Chapter 4

Materials and Methods
4.1 Equipments

1. Autoanalyzer ERBA CHEM 5 + TRANSASIA BIOMEDICALS Ltd Mumbai
2. Automated Hematology Analyzer Sysmex TOA Medical Electronics Co Ltd K-1000 KOBE, Japan.
3. Binary pump HPLC Shimadzu, Japan
5. Chromatography module Indtech IndTech, Mumbai
6. Column (HPLC) YMC ODC-AM 303 YMC, Japan
7. Distillation Assembly Lab Glass distillation assembly.
8. Dynamic mixer (HPLC) SUS mixer Shimadzu, Japan
9. HPLC-grade water Unit Millipore, USA
10. Manual injector Rheodyne 20 μl Rheodyne, USA
11. Microliter syringe (25μl) Hamilton, USA
13. Microscope (triocular, with photomicrographic Arrangement) Olympus optical Co., Japan
14. Motic Image Photomicrography Unit Motic, Italy
15. Plethysmometer (Transducer) 7140 UGO Basile, Biolog. Research App.,Italy
16. Rotary microtome Yorko Scientific Indst., Delhi
17. Software (HPLC system) WinChrom IndTech, Mumbai
18. Solute filtration assembly Hamilton. USA
19. Solvent delivery module LC-10AT vp Shimadzu, Japan
20. Solvent Filtration Pump Spinco Biotech, India
21. Soxhlet Assembly Rama Scientific Works, New Delhi
23. UV-Vis Detector (HPLC) SPD-10Avp Shimadzu, Japan
24. UV-Vis Spectrophotometer 1601 Shimadzu, Japan

4.2 Drugs and Chemicals

1. Aspirin Mano Pharma, Chennai.
2. Bradykinin Sigma, USA
3. Carrageenin  
4. Chlorpheniramine maleate  
5. Cyproheptadine  
6. Hyaluronidase  
7. Phenylbutazone  
8. Prostaglandin E₂ (Prostodin)  
9. Rutin (~90% pure)  
10. Serotonin

Sigma, USA
Cadila Healthcare, Ahmedabad.
Cipla Ltd., Mumbai
Rallis India Ltd. Mumbai
Sigma, USA
Astra IDL, Banglor
SD Fine Chemicals, Mumbai
Sigma, USA

4.3 Chemicals and Reagents

Either analytical grade or of highest purity grade available commercially were used.

1. Acetonitrile for HPLC  
2. Alcohol (Spectroscopy)  
3. Carboxy methyl cellulose  
4. Diethyl ether  
5. Dragendorff's Reagent  
6. Formaline  
7. Fehlings Solutions A & B  
8. Glass distilled water  
9. Hager's Reagent  
10. Isopropanol  
11. Mayer's reagent  
12. Molisch Reagent  
13. Ninhydrin reagent  
14. Sodium chloride  
15. Sodium citrate  
16. Stain, Alcoholic Eosin  
17. Tween 80  
18. Water for HPLC

SD Fine Chemicals, Mumbai
SD Fine Chemicals, Mumbai
SD Fine Chemicals, Mumbai.
CDH, Mumbai
CDH, Mumbai
Qualigens, Mumbai
CDH, Mumbai
Lab Distillation Assembly
CDH, Mumbai
SRL, Mumbai
CDH, Mumbai
CDH, Mumbai
CDH, Mumbai
E. Merck, Mumbai
Qualigens Fine Chem., Mumbai
Qualigens Fine Chem., Mumbai
Thomas Baker (Chemicals) Ltd., Mumbai.
E. Merck, Mumbai
4.4 Diagnostic Kits

1. GGTP (γ GTP) AUTOPAK Bayer Diagnostics, Baroda, India.
2. SGOT / AST AUTOPAK Bayer Diagnostics, Baroda, India.
3. SGPT / ALT AUTOPAK Bayer Diagnostics, Baroda, India.

4.5 Animals used

<table>
<thead>
<tr>
<th>Animal</th>
<th>Procured from Central Animal House, Jamia Hamdard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albino rats</td>
<td>Procured from Central Animal House, Jamia Hamdard</td>
</tr>
<tr>
<td>Sex</td>
<td>Either</td>
</tr>
<tr>
<td>Weight</td>
<td>120 – 200 g</td>
</tr>
<tr>
<td>Age</td>
<td>Adult</td>
</tr>
<tr>
<td>Strain</td>
<td>Wistar</td>
</tr>
</tbody>
</table>

4.6 Animal diet

Palet Rat Feed Amrut, Pune, Maharashtra.

4.7 Design of Research Work

The following experiments were designed to study the pharmacological actions of the three extracts of *Lawsonia inermis* Linn., viz.; Aqueous, Alcoholic and Chloroform extracts.

4.7.1 Preparation of the extracts of leaves of *Lawsonia inermis* Linn.

- Collection of leaves of *Lawsonia inermis* Linn.
- Authentication of the leaves
- Drying of leaves
- Preparation of the extracts
  - Aqueous extract.
  - Alcoholic extract.
  - Chloroform extract.
4.7.2 Anti-inflammatory activity

The literature on Lawsonia revealed its anti-inflammatory activity among several other pharmacological actions. Accordingly investigations on the three extracts were planned as per the following details:

4.7.2.1 Action against edema

➢ Carrageenin induced paw edema in rats
➢ Paw edema induced by different phologistic agents viz.;
  ◦ Histamine
  ◦ Serotonin
  ◦ Bradykinin
  ◦ Prostaglandin E₂
  ◦ Hyalluronidase in rats

4.7.2.2 Action against pain

➢ Tail immersion method
➢ Eddy's Hot-plate method

4.7.3 Toxicity study

➢ Hematological toxicity
➢ Ulcerogenic activity
➢ Body weight
➢ General behaviour
➢ Serum markers of tissue damage / inflammation
➢ Histopathology of kidney and liver

4.7.4 Standardization of the extracts

➢ Preliminary phytochemical screening
4.8 Experimental Protocol

4.8.1 Preparation of extracts of *Lawsonia inermis* Linn. leaves

4.8.1.1 Collection and authentication of plant material

*Lawsonia inermis* Linn. leaves were collected from Faridabad, Haryana and identified by Mr. Shoaib Ahmad, Lecturer, Dept. of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Jamia Hamdard. A voucher specimen (SA-RM-001/2005) of the plant has been retained. The leaves were air-dried.

