9. **In vivo study**

One of intestinal pathological state such as “Melanosis coli” develops in the colonic region due to prolonged intake of anthraquinone laxatives. Some investigators have suggested that increase in apoptosis of colonic mucosa by anthraquinone laxatives increased the risk of colonic cancer. Treatment of melanosis coli has not been recognized yet (Kew et al., 2012, Freeman et al., 2008). Often, a recommendation is made to manage symptomatic constipation with fiber containing foods or substances with mucilage, including psyllium, along with avoidance of anthraquinone cathartics. Therefore, considering *Melanosis Coli* as a precancerous stage, it became important to develop a delivery system that successfully delivers the drug to large intestine (colon). Present study describes the *in vivo* localization of developed CUR loaded self emulsifying nanocapsules to inflammed colon. The protocol followed in the study was approved by the institutional animal ethical committee of M.M. College of Pharmacy (MMCP/IAEC/11/23).

9.1. **Induction of melanosis coli**

Overnight fasted guinea pigs, 250-300 g (n=5) were divided randomly into six groups (n = 5) as defined in Table 39. They were fed with pre-weighed fixed normal guinea pig’s diet and were allowed free access to water. All groups were fed with 25 mg/kg sennosides (mixture of isomer A, B, C and D) except group VI (control group). Known weight of sennosides was suspended in 4% sucrose solution and administered orally in 2 ml daily doses to animals. Animals were kept under observation for 30 days.

9.2. **In vivo localization of optimal formulation to inflammed colon**

*In vivo* localization of CUR loaded nanocapsule optimal formulation to inflammed colon was done using a colitis guinea pig model to mimic melanosis environments. Test formulation (100 mg/kg equivalent weight of PSN formulation; P5 daily) was suspended in 4% sucrose solution and administered orally in 2 ml daily doses using polyethylene tubing. Reference formulation was also administered using polyethylene tubing to animals used for study. Animals kept under Group II (prevention group), were administered test formulation simultaneously with sennosides for 30 day. In contrast, group III were administered test formulation after 15 days intake of sennosides. Animals kept under standard group V, were administered reference formulation (prepared in section 7.2); for 16th to 30th day, after 15 days intake of sennosides. Whereas, animals kept under Group IV were administered standard formulations simultaneously with sennosides for 30 day. All the animals were kept under observation for 30 days. Chronological protocol followed in dosing to various animals groups is given in Table 39.
Table 39: Protocol of dosing to various animal groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Coding</th>
<th>Schedule (day)</th>
<th>Dose Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Positive Control</td>
<td>0-30</td>
<td>Sennosides</td>
</tr>
<tr>
<td>II</td>
<td>Prevention</td>
<td>0-30</td>
<td>Sennosides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-30</td>
<td>PSN Formulation</td>
</tr>
<tr>
<td>III</td>
<td>Treatment</td>
<td>0&lt;sup&gt;th&lt;/sup&gt;-15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Sennosides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16&lt;sup&gt;th&lt;/sup&gt;-30&lt;sup&gt;th&lt;/sup&gt;</td>
<td>PSN Formulation</td>
</tr>
<tr>
<td>IV</td>
<td>Standard (Prevention)</td>
<td>0-30</td>
<td>Sennosides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-30</td>
<td>Reference Formulation</td>
</tr>
<tr>
<td>V</td>
<td>Standard (Treatment)</td>
<td>0&lt;sup&gt;th&lt;/sup&gt;-15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Sennosides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16&lt;sup&gt;th&lt;/sup&gt;-30&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Reference Formulation</td>
</tr>
<tr>
<td>VI</td>
<td>Control</td>
<td>0-30</td>
<td>Vehicle</td>
</tr>
</tbody>
</table>

9.3. Characterization of colitis in guinea pig

9.3.1. Physical observation
Treated animals were kept under observation for 30 days. Their dietary intake, body weight and stool consistency were recorded regularly.

9.3.2. Histopathogical evaluation
Animals were sacrificed after 30 days of test solution intake and entire GIT section (stomach, intestine, rectum, ileum) was removed for pathological analysis. Tissue samples were cleaned with repeated saline washing, fixed with 10% formalin and preserved. Samples were processed and fixed in paraffin followed by hematoxylin and eosin staining.

