# CHAPTER-3

## THEORETICAL ANALYSIS

### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the sub-title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Theoretical analysis</td>
<td>43</td>
</tr>
<tr>
<td>3.1</td>
<td>Animal models for anti-nociceptive activity</td>
<td>43</td>
</tr>
<tr>
<td>3.2</td>
<td>Animal models for anti-inflammatory activity</td>
<td>45</td>
</tr>
<tr>
<td>3.3</td>
<td>Free radical scavenging and hepatoprotective activity</td>
<td>50</td>
</tr>
<tr>
<td>3.4</td>
<td>Aim of the study</td>
<td>51</td>
</tr>
<tr>
<td>3.5</td>
<td>Plan of work</td>
<td>52</td>
</tr>
</tbody>
</table>
2. **Theoretical Analysis**

As mentioned earlier in the introductory section and as evident from the literature review, it is evident that *Cuscuta reflexa* is a plant with several claims; however, there is a need for the systematic investigation of pharmacological and toxicological properties is lacking. To fulfill this research need, we decided to study biochemical, pharmacological and toxicological properties of this parasitic plant.

Looking at the rich phenolic and flavonoid constituents of *Cuscuta reflexa*, which may possess antioxidant of free radical scavenging property and as it has been largely established that generation of reactive oxygen species plays an important role in pathology of various conditions like inflammation, hepatotoxicity and cancer; it was decided to evaluate analgesic, anti-inflammatory, cytotoxic, antioxidant and hepatoprotective activities of various extracts.

3.1 **Animal models for anti-nociceptive activity**

Methods involving tests on intact rodents like mice or rat are necessary in research of analgesics before a compound can be given to man. Several tests are available for determination of analgesic activity, such as

- HAFFNER’s tail clip method in mice,
- Tail flick or other radiant heat methods,
- Tail immersion tests,
- Hot plate methods in mice or rats,
- Electrical stimulation,
Writhing test,

Formalin test in rats

3.1.1 **Hot plate method**

This test has been used by many researchers for evaluation of centrally acting analgesics. Mice and rats can be used as it has been observed that these animals are particularly sensitive to the analgesia induced by hot stimulus at 50-60°C. When an animal is put on a hot surface, it cannot tolerate the thermal nociception and starts jumping-off. Administration of centrally acting analgesics like pentazocin prolongs the latency time, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses\(^5\).

3.1.2 **Acetic acid induced writhings**

Writhing responses are characterized by abdominal contractions followed by extension of hind limbs, induced by intraperitoneal injection of acetic acid. The writhing test is simple and sensitive tool for rapid evaluation of mild analgesic non-steroidal antiinflammatory drugs. Intraperitoneal administration of acetic acid causes release of inflammatory mediators like cyclooxygenase(COX), lipoxygenase (LOX), prostaglandins(PGs), histamine, serotonin, bradykinin, substance-P, IL-1b, IL-8, TNF-\(\alpha\) in the peripheral tissue fluid. Increased level of these mediators causes the sensitization of primary afferent nociceptors entering dorsal horn of the central nervous system. These mechanisms are responsible for the development of inflammatory pain and
abdominal constriction and this effect is supposed to be a peripheral pathway\textsuperscript{52}.

### 3.1.3 Formalin test

Formalin test involves sub cutaneous injection of formalin in plantar surface of paws in mice which produces its response in two different phases and the effect produced by central analgesics and peripheral analgesics is different and hence the this test serves as a good tool to differentiate between different mechanisms involved in analgesic activity of any drug\textsuperscript{53}. Early phase is characterized by neurogenic pain, which is induced by direct chemical stimulation of the nociceptors, particularly C-fibers. The involvement of substance-P and bradykinin has also been reported. Centrally acting drugs like opioids inhibit both phases equally. In the late phase on the other hand, inflammatory pain is induced by production of different inflammatory mediators like prostaglandins (PGs), histamine, bradykinin and serotonin in peripheral tissues\textsuperscript{54}.

