4. Results

4.1. Preliminary pharmacological evaluation

4.1.1. Selection of Plant steroid

Topical application of Croton oil to the ears of mice resulted in significant increase in the weight of the treated left ear when compared with the untreated right ear. The topical application of 20 µl of SS, BA, GS, DG, WF, GA, SG and HG at a dose of 50 µg/mice significantly (p<0.01 & p<0.001) inhibited the ear edema (108.41%, 92.85%, 82.63%, 72.05%, 67.87%, 56.46%, 56.61%, 50.46% respectively) when compared to CO treated group (143.25%) respectively. FC (50 µg/mice) a steroidal anti-inflammatory drug exerted elevated ear edema inhibition (43.06%, p<0.001). The left ears received only vehicle acetone did not exhibit visible edema (Figure 4.1).

![Figure 4.1](image)

**Figure 4.1** Effect of Plant steroids on % inhibition on croton oil induced ear edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. b*p<0.05, c*p<0.01, a*p<0.001 represents the significance level compared with CO group.

CO = Croton oil, FC = Fluticasone, SS = Solasodine, BA = Boswellic acid, GS = Guggulsterone, DG = Diosgenin, WF = Withaferin, GA = Glycyrrhizic acid, SG = Sarsapogenin, HG = Hecogenin
4.2. Primary pharmacological evaluation

4.2.1. Croton oil induced Ear edema in mice

Effect of HG and combination on Δ Ear weight
Topical application of CO to the ears of mice resulted in significant increase in the weight of the treated right ear when compared with the untreated left ear. The topical application of HG (25 and 50 µg/mice) and combination (12.5 and 25 µg/mice, each) significantly (p<0.001) inhibited the ear weight as compared to CO treated group (35 & 55 % and 32 & 72 %), respectively. FC (25 and 50 µg/mice) a steroidal anti-inflammatory drug exerted significant ear weight inhibition (65 and 83%, p<0.001). The left ears that received only acetone as a vehicle did not show visible edema (Figure 4.2 and 4.3).

Figure 4.2 Effect of HG (25 µg) and combination on Δ ear weight in croton oil induced ear edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^{a}p<0.001\) represents the significance level compared with CO group and \(^{b}p<0.01\) compared with FC.

CO = Croton oil, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
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Figure 4.3 Effect of HG (50 μg) and combination on Δ ear weight in croton oil induced ear edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^{a}p<0.001\) represents the significance level compared with CO group and \(^{b}p<0.05\) and \(^{c}p<0.01\) compared with FC.

CO = Croton oil, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on MPO level

The effect of HG and combination treatment on ear MPO activity is shown in Figure 4.4. The MPO activity of normal control mice treated with acetone was found to be 1.15 ± 0.08 U per mg of tissue. The increase in MPO level in Croton oil group (3.65 ± 0.29 U per g of tissue) was found to be significantly prevented by both HG and combination treatment (2.66 ± 0.18 and 1.24 ± 0.11 U per g of tissue, \(p < 0.001\)), respectively.
Figure 4.4 Topical effect of HG and combination on MPO in croton oil induced ear edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \( ^a p<0.001 \) represents the significance level compared with NC group, \( ^b p<0.001 \) compared with CO; \( ^c p<0.05 \) and \( ^d p<0.001 \) compared with FC group .

CO = Croton oil, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on histopathological analysis of ear tissues

The histopathological analysis of mice ear tissues had revealed a significant increase in the thickness of dermis that was characterized by loosening of connective tissue and disorganization of extracellular matrix fibers. The increased numbers of inflammatory cells were also observed in the inflamed (right) ear tissue treated with CO (2.5 % v/v) (Figure 4.5B), when compared to non-inflamed (left) ear treated with acetone (Figure 4.5A). The treatments of ears with HG, FC and combination showed a significant reduction in infiltration of inflammatory cells and decrease in the thickness of dermis tissue (Figure 4.5C, 4.5D and 4.5E) when compared to the inflamed (right) ear treated with CO (Figure 4.5B). The mice ear sections treated with combination showed more evident effect than the individual drug treatment alone (Figure 4.5E).
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Figure 4.5 Photomicrograph of transverse sections of mice ears sensitized with topical application of croton oil (2.5% v/v) in acetone, stained with H & E and examined under light microscopy (100 X). Treatments: Acetone (A) Croton oil 2.5% v/v (B) HG (50 µg/mice) (C) FC (50 µg/mice) (D) HG + FC (25 µg/mice, each) (E). The shown sections are representative of six animals per group.

CO = Croton oil, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

4.2.2. Cotton pellet induced Granuloma in rats

Effect of HG and combination on percent inhibition (PI)
The effects of HG and combination on weight of dry cotton pellets are shown Figure 4.6. The effects of HG and combination on the proliferative phase of inflammation were calculated depending on the moist and dry weight of cotton pellets and summarized in Table 4.1. According to these results, the anti-proliferative effects of moist cotton pellets of HG and combination were found to be 78.84% and 80.76% (P<0.001) of granuloma inhibition, respectively. After they were dried, the anti-proliferative effects were calculated on the basis of dry weight of pellets and found to be 61.81% and 70.00% (P<0.001) inhibition of granuloma formation respectively. A synthetic glucocorticoids, FC showed a significant (P<0.001) inhibition of granuloma formation of 84.61% (moist pellets) and 74.54% (dry pellets) respectively.