4.8.1.2 Extraction of the plant material

Experiment 1
The leaves of *Lawsonia inermis* Linn., collected from Faridabad, were dried at room temperature and their aqueous, alcoholic and chloroform extracts were prepared.

Preparation of the aqueous extract of leaves

The air-dried leaves (1kg) were reduced to fine powder and packed in Soxhlet apparatus (5 L) in the form of a thimble. The drug was then extracted using 3 liters of water for 6 hours. The crude extract from the round bottom flask of Soxhlet apparatus was evaporated to dryness under reduced pressure. A sticky mass (31.0g or 31.0% w/w) was obtained.
Preparation of the alcoholic extract of leaves

The air-dried leaves (1kg) were reduced to fine powder and packed in Soxhlet apparatus (5 L) in the form of a thimble. The drug was then extracted using 3 liters of alcohol for 6 hours. The crude extract from the round bottom flask of Soxhlet apparatus was evaporated to dryness under reduced pressure. A sticky mass (330g or 33.0% w/w) was obtained.

Preparation of the chloroform extract of leaves

The air-dried leaves (1kg) were reduced to fine powder and packed in Soxhlet apparatus (5 L) in the form of a thimble. The drug was then extracted using 3 liters of chloroform for 6 hours. The crude extract from the round bottom flask of Soxhlet apparatus was evaporated to dryness under reduced pressure. A sticky mass (70g or 7.0%) was obtained.

4.8.2 Animal study

Albino rats (Wistar strain) of 120 - 200 g body weights, bred in the Central Animal House Facility, Jamia Hamdard, were used. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Hamdard University and CPCSEA, India. The animals were kept under standard laboratory conditions and allowed free access to a commercial pellet diet (Amrut Rat Feed, Pune, India) and water ad libitum. The room temperature was maintained at 25 ± 1°C.

4.8.2.1 Experiment 2 Carrageenin induced paw edema

Albino rats (Wistar strain) of 120 - 200 g body weight were grouped into nine groups of six animals each. Group N served as Normal control for biochemical and histopathological parameters. Animals of the groups I to VIII were given drug pre-
treatment for 3 days as per the details shown in Table 6. Exactly 1 hr after the 3rd day dose, rats of group I to VIII were injected 0.1 ml of 1% freshly prepared carrageenin in sterile normal saline into the sub-planter surface of the right hind paw to induce inflammation, according to the method of Winter et al. (1962). The foot volume was measured by the transducer type plethysmographic method, at intervals of 1 hr upto 6 hrs after carrageenin injection (Ghosh, 1974). The animals were then sacrificed under light ether anaesthesia by carotid bleeding, for histopathological study. Blood sample were collected for biochemical estimations.

On third day (1 hr after the 3rd day dose as given for different treated groups in Table 6) Carrageenin (0.1ml of 1%) was injected in the sub-planter surface of the right hind paw.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (For 3 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group N</td>
<td>Normal control. Normal diet. (Only for basal Biochemical values)</td>
</tr>
<tr>
<td>Group I</td>
<td>Toxic control</td>
</tr>
<tr>
<td>Group II</td>
<td>Standard drug</td>
</tr>
<tr>
<td>Group III</td>
<td>Test drug 1</td>
</tr>
<tr>
<td>Group IV</td>
<td>Test drug 1</td>
</tr>
<tr>
<td>Group V</td>
<td>Test drug 2</td>
</tr>
<tr>
<td>Group VI</td>
<td>Test drug 2</td>
</tr>
<tr>
<td>Group VII</td>
<td>Test drug 3</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Test drug 3</td>
</tr>
</tbody>
</table>
Solutions and reagents required

For treatment

1. Normal saline – 0.9g of NaCl was dissolved in 100ml of glass distilled water.
2. Carrageenin solution – 10 mg of carrageenin dissolved in 1 ml of normal saline.
3. Indomethacin solution – Indomethacin 25 mg was dissolved in 10 ml of normal saline.
4. Aqueous extract (0.075 g/ ml), alcoholic extract (0.075 g/ ml), chloroform extract (0.075 g/ml).

For estimations

Biochemical

Serum
- AST / SGOT estimation kit
- ALT / SGPT estimation kit
- γ GTP / GGTP estimation kit

Histopathological studies

10% buffered formal saline: 10 ml of formaline was mixed with 90 ml of buffered normal saline.

All rats were sacrificed under light ether anaesthesia by carotid bleeding. Before sacrificing, required amount of blood was collected through retro-orbital plexus using a microcapillary tube, liver was removed and washed with normal saline and fixed in 10% formal saline (1 part of the tissue by weight in 20 parts of 10% formal saline by volume)

4.8.2.2 Experiment 3  Inflammatory mediators / Different phologistic agents induced edema

For each of the five phologistic agents; histamine (10⁻³ g/ml, 0.1 ml); serotonin (10⁻³ g/ml, 0.1 ml); prostaglandin E2 (10⁻⁶ g/ml, 0.1 ml); bradykinin (2 x 10⁻⁵ g/ml, 0.1 ml) and hyaluronidase (2400 IU/ml, 0.1 ml) (Parmar and Ghosh, 1978), thirty six
albino rats (Wistar strain) of 120 – 200 g body weight were grouped into six groups of six animals each. Group N served as Normal control for biochemical and histopathological parameters. Animals of the groups I to V were given drug pre-treatment for 3 days as per the details shown in Table 7. Exactly 1 hr after the 3rd day dose, rats of group I to V were injected 0.1 ml of the respective phlogistic agent into the sub-planter surface of the right hind paw according to the method of Winter et al. (1962). The foot volume was measured by the transducer type plethysmographic method, upto 6 hrs after phlogistic agent injection at intervals of 1 hr (Ghosh, 1974). Blood samples were collected from the retro orbital plexus for biochemical estimations.

