CHAPTER 3: ANTI-HEMOLYTIC ACTIVITY STUDY

3.1. INTRODUCTION

In the previous chapter, based on the characterization results we concluded that kāntam formulations (KC and KP) are completely devoid of organic substances. Moreover, they differ in their elemental and chemical nature when compared with negative control (KNC). But the significance of these variations will be valid only if KP and KC are proved to be pharmacologically potent compared with KNC. So in order to demonstrate the difference in the pharmacological activity exhibited by KC and KP (both prepared by strictly adhering to the traditional protocol) compared with KNC (prepared with deviant protocol), a detailed study on animal model is mandated. Kāntam formulations have been traditionally used for many disorders such as dropsy, gastro-intestinal ulcers, leucorrhoea, metabolic disorders, eye disorders, jaundice, mental disorders, menorrhagia, fever, respiratory disorders, hemolytic complications and cervical cancer. Among this, different kāntam formulations are administered with different vehicles at different doses for treatment of various disorders. However, almost all the kāntam formulations are indicated for management of hemolytic complications (represented as “viṣappāṇṭu, erikuṇmpāṇṭuete” in Siddha literature) with honey as vehicle. Hemolysis is a relatively rare pathological mechanism that involves rupture of erythrocytes commonly seen in some types of poisoning, snakebites, infections, blood transfusions and autoimmune disorders. However, in the recent past, hemolysis has been frequently noted during treatment with a variety of drugs such as cephalosporins, nonsteroidal anti-inflammatory agents, levaquin, oxaliplatin, teicoplanin, fludarabine, primaquine, phenazopyridine, nitrofurantoin, ribavirin, levodopa, mfenamic acid, and diclofenac, procainamide, penicillin, dapsone, rasburicase etc. It is also reported that hemolysis occurs even in regular
smokers. The associated pathologies of hemolysis mainly include fever, spleenomegaly, hepatomegaly and anemia. The conventional management of autoimmune hemolysis includes corticosteroids and immunosuppressive drugs. Whereas, management of drug/agent-induced hemolytic anemia includes the immediate removal of the offending agent followed by supportive care along with the conventional therapies. Currently various approaches have been proposed to reduce drug-induced hemolysis such as drug polymer conjugation, modification in molecular chemistry of drug molecules, co-administration of botanical agents etc. However when the hemolysis, irrespective of the cause, gets severe the treatment is a very challenging task and may result in splenectomy if the patient is not responsive to first line of conventional therapies. Hence the requirement for other alternative drugs is wide open in managing hemolytic complications. Therefore, a detailed study on these kāntam formulations for their anti-hemolytic activities can help in recognizing them as an alternative for management of hemolytic complications. With this motivation the anti-hemolytic activity of kāntam formulations was selected for this study.

Among the different hemolytic animal models, Phenylhydrazine induced model is used for the induction of haemolysis and the study of its mechanism in many species such as rabbit, rat, mouse, calf, chicken, duck, rainbow trout, Xenopus and goldfish. Hence an acetylphenylhydrazine induced hemolytic rat model was selected to demonstrate the difference in pharmacological activities of KC and KP compared with KNC in managing hemolytic complications.

3.2. MATERIALS AND METHODS

3.2.1. Animals
Adult Wistar rats (6-8 weeks) of both sexes, procured from the Central Animal
Facility, SASTRA University campus, Tanjavur, India were used for this study. The animals were kept at 22±2 °C with a 12 h light/dark cycle, with free access to standard rat dry pellet diet (Altromin 1324, Germany) and water ad libitum. The experimental protocols were performed after obtaining the necessary approval (CPCSEA approval number: 260/SASTRA/IAEC/RPP) from the Institutional Animal Ethical Committee (IAEC) of SASTRA University.

3.2.2. Dose concentration
As the human therapeutic dose of Kāntam formulations is already established in the Siddha texts (65 mg/dose) for normal adult with 3 times a day, animal equivalent dose was calculated using allometric dose translations. Considering the weight of a normal adult as 60 kg, animal equivalent was calculated as

\[ \text{Animal equivalent dose (rat)} = \text{Human dose} \times \frac{\text{Human } Km}{\text{Animal } Km} \]

\[ \text{Animal equivalent dose (rat)} = 3.25 \times \frac{37}{6} = 20.0 \text{ mg/day} \]

Both KC and KP along with KNC were administered at 20.0 mg/day using honey as vehicle.

3.2.3. Experimental procedure
Animals were totally divided into 6 groups with 8 animals in each group. Except the normal control group, hemolysis was induced in all other groups by intra-peritoneal injection of 60 mg/kg of 20 mg/ml acetyl phenyl hydrazine (APH) (Sigma-Aldrich A4626, USA) dissolved in 20% (v/v) ethanol (Changshu Yangyuan Chemical Co., Ltd., China) on the first experimental day. Folic acid is the most commonly used drug currently for the management of hemolysis. Hence this was used as the drug in standard control group to determine whether the test drugs KC and KP are effective compared to folic acid. The test drugs were administered according to the Table 3.1
shown below. From previous works it was found that the hemolysis happens within 24 hours of administration of APH\textsuperscript{28}. So, the treatment drug administration was started 24 hours after the initial injection of APH for a period of 20 days.