Figure 4.6 Effects of HG and combination on weight of dry pellets in cotton pellet induced granuloma in rats. HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Table 4.1: Effects of HG and combination on mean weight of moist and dry cotton pellets and inhibition of exudates (%)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose (40 µl/ Pellet) (Topical)</th>
<th>Wt. of Moist Pellet (mg)</th>
<th>% Inhibition</th>
<th>Wt. of Dry Pellet (mg)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>Acetone</td>
<td>520.2 ± 32.3</td>
<td>0</td>
<td>110.6 ± 8.12</td>
<td>0</td>
</tr>
<tr>
<td>HG</td>
<td>50 µg/Rat</td>
<td>110.4 ± 9.10 &lt;sup&gt;a&lt;/sup&gt;, ns</td>
<td>78.84</td>
<td>42.4 ± 3.80 &lt;sup&gt;a&lt;/sup&gt;, ns</td>
<td>61.81</td>
</tr>
<tr>
<td>FC</td>
<td>50 µg/Rat</td>
<td>80.2 ± 6.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.61</td>
<td>28.8 ± 1.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.54</td>
</tr>
<tr>
<td>HG + FC</td>
<td>25 µg/Rat, Each</td>
<td>100.2 ±7.12&lt;sup&gt;a&lt;/sup&gt;, ns</td>
<td>80.76</td>
<td>33.8 ± 2.80&lt;sup&gt;a&lt;/sup&gt;, ns</td>
<td>70.00</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. <sup>a</sup>P<0.001 represents the significance level compared with DC and non-significant (ns) compared with FC.

DC = Disease control, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on adrenal and thymus gland index**

The effects of HG and combination on adrenal and thymus gland indices were studied in cotton pellets induced granuloma in rats as shown in Figure 4.7 and 4.8. The treatment of rats with acetonised cotton pellet (40 µl acetone) caused significant suppression of adrenal and thymus gland Index as compared to NC rats. Rats treated with HG, FC and combination caused significant (p<0.001) increment in the adrenal and thymus gland index as compared to DC rats treated with acetone.

![Graph showing the effect of HG and combination on adrenal gland index in cotton pellets induced granuloma in rats.](image)

**Figure 4.7** Effect of HG and combination on adrenal gland index in cotton pellets induced granuloma in rats.
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granuloma in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test.  

\[ \text{a} p < 0.001 \] represents the significance level compared with NC and \[ \text{b} p < 0.01, \ \text{c} p < 0.05 \] and non-significant (ns) compared to FC.

NC = Normal control, DC = Disease control, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

![Figure 4.8](image)

**Figure 4.8** Effect of HG and combination on thymus index in cotton pellets induced granuloma in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test.  

\[ \text{a} p < 0.001 \] represents the significance level compared with NC and \[ \text{b} p < 0.01 \]; non-significant (ns) compared to FC.

NC = Normal control, DC = Disease control, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum TNF-α level**

The rats treated with acetonised cotton pellets had significantly (p<0.001) elevated the level of TNF-α as compared to normal control group. Whereas, the rats treated with HG and combination showed significant (p<0.001) decrement in the TNF-α levels compared to acetonised cotton pellets rats as shown in Figure 4.9.
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Figure 4.9 Effect of HG and combination on serum TNF-α level in cotton pellet induced granuloma in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \( ^a \text{p}<0.001 \) represents the significance level compared with NC group; \( ^b \text{p}<0.001 \) compared with acetonised cotton pellet treated rats and \( ^c \text{p}<0.001 \) compared with FC.

NC = Normal control, DC = Disease control, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on serum IL-12 levels

The rats treated with acetonised cotton pellets had significantly (\( \text{p}<0.001 \)) elevated the levels of IL-12 as compared to NC group. Whereas, the animals treated with HG and combination showed significant (\( \text{p}<0.001 \)) decrement in the IL-12 levels compared to rats treated with acetonised cotton pellets as shown in Figure 4.10.

Figure 4.10 Effect of HG and combination on serum IL-12 levels in cotton pellet induced granuloma in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way
Results, Pharmacological evaluation

ANOVA followed by Tukey’s Kramer test. a\(p<0.001\) represents the significance level when compared with NC group, \(b\,p<0.001\) compared with acetonised cotton pellet treated rats, \(c\,p<0.05\) and \(d\,p<0.01\) compared with FC.

NC = Normal control, DC = Disease control, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

4.3. Secondary pharmacological evaluation

4.3.1. TNBS induced Colitis in rats

Effect of HG and combination on CW and CW/CL

The trans-rectal instillation of TNBS in rats shows the presence of diarrhoea along with decrease of colon weight (CW) and colon weight/length ratio (CW/CL), an indicator of the ongoing colon inflammation. The representative colon samples of each group rats were shown in Figure 4.11. The trans-rectal administration of HG and combination significantly elevates the colon weight and CW/CL ratio as compared to TNBS treated rats (Figure 4.12 and 4.13).

Figure 4.11 Representative colon tissue from each groups in TNBS induced colitis in rats.

NC = Normal control, TNBS = Tri-Nitro-Fluro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

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Figure 4.12 Effect of HG and combination on colon weight (mg) in TNBS induced colitis model. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test. \(^aP<0.001\) represents the significance level as compared to NC group & \(^bP<0.001\) compared to TNBS group, non-significant (ns) compared with FC.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Figure 4.13 Effect of HG and combination on colon weight to colon length ratio in TNBS induced colitis model. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test. \(^aP<0.001\) represents the significance level as compared to NC group & \(^bP < 0.001\) compared to TNBS group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on lesions score, diarrhea score and adhesion score
The trans-rectal instillation of TNBS in rats shows the presence of diarrhoea as an indicator of the colon inflammation. The rats also suffered from a marked colonic mucosal damage, edema, deep ulcerations and hemorrhage. The trans-rectal administration of HG and combination significantly elevates the severity and extent of colonic injury and diarrhoea status as compared to TNBS treated rats as shown in Table 4.2.
Results, Pharmacological evaluation

Table 4.2: Effect of HG and combination on lesions, diarrhoea and adhesion score in TNBS induced colitis in rats

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Macroscopic (Lesion) score (0-4)</th>
<th>Diarrhoea score (0-1)</th>
<th>Adhesion score (0-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>TNBS</td>
<td>4.01 a ± 0.03</td>
<td>1.06 a ± 0.00</td>
<td>1.02 a ± 0.00</td>
</tr>
<tr>
<td>HG</td>
<td>2.04 c, ns ± 0.01</td>
<td>1.01 ± 0.00</td>
<td>1.02 ± 0.00</td>
</tr>
<tr>
<td>FC</td>
<td>1.02 b ± 0.01</td>
<td>1.00 ± 0.00</td>
<td>1.01 ± 0.00</td>
</tr>
<tr>
<td>HG + FC</td>
<td>2.00 c, ns ± 0.01</td>
<td>1.01 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. aP < 0.001 represents the significance level compared with NC group; bP < 0.001 & cP < 0.05 compared to TNBS group; non-significant (ns) compared with FC group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on adrenal, spleen and thymus gland indices