### Table 7: Effect of Lawsonia extracts on Phlogistic agents induced inflammation (Animal grouping and Treatment)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (For 3 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group N</td>
<td>Normal control. Normal diet. (Only for basal Biochemical values)</td>
</tr>
<tr>
<td>Group I</td>
<td>Toxic control</td>
</tr>
<tr>
<td>Group II</td>
<td>Standard drug</td>
</tr>
<tr>
<td>Group III</td>
<td>Test drug 1</td>
</tr>
<tr>
<td>Group IV</td>
<td>Test drug 2</td>
</tr>
<tr>
<td>Group V</td>
<td>Test drug 3</td>
</tr>
<tr>
<td></td>
<td>Normal saline (1 ml/100g p.o.)</td>
</tr>
<tr>
<td></td>
<td>Standard drug (Prescribed dose)</td>
</tr>
<tr>
<td></td>
<td>Aqueous extract (1.5 g/kg p.o.)</td>
</tr>
<tr>
<td></td>
<td>Alcoholic extract (1.5 g/kg p.o.)</td>
</tr>
<tr>
<td></td>
<td>Chloroform extract (1.5 g/kg p.o.)</td>
</tr>
</tbody>
</table>

### Solutions and reagents required

For treatment

1. Normal saline – 0.9g of NaCl was dissolved in 100ml of glass distilled water.
2. Histamine (10^-3 g/ml); serotonin (10^-3 g/ml); prostaglandin E2 (10^-6 g/ml); bradykinin (2 x 10^-5 g/ml) and hyalluronidase (2400 IU/ml)
3. Chlorpheniramine maleate (0.8 mg/ ml), Cyproheptadine (1 mg/ ml), Indomethacin solution (2.5 mg/ ml), Aspirin (10 mg/ ml).
4. Aqueous extract (0.075 g/ml), alcoholic extract (0.075 g/ml), chloroform extract (0.075 g/ml).

For Biochemical estimations

Serum
- SGOT / AST estimation kit
- SGPT / ALT estimation kit

4.8.2.3 Experiment 4 Analgesic effect

For each of the two methods thirty albino rats (Wistar strain) of 120 – 200 g body weight were grouped into five groups of six animals each. Group N served as Normal control. Animals of the groups I to IV were given drug pre-treatment for 3 days as per the details given in Table 8. Exactly 1 hr after the 3rd day dose, the animals were subjected to respective algesic stimuli. Responses were recorded before administration of drugs, at 0 (immediately after administration of drug), 30, 60, 90, 120 and 150 min after drug administration.

Pain stimulus was induced by:

- Eddy's hot plate – Animals were placed on the hot plate maintained at 55±0.5°C. The reaction time was noted, which is the time between placing the animal on the hot plate and its jumping or licking of the paws. To protect the animal from any tissue damage or thermal injury a cut off time of 10 sec was strictly followed.
- Tail immersion - With the animal properly restrained, the tail (up to 5 cm) was dipped in a beaker of water maintained of 55±0.5°C. The time in seconds to withdraw the tail clearly out of the water was taken as the reaction time. To protect the animal from any tissue damage or thermal injury a cut off time of 10 sec was strictly followed.
Table 8: Effect of Lawsonia extracts on pain stimuli
(Animal grouping and Treatment schedule)

<table>
<thead>
<tr>
<th>Group</th>
<th>N=6</th>
<th>Treatment (For 3 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Toxic control</td>
<td>Normal saline (1 ml/100g p.o.)</td>
</tr>
<tr>
<td>Group II</td>
<td>Standard drug</td>
<td>Aspirin (100mg /kg p.o.)</td>
</tr>
<tr>
<td>Group III</td>
<td>Test drug 1</td>
<td>Aqueous extract (1.5 g/kg p.o.)</td>
</tr>
<tr>
<td>Group IV</td>
<td>Test drug 2</td>
<td>Alcoholic extract (1.5 g/kg p.o.)</td>
</tr>
<tr>
<td>Group V</td>
<td>Test drug 3</td>
<td>Chloroform extract (1.5 g/kg p.o.)</td>
</tr>
</tbody>
</table>

Solutions and reagents required

For treatment
1. Normal saline – 9g of NaCl was dissolved in 1000ml of glass distilled water.
2. Aspirin – 200 mg of acetyl salicylic acid was dissolved in 20 ml of glass distilled water.
3. Aqueous extract (0.075 g/ml), alcoholic extract (0.075 g/ml), chloroform extract (0.075 g/ml).

4.8.2.4 Experiment 5 Subacute Toxicity

Twenty four albino rats (Wistar strain) of 120 – 200 g body weight were grouped into four groups of six animals each. Group N served as Normal control for biochemical and histopathological parameters. Animals of the groups I to III were given drug pre-treatment 15 days as per the details given in Table 9. The body weight and general behaviour of the animals were monitored. On the 15th day the animals were sacrificed under light ether anaesthesia by carotid bleeding. Blood samples were collected for biochemical and hematological estimations. Liver and kidney were removed and preserved for evidence of any histopathological abnormality. Stomach were removed and incised on the greater curvature to observe for any ulcers or erosions (Turner, 1965).
Table 9: Sub-acute toxicological effects of Lawsonia extracts
(Animal grouping and Treatment schedule)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=6</td>
<td>(For 15 days)</td>
</tr>
<tr>
<td>Group I</td>
<td>Normal control. Normal saline (1 ml/100 g p.o.)</td>
</tr>
<tr>
<td>Group II</td>
<td>Test drug 1 Aqueous extract (2 g/kg p.o.)</td>
</tr>
<tr>
<td>Group III</td>
<td>Test drug 2 Alcoholic extract (2 g/kg p.o.)</td>
</tr>
<tr>
<td>Group IV</td>
<td>Test drug 3 Chloroform extract (2 g/kg p.o.)</td>
</tr>
</tbody>
</table>

Solutions and reagents required

For treatment
1. Normal saline – 9g of NaCl was dissolved in 1000ml of glass distilled water.
2. Aqueous extract (0.1 g/ml), Alcoholic extract (0.1 g/ml), Chloroform extract (0.1 g/ml).