### 3.2 Animal models for induction of inflammation

Inflammation is a series of events induced by numerous stimuli, e.g., infectious agents, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury. Inflammation is characterized by clinical signs of erythema, edema, hyperalgesia, pain and loss of function. Inflammatory responses involve three distinct phases, involving different mechanisms; acute inflammation, characterized by local vasodilatation and increased capillary permeability, subacute inflammation, characterized by infiltration of leukocytes
and phagocytic cells and chronic granulomatous inflammation, in which tissue
degeneration and fibrosis occur\textsuperscript{46}.

According to these phases, pharmacological methods have been
developed\textsuperscript{51}.

Methods inducing acute and subacute inflammation are:

- Ultraviolet rays induced skin hyperemia
- Effect on vascular dilatation
- Irritant mediated ear edema
- Pedal inflammation in rats (various modifications and various irritants)
- Pleurisy tests
- Granulomatous inflammation

The proliferative granulomatous inflammation is induced by:

- Cotton wool granuloma
- Glass rod granuloma
- PVC sponge granuloma.

Furthermore, methods for testing immunological factors have been
developed, such as:

- Adjuvant arthritis in rats.

3.2.1 Paw edema

Though many methods are available for screening of antiinflammatory
drugs, one of the most frequently used models is carrageenan induced paw
edema. Subcutaneous injection of carrageenan induces acute inflammation,
characterized by vasodilation and leukocyte infiltration resulting in
development of paw edema. Edema can be measured by recording the volume of the paw is measured before and after injection of the irritant.

Various devices have been developed for recording volume of the paw edema.

Many irritant substances including inflammatory mediators like histamine and serotonin, yeast, formalin, dextran, egg white, etc can also be used to induce paw edema\textsuperscript{52}.

\subsection*{3.2.2 Cotton pellet granuloma}

Meier and co-workers\textsuperscript{55} showed that surgical implantation of sterilized cotton pellet can induce foreign body granulomtous inflammation in rats, characterized by accumulation of giant cells and undifferentiated connective tissue along with fluid infiltration. The extent of granulomaformation can be estimated from the difference in the weight of cotton pellets before and after the test. This method has been successfully used for evaluation of steroidal and nonsteroidal anti-inflammatory drugs from several years.

\subsection*{3.2.3 Gastric ulcerogenicity test}

Adverse effects associated with the use of conventional NSAIDS include, gastrointestinal side effects e.g. gastrointestinal ulceration, bleeding and perforation in some rare cases. These local side effects may result from inhibition of biosynthesis of protective prostaglandins. Ulcerogenicity test in rats determines gastric irritation properties of orally administered compounds in fasted rats. For this animals are sacrificed after predetermined time intervals after the treatment and the stomachs are inspected for irritation and ulcers.
after removal. There is a good correlation between gastro-intestinal side effects in man and the ulcerogenic effects in rats and hence this test is a good tool to determine adverse effects as well as a probable mechanism of new drug\textsuperscript{52}.

### 3.2.4 Acetic acid induced vascular permeability

This test is frequently used to determine the mechanism involved in antiinflammatory effect of new drugs. The method involves evaluation of the inhibition of vascular permeability induced by intraperitoneal injection of acetic acid which causes mast cells to release histamine and other mediators of inflammation, resulting in increased permeability of the vascular bed. The overall effect is accumulation of fluid and plasma proteins giving rise to the formation of edema. Due to these changes, the dye solution administered intravenously is leaked in to the peritoneal cavity\textsuperscript{52}.

### 3.2.5 Leukocyte migration test

Exudative inflammation can also be induced by administration of compounds like dextran. Leukocytes migration induced by dextran, is used to analyze the effect of new drugs on leukocyte migration. Dextran induced leukocyte migration causes extrusion of fluid rich in proteins and leukocytes in the peritoneal cavity of an animal\textsuperscript{56}. Animals pretreated with drugs and challenged by intra peritoneal injection of dextran are sacrificed at the end of the study and the fluid in peritoneal cavity is collected for measuring the white blood cell number in the exudates using a hematocytometer\textsuperscript{56}.