The effects on adrenal, thymus and spleen gland indices were tested on rats after trans-rectal administration of TNBS (10 mg in 0.25 ml of 50 % ethanol) as shown in Figure 4.14. The treatment of rats with FC shows suppression of adrenal, thymus and spleen gland indices as compared to normal control rats. The rats treated with HG and combination caused significant (p<0.01) increment in the adrenal, thymus and spleen gland indices as compared to FC.
Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals

**Figure 4.14** Effect of HG and combination on adrenal, thymus and spleen gland indices in TNBS induced colitis model. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^aP<0.05\) & \(^bP<0.001\) represents the significance level compared to NC group and \(^cP<0.001\) and non-significant (ns) compared to FC group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on body weight and blood glucose measurement**

The effects on body weight and blood glucose level were tested on rats after trans-rectal administration of TNBS (10 mg in 0.25 ml of 50 % Ethanol). The transactivation-mediated increase in blood glucose concentration by GCs reflects the risk for induction of diabetes mellitus. Trans-rectal administration of FC showed significant increase in body weight and blood glucose level in rats. The treatment of rats with TNBS, HG and combination showed significant (\(p<0.05\); \(p<0.01\)) decrement in the body weight and blood sugar level as compared to FC (Figure 4.15 and 4.16).
Figure 4.15 Effect of HG and combination on body weight in TNBS induced colitis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test. $^a$P<0.001 represents the significance level compared to NC group and $^b$P<0.05 compared to FC group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Figure 4.16 Effect of HG and combination on blood sugar level in TNBS induced colitis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test. $^a$P < 0.001 represents the significance level compared to NC group and $^b$P < 0.05, $^c$P < 0.01 compared with FC group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
**Effect of HG and combination on MPO level**

The effect of HG and combination treatment on colon MPO activity is shown in Figure 4.17. The MPO level in colon tissue was significantly (p<0.001) increased in TNBS colitic group when compared with NC group. The treatment of rats with HG and combination shows significant (p<0.001) reduction in the elevated MPO level as compared to TNBS treated rats.

![Figure 4.17](image-url)

**Figure 4.17** Effect of HG and combination on MPO level in TNBS induced colitis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^{a}p<0.001\) represents the significance level compared with NC group; \(^{b}p<0.001\) compared with TNBS; \(^{c}p<0.05\) and non-significant (ns) compared with FC group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum TNF-α levels**

The treatment of rats with TNBS had significantly (p<0.001) elevated the levels of TNF-α as compared to NC group rats. Whereas, the animals treated with HG and combination showed significant (p<0.001) decrement in the TNF-α levels as compared to TNBS treated rats as shown in Figure 4.18.
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Figure 4.18 Effect of HG and combination on serum TNF-α level in TNBS induced colitis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with TNBS; \(^c\)p<0.05 & \(^d\)p<0.01 compared to FC group.

NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on serum IL-12 levels

The treatment of rats with TNBS had significantly (p<0.001) elevated the levels of IL-12 as compared to NC group. Whereas, the animals treated with HG and combination showed significant (p<0.001) decrement in the IL-12 levels compared to TNBS treated rats respectively as shown in Figure 4.19.

Figure 4.19 Effect of HG and combination on serum IL-12 levels in TNBS induced colitis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by
Tukey’s Kramer test. \( ^a p<0.001 \) represents the significance level compared with NC group; 
\( ^b p<0.001 \) compared with TNBS; \( ^c p<0.001 \) compared with FC group.
NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on histopathological analysis of colon tissues**

The histopathological photographs of normal control rat colon tissue represents with normal intestinal cytoarchitecture, epithelial cell layer accompanied by the presence of goblet cells and normal number of cells in lamina propria (Figure 4.20A and 4.21A). On the other hand, the TNBS treated rat group showed severe disruption of the normal architecture of colon with extensive ulceration and inflammation with necrosis, edema and diffuse inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and eosinophils) infiltration in the mucosa and distortion of crypts architecture (Figure 4.20B and 4.21B). The treatment of tissue samples with HG, FC and combination showed a significant reduction in edema, infiltration of diffuse inflammatory cells and normalization of crypt architecture (Figure 4.20C, 4.21C, 4.20D, 4.21D, 4.20E and 4.21E) compared to TNBS treated rats.
Figure 4.20 Photomicrograph of colon tissue stained with H & E in TNBS induced ulcerative colitis in rats and examined under light microscopy (100X). Treatments: Acetone (A) TNBS 10 mg dissolved 0.25 ml 50% ethanol (B) HG (50 µg/rat) (C) FC (50 µg/rat) (D) combination (25 µg/rat, each) (E). The shown sections are representative of six animals per group.
NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Figure 4.21 Photomicrograph of colon tissue stained with Giemsa in TNBS induced colitis in rats and examined under light microscopy (100X). Treatments: Acetone (A) TNBS 10 mg dissolved 0.25 ml 50% ethanol (B) HG (50 µg/rat) (C) FC (50 µg/rat) (D) HG + FC (25 µg/rat, each) (E). The shown sections are representative of six animals per group.
NC = Normal control, TNBS = Tri-Nitro-Fluoro-Benzene Sulphonic acid, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
4.3.2. DNFB induced Dermatitis in mice

Effect of HG and combination on ear thickness

The repeated application of DNFB results into ear swelling is a main characteristic of AD. The increases in ear thickness of DNFB treated rats were effectively inhibited by topical application of HG and combination. The inhibitory effects of FC on ear thickness were more effective than those observed in HG and combination group. The ear thickness of the HG, FC and combination group was found to be $0.51 \pm 0.02$ mm ($p<0.001$), $0.41 \pm 0.01$ mm ($p<0.001$) and $0.48 \pm 0.02$ mm ($p<0.001$) while that of DNFB treated group was found to be $0.87 \pm 0.03$ mm ($p<0.001$) as shown in Figure 4.22.