For biochemical estimations

Serum
• SGOT / AST estimation kit
• SGPT / ALT estimation kit
• GGTP / γ GTP estimation kit

4.8.3 Paw volume measurement with Plethysmometer

UGO Basile Transducer Plethysmometer 7140 is a volume meter. It is used to measure the rat paw volume / edema. The rat paw is dipped in water filled Perspex cell, the transducer records differences in water level caused by water displacement. A graphic LCD read-out shows the exact volume of the dipped paw.
Principle of Operation
Transducer measures the conductance between two vertical Pt - Ir wire electrodes in the small tube. Conductance is proportional to; the water level, the water conductivity (ion content, temperature)

Operation
Set up the apparatus, connect the transducer cable, "Hold" pedal. The reservoir is filled with freshly prepared solution (2 – 3 ml / lit of wetting compound, 0.4 – 0.5 g/ lit of NaCl)

Calibration
Calibration is essentially done as per the requirement of the study. Calibration is done with appropriate volume probe, for rats 4 cm³.

Measurement
After calibration the Zero key is depressed. Paw of the animal is dipped in the cell. The pedal switch is depressed to hold the device until the reading is observed and recorded manually or sent to printer or the attached Computer. Paw can be withdrawn. Pedal switch can then be released. The instrument is zeroed to make it ready for the next measurement.

4.8.4 Separation of serum

About 3-5 ml of blood was collected in a sterile centrifuge tube and left undisturbed at 37°C for 1 hr, till the formation of clot. The serum was aspirated using a sterile pipette after centrifugation at 3000 rpm for 15 min. Serum collected was analyzed for enzyme serum levels immediately or within 24 hrs after storing at 0-4 ºC (Guru, 1990). Hemolysed or grossly contaminated samples were rejected.
Precautions
i. Serum sample must be completely free from hemolysis, since RBCs are very rich in this enzyme. Hemolysis leads to the release of large amount of the enzyme and hence gives erroneous results.
ii. Avoid the use of detergents to clean the glassware.
iii. Use clean and dry glassware.
iv. Reagents are corrosive, avoid contact with skin or eyes.

4.8.5 Methods for biochemical estimations

In Serum
- GOT Glutamate Oxaloacetate Transaminase or Aspartate Transaminase (AST)
- GPT Glutamate Pyruvate Transaminase or Alanine Transaminase (ALT)
- γ GTP Gamma Glutamyl Transpeptidase (GGTP)

Principle: Transamination is the process by which an amino group of an amino acid is transferred to an alpha keto acid with the formation of keto acid corresponding to the original amino acid.

4.8.5.1 Method for estimation of SGOT / AST by UV kinetic (IFCC) method

Principle

\[
\text{GOT} \quad \begin{array}{c}
\text{L-Aspartate} + \alpha \text{Ketoglutarate} \\
\text{Oxaloacetate} + \text{L-Glutamate}
\end{array}
\]

\[
\text{MDH (Malate dehydrogenase)} \\
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \quad \longleftrightarrow \quad \text{L-Malate} + \text{NAD}^+
\]

AST = Aspartate aminotransferase. MDH = Malate dehydrogenase.
There is a decrease in absorption at 340 nm as NADH is converted to NAD. The rate of decrease in absorbance is measured and is proportional to AST activity in the sample.

Reagents (Supplied in the kit)

Reagent 1 (Enzymes)
- MDH ≥ 600 U/L
- LDH ≥ 900 U/L
- NADH 0.20 mmol/L
- α-Ketoglutarate 12 mmol/L

Reagent 1A (Buffer)
- Tris buffer, pH 7.80 88mmol/L
- L-Aspartate 260 mmol/L

Reagent reconstitution / Preparation of the working solutions
The reagents were allowed to attain room temperature. The contents of one bottle of reagent 1 were dissolved with one bottle of reagent 1A. The contents were mixed by gentle swirling and used after 5 mints.

The reconstituted reagent is stable for 4 weeks when stored at 2 – 8 °C.

Procedure
1. Autoanalyzer was set to the software for the test as per parameters for SGOT, shown in the table below and was calibrated using distilled water. The samples and the reconstituted reagent were brought to room temperature prior to use.
3. The working solution and serum were taken as shown below

<table>
<thead>
<tr>
<th>Dispense into test tube</th>
<th>Reconstituted reagent</th>
<th>1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td></td>
<td>100 μL</td>
</tr>
</tbody>
</table>
After adding the working solution to the tube containing the serum proper mixing was done and absorbance was read immediately.

Note
Samples with a very high SGOT activity cause an excessive consumption of NADH, resulting in a very low initial absorbance and/or non-linear reaction. When this occurs the assay should be repeated with a diluted sample.

4.8.5.2 Method for estimation of SGPT / ALT by UV kinetic (IFCC) method

Principle

\[
\text{GPT} \\
\text{L-Alanine + } \alpha \text{ Ketoglutarate } \xrightarrow{\text{GPT}} \text{L-Glutamate + Pyruvate}
\]

\[
\text{LDH} \\
\text{Pyruvate + NADH + H}^+ \xrightleftharpoons{\text{LDH}} \text{L-Lactate + NAD}^+
\]

There is decrease in absorption at 340 nm as NADH was converted to NAD. The rate of decrease in absorbance was measured and was proportional to SGPT activity in the sample.