### 3.2.6 Membrane stabilization study
Human red blood cell stabilization assay is frequently used to test the effect of drugs on lysosomal membrane stabilization. It is well known that agents that inhibit hypotonicity induced lysis of RBC’s are capable of stabilizing the lysosomal membrane which exerts protective effect against inflammation\textsuperscript{56}.

3.2.7 Experimental arthritis

Experimental polyarthritis can be induced in rats by injection of irritants like formaldehyde and immunological preparation like Freunds Complete Adjuvant. Both formaldehyde and FCA induce arthritis in rats that has many similarities to human rheumatoid arthritis. Subcutaneous injection of FCA into the rat paw causes edema as primary lesion within 3 to 5 days followed by secondary lesions occurring after a delay of approximately 11 to 12 days after FCA challenge. Secondary lesions are characterized by inflammation of non-injected sites like forepaws, ears, nose and tail, a decrease of body weight and immune responses. Anti-inflammatory compounds inhibit progression of paw edema\textsuperscript{57}.

3.3 Free radical scavenging and hepatoprotective activity

CCl\textsubscript{4} induced hepatotoxicity is a widely and extensively used animal model for assessment of hepatoprotective activity. CCl\textsubscript{4} causes lipid peroxidation in hepatocytes producing a toxic effect and a subsequent injury to the hepatocytes. CCl\textsubscript{4} is metabolized to produce the trichloromethylradical through the cytochromeP450monooxygenase system, which then reacts with oxygen to form the trichloromethyl-peroxylradical\textsuperscript{58}. Radicals generated in the metabolism of CCl\textsubscript{4} causes damage to cell organelles like proteins or lipids,
there by leading to lipid peroxidation and cell necrosis in parts of the liver. Injury to the hepatocytes is characterized by elevation of metabolic enzyme markers e.g. ALT, AST and ALP. Serum total bilirubin (TB) which is an index of normal hepatic metabolism is found to be increased in toxic rats. Various free radical scavengers, dietary antioxidants and medicinal plants are found to inhibit these deterioration related changes and exert hepatoprotective effect.

### 3.4 Aim of study

The aim of present work is to investigate the biochemical, pharmacological and toxicological effects of this parasitic plant *Cuscuta reflexa* on experimental animals. We hypothesize that as the plant is known to contain abundant phenolics and flavonoids, it will have beneficial effects on pathological states involving pain, inflammation and other conditions involving damage due to reactive oxygen species. We further hypothesize that plant extracts will have beneficial effects on conventional animal models of pain, inflammation, arthritis and hepatoprotective by virtue of its antioxidant property.

Following were the objectives identified to achieve the above cited aim of the work

1. To investigate the usefulness of extracts of *Cuscuta reflexa* in animal models of analgesic, antiinflammatory, antiarthritic and anthelmintic activity.
2. To investigate the effect of the plant extracts on biochemical parameters in experimental animal models of analgesic, antiinflammatory, antiarthritic and hepatoprotective activity.

3. To investigate toxicological effects of plant extracts on animal models of various toxicity tests, such as acute and subacute toxicity study.

3.5 Plan of work

Present study is aimed to explore biochemical, pharmacological and toxicological effects exerted by extracts of the plant *Cuscuta reflexa* on experimental animals according to following action plan.
Plan of work

1. Collection of Plant material
2. Preparation of extracts
3. Phytochemical investigations
4. Assay for total phenolics
5. Assay for total flavonoids
6. HPTLC fingerprinting of extracts of Cuscuta reflexa
7. Isolation and characterization of phytoconstituents
8. Toxicological investigations
9. Acute oral toxicity study
10. Sub-acute oral toxicity study
11. Pharmacological and biochemical investigations
12. Anti-nociceptive activity
13. Anti-inflammatory activity
14. Anti-arthritic activity
15. Antioxidant and hepatoprotective Activities
16. Anthelmintic activity