**Figure 4.22** Effect of HG and combination on ear thickness (mm) in DNFB induced AD in Balb/c mice. Values expressed as Mean ± SEM ($n = 6$) & analysed by One-way ANOVA followed by Tukey’s Kramer test. $^a$$p<0.001$ represents the significance level compared with NC group; $^b$$p<0.001$ compared with DNFB group and $^c$$p<0.05$ compared with FC group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on ear weight

The effects of HG and combination on ear swelling were evaluated by measuring the ear weights of left and right ear after the drug treatments. The representative photographs of ear tissue from each group are shown in Figure 4.23. Repeated painting of DNFB shows increased ear weight up to $35.4 \pm 1.71$ mg ($p<0.001$) in the DNFB treated group. These increases in ear tissue weight were effectively inhibited upto $22.8 \pm 1.47$ mg ($p<0.001$) by treatment with HG and up to $19.0 \pm 1.14$ mg ($p<0.001$) by combination. The treatment of
mice with FC significantly inhibited the ear weight upto 18.4 ± 1.59 mg (p<0.001) as shown in Figure 4.24.

Figure 4.23 Representative photograph of ear tissue in each group in DNFB induced AD in Balb/c mice.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Figure 4.24 Effect of HG and combination on ear weight (mg) in DNFB induced AD in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with DNFB group; \(^c\)p<0.05 and non-significant (ns) compared to FC group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on ear erythema score

The representative photographs of different groups captured at the end of DNFB treatment were shown in Figure 4.25. The ear erythema score of DNFB treated mice was found to be 4 (p<0.001) which was compared with ear erythema score of NC mice having 0 score. The treatment of mice with HG (Score = 2; p<0.001), FC (Score = 2; p<0.001), and combination
(Score = 1; p<0.001), was significantly inhibited the erythema score as compared to DNFB treated mice (Figure 4.26).

**Figure 4.25** Effect of HG and combination in DNFB induced AD in Balb/c mice. Representative photographs of each group obtained after completion of DNFB treatment. Six animals were allocated to each group. NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Figure 4.26** Effect of HG and combination on ear erythema score in DNFB induced AD in Balb/c mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\\)p<0.001 represents the significance level compared with NC group; \(^b\\)p<0.001 compared with DNFB group; \(^c\\)p<0.01 and non-significant compared to FC group. NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Effect of HG and combination on MPO level

The effect of HG and combination treatment on ear MPO activity is shown in Figure 4.27. The MPO level in ear tissue (U/g) was significantly (p<0.001) increased in DNFB group when compared with normal control group. When compared with DNFB group, the elevated MPO level was significantly (p<0.001) decreased by HG and combination showed significant reduction (p<0.001) in the elevated MPO level.

![Figure 4.27](image)

**Figure 4.27** Effect of HG and combination on MPO level in DNFB induced AD in Balb/c mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. *p<0.001 represents the significance level compared with NC group; ^p<0.001 compared with DNFB group; †p<0.05 and non-significant (ns) compared to FC group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on serum TNF-α level

The treatment of animals with DNFB had significantly (p<0.001) elevated the levels of TNF-α as compared to NC group. Whereas, the animals treated with HG and HG + FC showed significant (p<0.001) decrement in the TNF-α levels as compared to DNFB treated animals as shown in Figure 4.28.
Figure 4.28 Effect of HG and combination on serum TNF-α level in DNFB induced AD in Balb/c mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with DNFB group and \(^c\)p<0.001 compared to FC group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on serum IL-12 level

The treatment of mice with treated with DNFB had significantly (p<0.001) elevated the levels of IL-12 as compared to NC group. Whereas, the animals treated with HG and combination showed significant (p<0.001) decrement in the IL-12 levels compared to DNFB treated mice as shown in Figure 4.29.
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**Figure 4.29** Effect of HG and combination on serum IL-12 level in DNFB induced AD in Balb/c mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with DNFB group and \(^c\)p<0.001 compared to FC group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on skin atrophy**
Skin atrophy is one of the most universal side effects of FC after long term topical treatment. The mice treated with FC shows reduced skin thickness than the normal mice. Whereas, treatment of Balb/c mice with HG and combination topically for 13 days significantly improved the skin thickness as compared to FC treated mice as shown in Figure 4.30.

**Figure 4.30** Effect of HG and combination on skin atrophy in DNFB induced AD in Balb/c mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.05 represents the significance level compared with NC group and \(^b\)p<0.001 compared with FC group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on histopathological analysis of ear tissues**
The mice ear tissue stained with H and E, showed normal architecture of ear tissues of normal control group (Figure 4.31A) whereas, DNFB treated mice showed severe edema and
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abundant inflammatory cell infiltration in the epidermis and dermis of ear tissue (Figure 4.31B). The treatment of mice with HG and combination decrease the ear thickness and inflammatory cell infiltration induced by DNFB. Treatment with combination was more effective than HG treatment alone (Figure 4.31C and 4.31D). The FC group mice displayed almost normal features, and there was some infiltration of immune cells (Figure 4.31E).

**Figure 4.31** Photomicrograph of ear tissue stained with H & E in DNFB induced AD in Balb/c mice and examined under light microscopy (100X). Treatments: Acetone (A) DNFB 0.2% DNFB (B) HG (50 µg/mice) (C) FC (50 µg/mice) (D) Combination (25 µg/mice, each) (E). The shown sections are representative of six animals per group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Mast cells are one of the most important inflammatory cells in the dermis region of skin of AD patients. These cells are stimulated by IgE allergen sensitized through their receptors on cell surface and secreting various inflammatory mediators, such as prostaglandin, leukotrienes and variety of pro-inflammatory cytokines. Thereby, we have studied the effect of HG and Combination on the infiltration and degranulation of mast cells, using Toluidine blue staining.
The numbers of infiltrating and degranulated mast cells were significantly reduced in the dermis of HG and combination group (Figure 4.32C and 4.32D) as compared to DNFB treated group Balb/c mice wherein, the infiltrating mast cells number was higher (Figure 4.32B). The FC group displayed almost normal features, and there was some infiltration of mast cells in the dermis region of mice ear tissue (Figure 4.32E).