Reagents

Reagent 1 (Enzymes)
- LDH \( \geq 1200 \text{ U/L} \)
- NADH 0.20 mmol/L
- \( \alpha \)-Ketoglutarate 16 mmol/L

Reagent 1A (Buffer)
- Tris buffer, pH 7.50 110 mmol/L
- L-Alanine 550 mmol/L
Reagent reconstitution / Preparation of the working solutions
The reagents were allowed to attain room temperature. The contents of one bottle of reagent 1 were dissolved with one bottle of reagent 1A. The contents were mixed by gentle swirling and used after 5 minutes. The reconstituted reagent is stable for 4 weeks when stored at 2 – 8 °C.

Procedure
1. Autoanalyzer was set to the software for the test as per parameters for SGPT, shown in the table below and was calibrated using distilled water. The samples and the reconstituted reagent were brought to room temperature prior to use.
2. The working solution and serum were taken as shown below

<table>
<thead>
<tr>
<th>Dispense into test tube</th>
<th>Reconstituted reagent</th>
<th>1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

After adding the working solution to the tube containing the proper mixing was done and the absorbance was immediately read at 340 nm.

Note
Samples with a very high SGPT activity cause an excessive consumption of NADH, resulting in a very low initial absorbance and/or non-linear reaction. When this occurs the assay should be repeated with a diluted sample.

4.8.5.3 Method for estimation of γ GTP / GGTP by UV Kinetic (Szasz) method

Principle
Gamma-Glutamyl Transpeptidase catalyses the transfer of the Gamma-Glutamyl group from the substrate Gamma-Glutamyl para-Nitroanilide (γ-glutamyl peptides) to Glycylglycine (other peptide and L-amino acids), releasing free p-Nitroaniline which absorbs light at 405 nm. In this L-γ-glutamyl-p-nitroanilide is used as a
substrate and glycylglycine as acceptor. Enzyme activity is proportional to the increase in absorbance at this wavelength

\[
\gamma\text{-GTP}
\]

\[
\text{GPNA} + \text{Glycylglycine} \rightarrow L-\gamma\text{-Glutamyl-Glycylglycine} + p\text{-Nitroaniline}
\]

\[
\text{GPNA} = L-\gamma\text{-Glutamyl-}p\text{-Nitroanilide}
\]

Reagents

Reagent 1 (Substrate)
- Glycylglycine 94 mmol/L
- L-\gamma - \text{Glutamyl-}p\text{-Nitroanilide} 3.2 mmol/L

Reagent 1A (Buffer)
- Tris buffer, pH 8.20 200 mmol/L
- Surfactant 0.2 %

Reagent reconstitution
The reagents were allowed to attain room temperature. 3 ml of reagent 1A was added to one bottle of reagent 1. The contents were mixed by gentle swirling for complete dissolution and used after 5 minutes.
The reconstituted reagent is stable for 3 weeks when stored at 2 - 8 °C.

Procedure

1. Autoanalyzer was set to the software for the test as per parameters for \( \gamma \) GTP / GGTP, shown in the table below and was calibrated using distilled water.
The samples and the reconstituted reagent were brought to room temperature prior to use.

2. The working solution and serum were taken as shown below

<table>
<thead>
<tr>
<th>Dispense into test tube</th>
<th>Reconstituted reagent</th>
<th>1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>100 ( \mu )L</td>
<td></td>
</tr>
</tbody>
</table>
After adding the working solution to the tube containing the serum proper mixing was done and the absorbance was read immediately.

4.8.5.4 Estimation of Blood Glutathion / GSH (Beutler et al., 1963).

Principle

The sulfhydryl compound of Glutathion, released from RBCs on haemolysis, is reacted with the DTNB. It gives yellow coloured product. Optical density of the product and the blank are measured at 412 nm.

Reagents

Precipitating solution: 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of sodium chloride were dissolved separately in minute quantity of distilled water. The three solutions were mixed together and volume was made upto 100 ml with distilled water. This solution was found stable upto 3 weeks at 5°C.

Phosphate solution (0.3 M) 23.10 g of disodium hydrogen phosphate was dissolved in distilled water and the volume was made upto 500 ml (stored at 4°C).

DTNB Reagent 1g of sodium citrate was dissolved in 100 ml of distilled water. Then, 40 mg of DTNB was added to the above solution and mixed until it dissolved.

Procedure

0.2 ml of fresh whole blood was collected from the animal and 1.8 ml distilled water and 3.0 ml precipitating solution were added to the above mixture. The mixture was allowed to stand for approximately 5 min and then filtered. 2.0 ml of filtrate was added to 8.0 ml of phosphate solution in a test tube. A blank was prepared with 8 ml of phosphate solution, 2.0 ml of dilute precipitating solution (3
parts to 2 parts of distilled water) and 1.0 ml of DTNB reagent. The O.D. was measured at 412 nm.

**Calculation**

Blood Glutathion reduced was calculated by the following formula and expressed as mg%:

\[ \text{GSH mg \%} = 31.04 \times E_1 \times \text{O.D.} \]

Where - O.D. is Optical Density at 412 nm and \( E_1 \) is the Correction factor (0.542)

**4.8.6 The Auto analyzer ERBA CHEM-5 Plus (TRANSASIA)**

It is a photometric, microprocessor based chromatic measurement equipment, used for analysis of substrate, enzyme and drug assays. Parameters of the desired chemistry; Absorbance, Concentration (linear and non-linear), Fixed time, Kinetic and Coagulation modes, can be accessed, changed, entered and stored.

Kinetic Mode is for the enzymatic biochemical estimations and has been used in the study. Where the change in absorbance over a given time is monitored and is used to calculate the result.

After feeding and storing the parameters for the chemistry operational steps were followed as below:

Distilled water was aspirated and the instrument is autozeroed using ZERO key. Reaction mixture (Reagent + Sample) to be analysed was aspirated. ENTER was pressed to start the delay time countdown.