9.3.3. Determination of tumour necrosis factor (TNF-α)
TNF-α level was detected in tissue (removed from each group’s animal) using sandwich ELISA method as per the instruction given in the test kit (BD biosciences, TNF-α ELISA kit). 100 µl of diluted captured antibody (anti human TNF-α) was added to each well and incubated at 4°C for overnight. Samples were aspirated and washed 5 times. 200 µl of assay diluent was added to each well to prepare block plates and incubated at 25°C for 1 h. Samples were aspirated and washed 5 times. 100 µl of diluted detection antibody (biotinylated anti human TNF-α) was added to each well to prepare block plates and incubated at 25°C for 2 h. Samples were aspirated and washed 5 times. 100 µl of diluted detection antibody was added to each well and incubated at 25°C for 1 h. Samples were aspirated and washed 5 times. 100 µl of diluted Streptavidine-Horseradish peroxidase conjugate was added to each well and incubated at 25°C for 30 min. Samples were aspirated and washed 7 times (with 30 sec to 1 min soaks). 100 µl of TMB substrate solution was added to each well and incubated at 25°C for 30 min in dark. 50 µl of
In-vivo study

stop solution (1M phosphoric acid) was added to each well and absorbance was observed within 30 min at 450 nm.

9.4. Results and discussion

Present study endorses the findings of Walker et al., 1988, that administration of sennosides induces melanosis coli in the intestinal region. It has been observed during study that treatment with sennoside initiated the inflammation, as shown by swollen intestinal region in various groups upon macroscopic examination. The results also confirm the salient findings of the study (Walker et al., 1988), which states that the orally administered anthraquinone laxatives may increase the accumulation of pigments in the epithelial cells followed by apoptosis of colonic epithelium.

9.4.1. Physical changes

Colonic inflammation was confirmed by persistent reduction of body weight of animals kept under observation throughout the study. All groups treated with sennosides had passed soft stool during the study, whereas, there was no such (laxative) effect seen in control group. A continuous decrease in body weight was observed in animals treated with sennosides (Group - I, III, IV and V), whereas a slight decrease in body weight was observed in group II animals (prevention group). In contrast, the animals of group VI (control) were observed to increase their body weight with time (as shown by Figure 55).

9.4.2. Macroscopic study

After completion of study at day 30, animals treated with sennosides were sacrificed and were found to have inflamed colon (swelling and brownish in colour), as shown in Figure 56. Intestinal region of group I animals were found to remain in its normal state (pink colour). In contrast, Intestine removed from the group I animals were found to be black in colour, whereas, No such changes were observed in group II animals. Group III animals were observed with brown patches on found to remain in its normal state (pink colour). Animals kept under group IV and V were found to have chronic inflammation on intestine as shown by Figure 56.

9.4.3. Histopathology study

Inflammation was further characterized by histopathological analysis of tissues removed from various animals kept under various groups. Autopsy of the tissues removed from animals (control group) signified that the lamina propria macrophages of colon were translucent. In contrast, animals received sennosides (group II) were found to have round (brown coloured) cells in the lamina propria and mucous membrane of colon. The proximal colon was lightly pigmented, whereas, pigmentation was more intense in cecum (
Figure 57). Mucous membrane in the control group was found to be uniform whereas, disrupted membrane was observed in the case of group I (Positive control) and group IV animals. Tissues were observed with brownish pigmentation in the epithelial cells of mucous membrane. Group II and III have negligible pigmentation in the cells, signifies maintenance of normal physiology of intestine. Group I was observed with highly pigmentation area represents chronic inflammation. Group IV and V were also observed with significant pigmentation.

9.4.3. Determination of TNF-α levels in tissues

Further, apoptosis of epithelium was confirmed by increased level of TNF–α in the tissues removed from various groups. The levels of TNF-α in tissue removed from animals of various groups were found to be higher in comparison to control group (Figure 58). Results were found to be significant (P<0.05 and P<0.01).

![Figure 55: Change in body weight of animals during study](image-url)
Figure 56: Physical examination of intestine removed from guinea pig of various group after treatment at day 30
Figure 57: H & E stained (paraffin sections) of the colon obtained from animals of; I). Positive control group (group I); II). Prevention group (group II); III). Treatment group (group III); IV). Standard prevention group (group IV); V). Standard treatment group (group V) and VI). I). Control group (group VI)
9.5. Conclusion

On the basis of observed results, it can be concluded that the intake of anthraquinone laxative significantly induced Melanosis coli in the intestinal region. Results were supported by higher level of TNF-α in positive control group, which further confirmed the induction of apoptosis in the tissues. Epithelial cells of mucosal membrane were observed to be appreciably disrupted with brownish pigmentation in various groups, which are not treated with test formulation. In contrary, normal physiology was found to be maintained in animals kept under prevention group treated with curcumin loaded Self-emulsifying nanocapsule formulation.