**Figure 4.32** Photomicrograph of ear tissue stained with Toludine blue in DNFB induced AD in Balb/c mice and examined under light microscopy (100X). Treatments: Acetone (A) DNFB 0.2% DNFB (B) HG (50 µg/mice) (C) FC (50 µg/mice) (D) Combination (25 µg/mice, each) (E). The shown sections are representative of six animals per group.

NC = Normal control, DNFB = 2, 4-Di-Nitro-Fluoro-Benzene, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

### 4.3.3. OVA induced Lung edema in mice

Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals
Effect of HG and combination on total and differential cell count in BALF

Mice were immunized with OVA and submitted to two OVA aerosol challenges show statistically significant (p<0.001) increase in total cells, monocytes, neutrophils, eosinophils in the BALF collected at 24th h whereas, decrease in lymphocytes when compared to NC mice. As compared to OVA, HG and combination on intra-nasal administration showed statistically significant (p<0.001) reduction in total cell (TC) count. However, the numbers of circulating neutrophils (NP) (p<0.001), eosinophils (EP) (p<0.001) and monocytes (MC) (p<0.001) were significantly decreased and lymphocytes (LP) (p<0.001) were significantly increased by HG and combination treated mice (Figure 4.33). Whereas, the FC treated mice proved to be more potent and significantly reduced the total cells and differential cell count in BALF in OVA induced lung edema in mice.

Figure 4.33 Effect of HG and combination on total and differential cell count in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. a*p<0.001 represents the significance level as compared to NC group; b*p<0.001 as compared to OVA group; c*p<0.05, d*p<0.01 and non-significant (ns) compared to FC group.
NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Effect of HG and combination on adrenal, spleen and thymus gland indices

The effects on adrenal, thymus and spleen gland weights were tested on rats after i.p. administration of OVA (50 µg in 0.2 ml of Normal saline) as shown in Figure 4.34. The adrenal, thymus and spleen indices are associated with immunological functions. The treatment of mice with FC shows significant decrease in the adrenal, spleen and thymus gland index as compared to normal control mice. Whereas, HG and combination treatment to mice caused significant (p<0.01) increment in the adrenal, thymus and spleen gland Indices when compared to FC mice.

Figure 4.34 Effect of HG and combination on adrenal, thymus and spleen gland indices in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. *p<0.001 compared to NC group and
b* p<0.01 and c* p<0.001 compared to FC group.
NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on body weight and blood glucose level

After trans-nasal administration of FC in mice, showed significant increment in body weight and blood glucose level. The treatment of mice with HG and combination showed significant (p<0.01) decrement in the body weight and blood sugar level as compared to FC treated mice as shown in Figure 4.35 and 4.36.
Figure 4.35 Effect of HG and combination on body weight in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.01 represents the significance level compared to NC group; \(^b\)p<0.01 compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Figure 4.36 Effect of HG and combination on blood glucose in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)P<0.01 compared to NC group; \(^b\)P<0.05 & \(^c\)P<0.01 compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Hecogenin + Fluticasone

**Effect of HG and combination on MPO level**

The effect of HG and combination on lung tissue is shown in Figure 4.37. MPO parameter reveals the stage of cell injury and extent of cell damage in the lungs. The MPO level in lung tissue (U/g) was significantly (p<0.001; 18.38 ± 0.17) increased in OVA group when compared with NC mice. The elevated MPO level was significantly decreased by HG (p<0.001; 11.11 ± 0.10), combination (p<0.001; 9.40 ± 0.23) and FC (p<0.001; 7.99 ± 0.50) as compared to OVA mice as shown in Figure 4.38.

![Figure 4.37](image1.png)

**Figure 4.37** Representative photograph of lung tissue in each group in OVA induced lung edema in Balb/c mice.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

![Figure 4.38](image2.png)

**Figure 4.38** Effect of HG and combination on MPO level in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with OVA; \(^c\)p<0.05 and \(^d\)p<0.01 compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
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Hecogenin + Fluticasone

**Effect of HG and combination on serum TNF-α level**

TNF-α is an amplifying agent in asthma and is produced in enhanced amounts in the asthmatic airways. It activates the proinflammatory transcription factors, viz. nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) which in turn activate many inflammatory genes in the asthmatic airway. The TNF-α (pg/ml) level were significantly (p<0.001) increased, in OVA mice when compared with NC mice. As compared to OVA group, the elevated TNF-α levels were significantly decreased (p<0.001) by HG, FC and combination treatments as shown in Figure 4.39.

![Figure 4.39](image)

**Figure 4.39** Effect of HG and combination on serum TNF-α level in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. *a*p<0.001 represents the significance level compared with NC group; *b*p<0.001 compared with OVA and *c*p<0.001 compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum IL-12 level**

IL-12 plays a key role in the allergic inflammatory response that results in the formation of IgE. The IL-12 levels were significantly (p<0.001) increased in the OVA group when compared with NC mice. When compared to OVA control, elevated IL-12 levels were significantly decreased by HG, FC and combination respectively as shown in Figure 4.40.
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Figure 4.40 Effect of HG and combination on serum IL-12 level in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a p<0.001\) represents the significance level compared with NC group; \(^b p<0.001\) compared with OVA and \(^c p<0.001\) compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on serum IL-6 levels

There was a significant (\(p<0.001\)) increased in IL-6 level in OVA treated mice as compared to the NC mice. Both HG and their combination showed significant (\(p<0.001\)) inhibitory effects on the production of IL-6 as compared with OVA mice. In addition, the level of IL-6 was significantly decreased in FC treated mice as shown in Figure 4.41.
Figure 4.41 Effect of HG and combination on serum IL-6 level in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with OVA and ns (non-significant) compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on serum TXB\(_2\) levels
The TXB\(_2\) level was significantly (p<0.001) increased in OVA treated mice as compared to the normal control mice. Both HG and combination showed significantly (p<0.001) decreased the level of TXB\(_2\) as compared with OVA mice as shown in Figure 4.42.

