was carried out for both the treated and untreated mice and it revealed that the wound healing process of the wounded tissue. Mice which receive topical application of 5% gel were comparably close to the standard drug. No healing was observed in control group. Sections of granulation tissue of mice treated with 5% gel showed less aggregation of macrophages, enhanced migration of fibroblasts cells, formation of new blood vessels and enhanced collagen deposition indicates nearly complete healing of wound in the 10th post wounding day in both excision model and incision model (Fig.5).

It also showed that the leaves extract (MLA) and active fraction (ELA) of Lawsonia alba leaves has significantly increase time dependently in mouse peritoneal macrophage cells as well as in total peritoneal exudates than untreated control group. So, it may strengthen the immune-stimulant effect. (Fig.6 and Fig.7).

The number of natural product derived drugs present in the total drug launches from 1981 to 2002 was recently analyzed and it was concluded that natural products are still a significant source of new drugs, especially in the anti-cancer and anti-hypertensive
therapeutic areas. Chemoprevention is a novel approach emphasizing on the prevention or delay of carcinogenesis by means of pharmacological, biological, and nutritional intervention and recently, cancer chemoprevention with strategies using foods and medicinal herbs has been regarded as one of the most visible fields for cancer control. It is likely that the MLA extract of LA leaves may also have protective effect of liver damage. \cite{5}

**CONCLUSION**

In conclusion, our results indicate that methanolic extract (MLA) as well as its active fraction (ELA) of *Lawsonia alba* Lam. leaves cures the wound, inflammation, pain. It also possesses anti-proliferative property. Further prospective studies will be needed to isolate the active compound and confirm the actual cellular mechanism of action of *Lawsonia alba* Lam. Leaves.

**ACKNOWLEDGEMENT**

The authors would like to express their gratitude to the CSIR, Indian Institute of Chemical Biology, (CSIR,IIICB) Kolkata, West Bengal for funding to perform the research work. Authors also thankful to the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi, Jharkhand for providing healthy animals.

**REFERENCE**