The acoustic beep indicated the end of analysis and the result was displayed. For the next sample the RUN TEST key was pressed followed by the distilled water aspiration.
### Table 10: Parameters of Autoanalyzer for the biochemical estimations

The parameters for each chemistry; SGOT, SGPT and GGTP were fed as per the required specifications of the kits, which are mention below:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SGOT</th>
<th>SGPT</th>
<th>GGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Mode (N) / Reaction type</td>
<td>KIN</td>
<td>KIN</td>
<td>KIN</td>
</tr>
<tr>
<td>ii. Reaction slope</td>
<td>Decreasing</td>
<td>Decreasing</td>
<td>Increasing</td>
</tr>
<tr>
<td>iii. Filter 1 / Wave length (nm)</td>
<td>340</td>
<td>340</td>
<td>405</td>
</tr>
<tr>
<td>iv. Filter 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>v. Factor</td>
<td>1746</td>
<td>1746</td>
<td>1111</td>
</tr>
<tr>
<td>vi. Standard</td>
<td>0000</td>
<td>0000</td>
<td>0000</td>
</tr>
<tr>
<td>vii. N. V. Max</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>viii. N. V. Min</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>ix. Temp °C</td>
<td>37</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>x. Delay Time (sec.)</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>xi. Rate Time</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>xii. Read Numbers</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>xiii. Interval (sec.)</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>xiv. Abs Min</td>
<td>800</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>xv. Lin Lim</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>xvi. Unit</td>
<td>U / L</td>
<td>U / L</td>
<td>U / L</td>
</tr>
<tr>
<td>xvii. Asp Vol (µl)</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>xviii. QC Value</td>
<td>0050 - 0076</td>
<td>0050-0076</td>
<td>0050-0076</td>
</tr>
<tr>
<td>xix. Reag (ml) / Samp Vol (µl)</td>
<td>1 / 100</td>
<td>1 / 100</td>
<td>1 / 100</td>
</tr>
<tr>
<td>xx. Path length (cm)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>xxi. Zero setting with</td>
<td>Dist water</td>
<td>Dist water</td>
<td>Dist water</td>
</tr>
</tbody>
</table>
4.8.7 Automated Hematology Analyzer K-1000, Sysmex

It is a quantitative hematology analyzer for in vitro diagnostic use in laboratories. Its working principle utilizes the electric resistance detection method. The aspirated, measured, diluted sample is fed into the Transducer Chamber. During passage of cells through transducer aperture, where a constant DC current flows from an internal electrode to an external electrode, there occurs change in the resistance of the conductive diluent. It is recorded as increase in the voltage between the electrodes, which is proportional to the size of cells.

Reagent System

The reagents required are a diluent, two lysing reagents and a detergent.

*Commercial diluent* contains; Sodium chloride 6.38 g/L, Boric acid 1.00 g/L, Sodium tetraborate 0.20 g/L, EDTA-2K⁺ 0.20 g/L

*WBC lysing reagent* lyses RBCs to eliminate RBC stroma for WBC count. Commercial WBC lysing reagent contains; organic quaternary ammonium salt 10.2 g/L, Sod chloride 2.0 g/L

*Hgb lysing reagent* lyses RBC and forms the complex hemoglobin/cyanide/cyanmethemoglobin. It contains Potassium cyanide 1.3 g/L, Organic quaternary ammonium salt 4.0 g/L, Sod sulphate anhydrous 11.0 g/L.

Desirable anticoagulant for sampled blood is EDTA.

Detection principles

<table>
<thead>
<tr>
<th>Component</th>
<th>Detection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>Electric Resistance Detection</td>
</tr>
<tr>
<td>RBC</td>
<td>Electric Resistance Detection</td>
</tr>
<tr>
<td>Hgb</td>
<td>Cyanmethemoglobin or SLS-hemoglobin at 540 nm</td>
</tr>
<tr>
<td>PLT</td>
<td>Electric Resistance Detection</td>
</tr>
</tbody>
</table>
Whole Blood mode was used. The sample requirement is 0.1 ml. The dilution ratio in this mode are as in the following Table 11.

Table 11: Sample (dilution) flow of Automated hematology analyzer

<table>
<thead>
<tr>
<th></th>
<th>WBC</th>
<th>RBC &amp; PLTL</th>
<th>HGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood sample with EDTA</td>
<td>12 μL</td>
<td>4 μL</td>
<td>6 μL</td>
</tr>
<tr>
<td>Dilution 1st</td>
<td>1:250</td>
<td>1:500</td>
<td>1:500</td>
</tr>
<tr>
<td>Diluent</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Dilution 2nd</td>
<td></td>
<td>40 μL of 1:500 → (1:50)</td>
<td>+</td>
</tr>
<tr>
<td>Diluent</td>
<td></td>
<td>2 ml</td>
<td>1 ml Hgb</td>
</tr>
<tr>
<td>Final dilution</td>
<td></td>
<td>1:25000</td>
<td>reagent</td>
</tr>
<tr>
<td>Lysing reagent</td>
<td>1 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol of diluted sample for count</td>
<td>0.4 ml</td>
<td>0.25 ml</td>
<td>Whole</td>
</tr>
<tr>
<td>No of cells counted</td>
<td>8000</td>
<td>50 000</td>
<td></td>
</tr>
<tr>
<td>Aperture size</td>
<td>100 μm dia</td>
<td>75 μm dia</td>
<td></td>
</tr>
<tr>
<td>Counting time</td>
<td>8 sec</td>
<td>9 sec</td>
<td></td>
</tr>
<tr>
<td>Absorbance</td>
<td></td>
<td></td>
<td>540 nm</td>
</tr>
<tr>
<td>Accuracy</td>
<td>± 3.0 %</td>
<td>± 2.0 %</td>
<td>± 5.0 %</td>
</tr>
</tbody>
</table>

Precautions

- Sample should be analysed within 12 hrs of collection.
- Cell count is based on the particle size count, changes in cell volume and particle density during storage after collection may affect the results.
- Conductivity of the diluent is critical, alteration in composition or make may influence the results.
- EDTA in the sample may affect the Platelet count.
- Roulex formation may give false results.
- Aggregated platelets may give false results.
- Higher temperature decreases conductivity thus influences the results.
Agglutinated protein particles may be interpreted as cells.