Figure 4.42 Effect of HG and combination on serum TXB\(_2\) level in OVA induced lung edema in mice. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with OVA and \(^c\)p<0.001 compared to FC group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on histopathological analysis of lung tissues
The histology of bronchi after staining with H & E and Giemsa revealed the normal architecture of bronchial epithelial layer and active mucus secreting goblet cells in NC group (Figure 4.43A and 4.44A). Whereas, in mice treated with OVA showed severe increase in
peri-bronchial inflammation, epithelial fragility, wall thickening and hyperplasia of goblet cells (Figure 4.43B and 4.44B). The treatment of mice with HG and Combination shows restoration in the changes occurs in the bronchi as compared with OVA treated mice (Figure 4.43C, 4.44D, 4.43C and 4.44D). However, similar observation was seen in mice treated with FC (Figure 4.43E and 4.44E).

**Figure 4.43** Photomicrograph of lung tissue stained with H & E and examined under light microscopy (100X). Treatments: Phosphate Buffer Saline (A) OVA 2.5% w/v OVA (B) HG (50 µg/mice) (C) FC (50 µg/mice) (D) HG + FC (25 µg/mice, each) (E). The shown sections are representative of six animals per group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
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Figure 4.44 Photomicrograph of lung tissue stained with Giemsa and examined under light microscopy (100X). Treatments: Phosphate Buffer Saline (A) OVA 2.5% w/v OVA (B) HG (50 µg/mice) (C) FC (50 µg/mice) (D) HG + FC (25 µg/mice, each) (E). The shown sections are representative of six animals per group.

NC = Normal control, OVA = Ovalbumin, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

4.3.4. CFA induced arthritis in rats

Effect of HG and combination on paw volume

The effect of HG and combination on rat paw swelling was shown Figure 4.45. In the primary phase of arthritis (form day 4 to 12), there was non-significant deceased in the paw volume was observed in CFA treated rats. There was significant (p<0.001) increase in the paw volume of the rats treated with CFA compared to NC rats. HG and combination treated groups has shown significantly (p<0.001) decrease in the paw volume from day 14 onwards upto 28 days as compared to CFA group. The rats treated with FC also showed significantly
decrease \((p<0.001)\) in paw volume from day 14 to 28 as compared to NC rats as shown in Figure 4.46.

**Figure 4.45** Effect of HG and combination on paw swelling in CFA induced arthritis in rats. A= NC, B = CFA, C= HG, D= FC and E= HG + FC

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Figure 4.46 Effect of HG and combination on paw volume in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001\) represents the significance level compared to NC group; \(^b\)p<0.001\) compared to CFA group; \(^c\)\(p<0.001\) and \(^d\)\(p<0.05\) compared to FC group.

NC=Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination onarthritic score

Subplantar administration of CFA results in significant increase \((p<0.001)\) in arthritic score in CFA treated rats as compared to normal control rats. This increase in the arthritic score had shown a biphasic response. There was decrease in the arthritic score from day 4 to 7, however, this change was not statistically significant. The arthritic score was significantly increased from day 7 to 12 in disease control rats which remained significantly increased up to 28th days as compared to NC rats. Treatment of rats with HG and combination showed significant decrease in arthritic score \((p<0.05, p<0.01, p<0.001\) respectively\) from day 12 onward up to 28 days as compared to CFA rats as shown in Figure 4.47.

![Graph showing the effect of HG and combination on arthritic score](image)

Figure 4.47 Effect of HG and combination on arthritic score in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)\(p<0.001\) represents the significance level compared to NC group;
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Effect of HG and combination on joint diameter

There was significant (p<0.001) increase in the joint diameter of rats treated with CFA was observed as compared to NC rats. The treatment of rats with HG and combination significantly (p<0.01 and p<0.001, respectively) showed decreased the joint diameter from day 14 to 28 as compared to CFA rats. The change in joint diameter of HG (2.1 ± 0.09) and combination (1.58 ± 0.04) was evident as compared to CFA rats (3.75 ± 0.22) on day 28 as shown in Figure 4.48.

Figure 4.48 Effect of HG and combination on joint diameter in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. a p<0.001 represents the significance level compared to NC group; b p<0.001 as compared to CFA group and c p<0.001 compared to FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Effect of HG and combination on paw swelling

The treatment of rats with CFA exhibited a marked peripheral edema of the injected paw, subsequently observable 24 h after the injection. After CFA immunization, the arthritis swelling in the right hind paws of rats was significantly (\(p<0.001\)) increases and maintained for 28 days compared with NC group. However, the treatment of rats with HG and combination significantly (\(p<0.001\)) inhibited the paw swelling as compared to CFA group. FC also significantly inhibited the paw swelling in rats (Figure 4.49).

![Figure 4.49](image)

**Figure 4.49** Effect of HG and combination on paw weight in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey's Kramer test. \(^a\)p<0.001 represents the significance level compared to NC group; \(^b\)p<0.001 compared to CFA group and non-significant (ns) compared to FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on spleen and thymus gland indices

The glucocorticoid such as FC produces various systemic side effects after long term treatment such as reduction of spleen and thymus gland weight. The oral treatments of rats with 40 µl of FC for 28 days exhibited significant reduction in spleen and thymus gland index as compared to NC rats. The treatments of rats with HG and combination induces significantly (\(p<0.01\)) less deterioration related to immune cell apoptosis such as reduction in spleen and thymus gland index as compared to FC treated rats as shown in Figure 4.50 and 4.51.
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**Figure 4.50** Effect of HG and combination on spleen index in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \( ^{a}p<0.05 \) represents the significance level compared to NC group; \( ^{b}p<0.01 \) and non-significant (ns) compared to FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Figure 4.51** Effect of HG and combination on thymus index in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \( ^{a}p<0.05 \) represents the significance level compared to NC group; \( ^{b}p<0.05 \) and non-significant (ns) compared to FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone
Effect of HG and combination on haematological parameters

The decreased levels of RBC and Hb and increased levels of WBC were observed in CFA treated rats as compared to normal control rats. These levels of RBC, WBC and Hb levels were significantly (p<0.001) altered with the treatments of rats with HG, combination and FC also as shown in Table 4.3.