Leukocytosis above 100,000 /μL, lipaemia, abnormal proteins in plasma falsely elevate Hgb result. Such plasma may be run as blank or eliminated.

4.8.8 Blood Clotting Time

Blood coagulation time was recorded according to the method described by Ghai, 1990. Under light anaesthesia blood was withdrawn from the orbital plexus of the eye of rat in a glass capillary tube. The time was noted and a small piece (about one cm) of the glass capillary tube was broken from one end and this was repeated every thirty seconds till fibrin threads of blood appeared between the broken ends.

4.8.9 General Behavioural study

The animals in the sub-acute toxicity study were observed for general behaviour after 1 hr of the 3rd day dose. Various profiles were observed by the method described by Turner, 1965. The effects were scored with nine degrees i.e. 0 to 8. For normal signs / effects score is 4. Score below 4 are sub-normal, and above 4 are super normal.

4.8.10 Histopathological studies (Belur and Kandaswamy, 1990)

The rats were sacrificed under light ether anaesthesia and livers were removed and washed with normal saline. Small pieces of liver section (tissues) were collected in 10% buffered formal saline for proper fixation. Fixed tissues were then subjected to staining procedure as follows.

Routine Hematoxylin and Eosin staining; various steps involved in this staining were:

a) Dehydration: The water content of the tissues was replaced using increasing concentrations of ethanol.

80% ethanol – 1 hr
95% ethanol – 1 hr (2 changes)
100% ethanol – 1 hr

b) Clearing: The reagent used for clearing must be miscible with dehydrant and paraffin. When the dehydrant was removed, the tissue cleared and became translucent signifying the completion of the process. Xylene was used as the clearing agent.

c) Impregnation: Complete removal of clearing agents by substitution was done by paraffin wax as it penetrates the tissue. Impregnation was done with 3 paraffin baths for 3 hrs. Paraffin with melting point of 56-58°C was used. Precaution was taken so that heating 5°C above the melting point of paraffin was avoided, which might shrink and harden the tissue. The tissues were then cast into blocks of paraffin wax.

d) The blocks were kept for freezing. The frozen blocks were carefully taken and sections of the tissue, 5-6 microns in thickness were cut with the help of a rotary microtome.

e) The section ribbons were made and floated on warm water and then placed on glass slides to remove wrinkles, slightly warmed and dried.

f) Hydration: The sections were hydrated with Xylene for 2 min and 70% alcohol for 1 min. They were then rinsed with distilled water.

g) Staining: Sections were stained with 1% hematoxylin (3 min) rinsed with distilled water and then 1% Eosin in 90% alcohol was added for 1 min, slides were then dried.

h) The stained sections were then covered with DNP mounting agent and cover slips were placed carefully on the sections, taking care that no air bubble entered.

4.8.11 Statistical Analysis (Kapoor and Gupta, 1978; Armitage and Berry, 1985)

Results of the biochemical parameters are reported as Mean ± SD. Total variation present in a set of data was estimated by one way Analysis of Variance (ANOVA). It was followed by Dunnett's 't' test, which was used to identify differences
between Means of groups. P values < 0.05 (95% confidence level) were considered significant. The F-ratio was also calculated. Percentage inhibition in the paw volume with respect to the Toxic control group was calculated.

4.9 Standardization of the extracts of leaves of *Lawsonia inermis* Linn.

Experiment 6 Qualitative phytochemical tests for the constituents in *Lawsonia inermis* Linn. leaf extracts.

These extracts were subjected to phytochemical analysis for the primary / secondary metabolites (Zafar et al, 2004) as mentioned below and the observations were recorded.

4.9.1 Alkaloids

5 ml of alcoholic leaf extract was evaporated to dryness and the resultant residue dissolved in dilute sulphuric acid, followed by addition of either of the following reagents:

1. Dragendorff's Reagent (Solution of potassium bismuth iodide)
   Reddish brown precipitate indicated positive result.
2. Hager's Reagent (Saturated solution of picric acid in water)
   Yellowish precipitate indicated the positive result.
3. Mayer's reagent (Solution of potassium mercuric iodide)
   White or yellow precipitate indicated positive result.

4.9.2. Amino Acids

Alcoholic leaf extract was concentrated, a few drops of Ninhydrin reagent was added and the resultant solution was heated. A pink violet color appeared indicating presence of the amino acids.
4.9.3  Anthraquinones (Borntrager test)

The Lawsonia leaves were extracted with benzene and filtered. To the filtrate, dilute ammonia solution was added which showed no appearance of pink color in the ammonical layer confirming the absence of free anthraquinones.

4.9.4  Carbohydrates

4.9.4.1  Molisch’s test

To about 2 ml of the alcoholic extract, few drops of Molisch Reagent (20% α-naphthol in ethyl alcohol) were added. This was followed by the addition of 1.5 ml of concentrated H₂SO₄ along the side of the tube. Red ring at the junction of two layers indicated the presence of carbohydrates.

4.9.4.2  Fehling’s test

About 2 ml each of Fehling Solutions A & B were added to the alcoholic extract and the solution was heated on a boiling water bath. Brick red precipitate indicated presence of reducing sugar.

4.9.5  Flavonoids

Aqueous extract was heated on a water bath and filtered. Dilute sulphuric acid was added to the filtrate, which was then heated for 15 minutes. It was extracted with chloroform. To chloroform layer, few pieces of zinc granules were added followed by the addition of a drop of concentrated hydrochloric acid. A pink color appeared indicating presence of flavonoids.
4.9.6 Phenolics

4.9.6.1 Ferric chloride test

A few ml of the alcoholic extract was evaporated to dryness. Blue or green color on addition of 5\% FeCl\(_3\) indicated the presence of phenolic compounds.

4.9.6.2 Lead acetate test

Addition of a few drops of lead acetate solution (5\%) to the aqueous extract resulted in production of yellow white precipitate. It indicated the presence of phenolics.

4.9.7 Proteins

Million's test: To the alcoholic extract, few drops of Million's reagent were added. Formation of the white precipitate, which turned red on heating, indicated presence of proteins.

4.9.8 Saponins

The dried powder of leaves was shaken with water. Production of soap like froth indicated presence of saponins.

4.9.9 Steroids

The alcoholic extract was evaporated to dryness and residues were extracted with petroleum ether and acetone. The insoluble residue left after extraction with petroleum ether and acetone was tested for sterols as follows:
Salkowski Reaction: To the extract, 2 ml of concentrated sulphuric acid was added. The appearance of a yellow ring at the junction which turned red after 1 minute confirmed the presence of sterols.

4.10 Qualitative HPLC analysis of Lawsonia leaf extracts for Rutin

The qualitative determination of rutin was performed by HPLC minor by modification of the method described by Duber & Kanfer, 2004, as mentioned below:

4.10.1 Preparation of standard solutions

5 mg rutin was dissolved in 5 ml of ethanol. 1ml of this solution was further diluted to 10 ml with ethanol to produce a solution of 100 μg/ml. The solution was filtered through 0.20μm sample filtration assembly.

4.10.2 Preparation of Lawsonia leaf extracts

500 mg of the dried extract was extracted with 50 ml HPLC grade ethanol on a water bath maintained at 60°C for 3 hours. The extract was cooled and filtered. Excess of the solvent in filtrate was evaporated and final filtrate volume adjusted to 10 ml. Hence, all the extracts had a final concentration of 50 mg/ml (dry weight basis). All the test samples were filtered through 0.20μm sample filtration assembly.

4.10.3.1 HPLC set-up

<table>
<thead>
<tr>
<th>Chromatographic system</th>
<th>Binary Gradient Shimadzu HPLC system with WinChrom software and UV-Visible detector operating at 220nm wavelength.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Chromatographic runs were performed on a YMC ODS-AM 303 column (300 x 4.6 mm i.d., 5μm).</td>
</tr>
</tbody>
</table>
Mobile phase It was constituted by water-acetonitrile (65:35) under gradient elution condition. The mobile phase was filtered through 0.45 \( \mu \text{m} \) filter in the HPLC solvent filtration assembly and then degassed.

4.10.3.2 Procedure

The equilibration time was 12.5 minutes for standard rutin solution and test samples. The flow rate was 0.5 ml/min and samples of 20 \( \mu \)l were injected.

4.11 To determine the flavonoid subtypes present in Lawsonia leaves

Lawsonia leaves had shown a positive test for flavonoids. Hence determination of the subtype/s of flavonols present was carried out by recording the spectra-scans for different extracts of Lawsonia.

Procedure

Spectrum scan from 200-400 nm was performed on the spectrophotometer for alcoholic, aqueous and chloroform extracts of Lawsonia leaves. The peaks were qualitatively matched with the reported peaks of flavonoids as reported by Harborne, 1997.

To record UV spectrum of rutin

This experiment was performed in order to find the absorption maxima of rutin on the spectrophotometer on which the quantification of flavonols with respect to rutin was to be performed.
Chapter 4

Material & Methods

Procedure

Spectrum scan from 200-400 nm was performed on the spectrophotometer for standard rutin solution. The absorption maxima peaks were noted.

To quantify the flavonol content of alcoholic extract of Lawsonia leaves

As the spectrum scan of alcoholic extract of Lawsonia leaves had revealed presence of flavonols the quantification of the flavonols was carried out.

Procedure

In view of the rutin absorption maxima at 268 nm (Experiment no. , page ), the Therapeutics Goods Administration method (Anonymous, 2005) was modified by preparing a standard curve for rutin.

The standards and test sample were stirred for 1 hour and volume was made to 8 ml with 0.05 M sodium hydroxide. After making the volume to 10 ml with sodium hydroxide, the contents were transferred to centrifuge tubes and subjected to centrifugation at 5000 rpm for 10 min. After 10 min, the supernatant liquid was decanted. One ml of the superlative liquid was made to 10 ml with 0.05 M NaOH. Thus, the stock solution of rutin 1 mg/ml (1000 μg/ml) in 0.05 M sodium hydroxide was obtained from which was diluted to prepare following working standards:

2.5 μg/ml in 0.05 M Sodium hydroxide.
5.0 μg/ml in 0.05 M Sodium hydroxide.
7.5 μg/ml in 0.05 M Sodium hydroxide.
Blank (0.05 M Sodium hydroxide) was labelled as 0 μg/ml rutin.

The absorption of test and standard solutions was noted.

A working standard curve was prepared by plotting the absorbance of rutin standards versus concentration at 268 nm. The concentration of the flavonols in alcoholic extract of Lawsonia leaves was found by extrapolation on the standard curve of rutin.
Evaluation of alcoholic extract of Lawsonia leaves for lawsone.

Lawsonia leaves had shown a positive test for phenolics and flavonoids (Experiment, Page). Hence, it was considered worthwhile to screen the alcoholic leaf extract of Lawsonia for the presence or absence of lawsone by recording the spectra-scans for alcoholic extract of henna.

Procedure

Spectrum scan from 400-700 nm was performed on the spectrophotometer for alcoholic extracts of Lawsonia leaves. The peaks (nm) were qualitatively matched with the reported peaks of lawsone as reported by Pratibha and Korwar, 1999 and Keck et al, 2002.