Table 4.3: Effect of HG and combination on RBCs, WBCs and Hb in CFA induced arthritis in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>RBC (X 10^6/µl)</th>
<th>WBC (X 10^3/µl)</th>
<th>Hb (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>8.34 ± 0.63</td>
<td>9.06 ± 0.69</td>
<td>15.56 ± 1.02</td>
</tr>
<tr>
<td>CFA</td>
<td>5.89 ± 0.49^a</td>
<td>19.33 ± 1.23^a</td>
<td>5.30 ± 0.68^a</td>
</tr>
<tr>
<td>HG</td>
<td>7.13 ± 0.51^b,c</td>
<td>12.99 ± 0.98^b,d</td>
<td>12.91 ± 0.98^b,c</td>
</tr>
<tr>
<td>FC</td>
<td>7.89 ± 0.62^b</td>
<td>5.76 ± 0.43^b</td>
<td>12.14 ± 0.91^b</td>
</tr>
<tr>
<td>HG + FC</td>
<td>7.59 ± 0.63^b,d</td>
<td>12.48 ± 0.81^b,d</td>
<td>13.01 ± 0.65^b,d</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. ^a p<0.001 represents the significance level compared to NC group and ^b p<0.001 as compared to CFA group; ^c p<0.01 and ^d p<0.001 compared to FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

4.3.4.7. Effect of HG and combination on biochemical parameters

In CFA treated arthritic rats showed significant (p<0.001) elevation of the serum AST, ALT and ALP levels. Treatment of CFA treated rats with HG and combination significantly (p<0.05; p<0.001) prevented the elevation of serum AST, ALT and ALP. However, treatment of rats with FC (p<0.05; p<0.001) elicited maximum inhibition of serum AST, ALT and ALP levels as shown in Figure 4.52.
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Figure 4.52 Effect of HG and combination on biochemical parameters in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. a p<0.01; b p<0.001 represents the significance level compared to NC group; c p<0.05 & d p<0.001 compared to CFA group; e p<0.001 & f p<0.01 compared to FC group

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

Effect of HG and combination on MPO level

The MPO levels were significantly (p<0.001) increased in joint tissue of CFA treated rats as compared to normal control rats. The treatment of rats with HG (p<0.001) and combination (p<0.01) significantly decreased the levels of MPO in joint tissue. The level of MPO in FC group also showed significant (p<0.001) reduction as shown in Figure 4.53.
**Figure 4.53** Effect of HG and combination on MPO level in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group; \(^b\)p<0.001 compared with CFA; \(^c\)p<0.01 and non-significant (ns) compared with FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum TNF-α level**

A significant elevation (p<0.001) in serum TNF-α level were observed in CFA treated arthritic rats. Whereas, the treatment of rats with HG, FC and combination significantly (p<0.001) prevented the elevation of serum TNF-α levels as compared to CFA rats. As shown in Figure 4.54.

**Figure 4.54** Effect of HG and combination on serum TNF-α level in CFA induced arthritis in rats Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level compared with NC group, \(^b\)p<0.001 when compared CFA group and \(^c\)p<0.001 compared with FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum IL-12 level**

A significant (p<0.001) elevation in serum IL-12 level were observed in CFA treated rats. Whereas, the treatment of arthritic rats with HG (50 µg/rat), FC (50 µg/rat) and combination (25 µg/rat, each) significantly (p<0.001) prevented the elevation of serum IL-12 levels as compared to CFA rats as shown in Figure 4.55.
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**Figure 4.55** Effect of HG and combination on serum IL-12 level in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level when compared with NC group, \(^b\)p<0.001 when compared with CFA group and \(^c\)p<0.001 compared with FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum IL-6 levels**

A significant (p<0.001) elevation in serum IL-6 level were observed in CFA treated rats. Whereas, the treatment of arthritic rats with HG, FC and combination significantly (p<0.001) prevented the elevation of serum IL-6 levels as compared to CFA rats as shown in Figure 4.56.
Chapter 4, Section 4.3

Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals

**Figure 4.56** Effect of HG and combination on serum IL-6 level in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level when compared with NC group, \(^b\)p<0.001 when compared with CFA group and ns (non-significant) as compared with FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on serum TXB\(_2\) levels**

The TXB\(_2\) levels were significantly (p<0.001) increased in the CFA treated group as compared with NC rats. As compared to CFA rats, elevated TXB\(_2\) levels were significantly decreased by HG, FC and combination respectively as shown in Figure 4.57.

**Figure 4.57** Effect of HG and combination on TXB\(_2\) level in CFA induced arthritis in rats. Values expressed as Mean ± SEM (n = 6) & analysed by One-way ANOVA followed by Tukey’s Kramer test. \(^a\)p<0.001 represents the significance level when compared with NC group, \(^b\)p<0.001 when compared with CFA group and \(^c\)p<0.001 compared with FC group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on radiological analysis of ankle joints**

The radiographic analysis was done at the end of 28 days of treatment and was compared for degree of erosion, joint space narrowing and bone destruction. CFA injected rats had developed joint space narrowing, soft tissue swelling and extensive joint erosion of ankle
The treatment of rats with HG showed moderate effect on joint space narrowing and bone erosion. Whereas, treatment of rats with combination and FC showed pronounced effect on joint space narrowing and bone erosion of ankle joint as shown in Figure 4.58.

**Figure 4.58** Effect of HG and combination on radiographic analysis level of ankle joints in CFA induced arthritis in rats. The shown radiographic films are representative of six animals per group.

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**Effect of HG and combination on histopathological analysis of ankle joints**

The histopathological analysis of ankle joint of NC rats showed normal connective tissue structure without inflammation, necrosis and intact synovial lining of bone (Figure 4.59A). Treatment of rats with CFA showed massive inflammatory cells influx, chronic inflammation and necrosis of bone, synovial hyperplasia and damaged synovial lining (Figure 4.59B). Treatment of rats with FC showed normal connective tissue structure with absence of lymphocytic infiltration and necrosis (Figure 4.59D). Whereas, rats treated with HG and
combination showed significant protection against normal structure of connective tissue, necrosis with low influx of inflammatory cells in the ankle joints (Figure 4.59C and 4.59E).

Figure 4.59 Photomicrograph of transverse sections of ankle joint tissue treated with CFA stained with H & E and examined under light microscopy (100X). Treatments: Acetone (A) CFA (0.1 ml) (B) HG (50 µg/rat) (C) FC (50 µg/rat) (D) HG + FC (25 µg/rat, each) (E). The shown sections are representative of six animals per group.
NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

4.3.4.15. Effect of HG and Combination on COX-2 mRNA expression by RT-PCR
The increased expression of COX-2 mRNA was observed in the rats treated with CFA (Figure 4.60H), where as a low levels of expression was detected in the HG (50 µg/rat) and HG + FC (25 µg/mice) groups (Figure 4.60J & 4.60L). Similarly, FC (50 µg/rat) also showed significant effect on COX-2 mRNA expression (Figure 4.60K). GAPDH expression was
observed in all groups of animals (Figure 4.60 B, C, D, E & F).

**Figure 4.60** Effect of HG and combination on COX-2 mRNA expression on joint tissue by using RT-PCR

**GAPDH:** B= NC, C=CFA, D= HG, E= FC, F= HG + FC;

**COX-2:** H= NC, I=CFA, J= HG, K= FC, L= HG + FC

NC = Normal control, CFA = Complete Freund Adjuvant, HG = Hecogenin, FC = Fluticasone, HG + FC = Hecogenin + Fluticasone

**4.4. Acute oral toxicity study**

The acute oral toxicity study of HG does not show any toxic symptoms and mortality and was found to be safe up to the dose of 2000 mg/kg, B.W. Therefore, the dose of 50 µg/animal was selected for further pharmacological studies depending on the basis of pilot study.

**4.5. Safety pharmacological study**

**Effect of HG on general appearance and behavioral patterns**

HG at a dose of 50 µg/mice had no adverse effect on the behavioral responses of the mice up to 14 days of observation. The behavioral patterns of animals were observed first 6 h and followed by 14 h after the administration and did not display significant changes in behavior, changes in skin, hair loss, eyes, mucous membrane, behavior patterns, tremors, salivation, diarrhoea, food intake and water consumption of mice (Table 4.4).
Table 4.4: Effect of HG on general appearance and behavioral observations in mice

<table>
<thead>
<tr>
<th>Observations</th>
<th>NC</th>
<th>HG</th>
<th>NC</th>
<th>HG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
<td>14 h</td>
<td>6 h</td>
<td>14 h</td>
</tr>
<tr>
<td>Skin</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Hair Loss</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Eyes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Mucous</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Behavioral pattern</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Tremors</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Not observed</td>
</tr>
<tr>
<td>Salivation</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Food intake</td>
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</tr>
<tr>
<td>Water</td>
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</tr>
</tbody>
</table>

Values were expressed as Mean ± SEM (n = 10)
NC = Normal control, HG = Hecogenin

Effect of HG on organ weights
HG at a dose of 50 µg/mice generally had no significant differences in the organ weights of mice (Table 4.5).

Table 4.5: Effect of HG on various organs weights in mice

<table>
<thead>
<tr>
<th>Organs</th>
<th>NC</th>
<th>HG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>3.71 ± 0.21</td>
<td>3.80 ± 0.15</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>0.83 ± 0.03</td>
<td>0.81 ± 0.03</td>
</tr>
<tr>
<td>Lung (g)</td>
<td>1.57 ± 0.09</td>
<td>1.62 ± 0.04</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.59 ± 0.01</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>1.49 ± 0.11</td>
<td>1.52 ± 0.06</td>
</tr>
</tbody>
</table>

Values were expressed as Mean ± SEM (n = 10) & analysed by students‘t’ test. P values less than 5 % were considered statistically significant (p<0.05).
NC = Normal control, HG = Hecogenin, FC = Fluticasone
Effect of HG on biochemical parameters
From the present study, it was seen that there was no significant change in the biochemical parameters in the HG group compared to NC group (Table 4.6).

**Table 4.6:** Effect of HG on biochemical parameters in mice

<table>
<thead>
<tr>
<th>Biochemical Parameters</th>
<th>NC</th>
<th>HG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/L)</td>
<td>25.67 ± 1.45</td>
<td>28.11 ± 1.19</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>30.19 ± 2.82</td>
<td>32.43 ± 2.25</td>
</tr>
<tr>
<td>Albumin/Globulin ratio (g/L)</td>
<td>0.85 ± 0.03</td>
<td>0.86 ± 0.35</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>217.98 ± 17.2</td>
<td>219.29 ± 20.17</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>162.35 ± 12.8</td>
<td>165.27 ± 14.35</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>37.24 ± 2.89</td>
<td>40.16 ± 3.91</td>
</tr>
</tbody>
</table>

Values were expressed as Mean ± SEM (n = 10) & analysed by student ‘t’ test. P values less than 5% were considered statistically significant (p<0.05).
NC = Normal control and HG = Hecogenin

Effect of HG on gross observation and histopathological analysis of various organs
HG at a dose of 50 µg/mice generally had no significant differences in the gross observation and histopathological examination of various organs stained with H & E (Figure 4.61 and 4.62).
Figure 4.61 Gross observations of systemic organs in safety pharmacological study; Liver (A1 & A2), Kidney (B1 & B2), Lung (C1 & C2), Heart (D1 & D2) and Spleen (E1 & E2) from NC and HG mice. The shown organs are representative of six animals per group. NC = Normal control and HG = Hecogenin

Figure 4.62 Histopathological analysis of systemic organs in safety pharmacological study; A: Heart, B: Kidneys, C: Liver, D: Lung and E: Spleen from NC and HG mice. The shown sections are representative of ten animals per group. NC = Normal control and HG = Hecogenin