Table 3.1 Experimental plan for evaluation of effect of KC and KP on APH induced hemolytic adult wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Administration Schedule from day1 to day20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Normal control)</td>
<td>Normal water</td>
</tr>
<tr>
<td>Group II (Disease control)</td>
<td>Normal water</td>
</tr>
<tr>
<td>Group III (Standard control)</td>
<td>1mg/kg of folic acid dispersed in water</td>
</tr>
<tr>
<td>Group IV (KNC treated)</td>
<td>20.0 mg/kg of KNC dispersed in honey</td>
</tr>
<tr>
<td>Group V (KC treated)</td>
<td>20.0 mg/kg of KC dispersed in honey</td>
</tr>
<tr>
<td>Group VI (KP treated)</td>
<td>20.0 mg/kg of KP dispersed in honey</td>
</tr>
</tbody>
</table>

3.2.4. Hematology and Biochemical analysis

Blood samples were collected on 21\textsuperscript{st} day from retro-orbital plexus before sacrifice. Hematology analysis was performed to analyze erythrocytes, leukocytes, lymphocytes, monocytes, neutrophils, hemoglobin\%, hematocrit value, reticulocytes, platelets count, MCV, MCH and MCHC in whole blood using GENESIS Veterinary Hematology System (Oxford Science, USA). In this technique, cells are injected through the counting orifice utilizing solid state syringes. The injected cells are surrounded by a sheath fluid that protects and ensures that they are counted one cell at a time. Sizing and counting of cells were performed using impedance technology and the complexity of cells are determined using laser technology\textsuperscript{21}.

The serum was separated by allowing the remaining blood sample to coagulate at
room temperature followed by centrifugation at room temperature using mini spin
(Eppendorf, Germany) at 605 rcf for 10 minutes. Biochemical analysis was performed
in this serum using A 15 auto analyzer (BioSystems, Spain)\(^6\). Blood smears are
prepared from fresh blood and fixed with 100% methanol for 2 minutes followed by
staining with 10% giemsa for 30 minutes.

**3.2.5. Histopathology analysis**

After sacrifice, the gross pathology was noted for all the organs and specific organs
were isolated, washed with cold saline, weighed (using GPA 3202, SARTORIUS,
Germany) and finally fixed in 10% buffered formalin solution for histopathological
studies. The fixed tissue was embedded in paraffin and the sections were cut into 3-5
\(\mu m\) thick slices and were stained using haematoxylin and eosin. The stained tissues
were observed under light microscope (Nikon Eclipse Ci with DS-Fi2 digital camera,
Nikon, Japan). The scoring was given to the pathological features seen in the slides
based on the four point grading system with 0 as normal, 1 as minimal, 2 as slight, 3
as moderate, 4 as marked and 5 as severe\(^{23}\).

**3.2.6. Statistical analysis**

Average of each groups values are expressed as mean \(\pm\) SD (\(n = 6-8\)). One way
ANOVA was performed to compare the means of different group as it was considered
to be the best method for experiments with more than two groups and is commonly
used in many studies. In order to compare the normal control with different groups,
Dunnet’s post hoc test was performed as it was commonly used method in various
animal experiments\(^1\).
3.3. RESULTS

3.3.1. Cage side Observations

No significant change in feed and water consumption was noted in any of the groups. A gradual decrease in body weight of animals from day 0 to day 9 followed by a gradual increase from day 9 to 18 was observed in all the APH treated groups. Oral ulcers (fig 3.1(A&B) showing the normal and disease control respectively) appeared in animals of all the groups injected with APH on the day1.

Table 3.2a: Body weight changes in animals of various groups (data are shown as percentage of increase/decrease with minus (-) sign indicating weight loss and absence of minus sign indicating weight gain)

<table>
<thead>
<tr>
<th>Animal No</th>
<th>Group</th>
<th>Day 5 % of weight loss/gain</th>
<th>Day 10 % of weight loss/gain</th>
<th>Day 15 % of weight loss/gain</th>
<th>Day 20 % of weight loss/gain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>1 7.5</td>
<td>0.6</td>
<td>4.9</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 5.7</td>
<td>4.5</td>
<td>3.6</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 8.7</td>
<td>2.8</td>
<td>5.1</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 4.0</td>
<td>5.1</td>
<td>0.4</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 12.8</td>
<td>5.6</td>
<td>1.7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 6.0</td>
<td>1.7</td>
<td>5.2</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 10.4</td>
<td>3.8</td>
<td>2.6</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 0.9</td>
<td>4.5</td>
<td>3.5</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1 -3.6</td>
<td>-4.5</td>
<td>1.4</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 -4.6</td>
<td>-10.1</td>
<td>-0.1</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 -3.5</td>
<td>-3.8</td>
<td>-0.2</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 -7.4</td>
<td>-5.8</td>
<td>-3.7</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 -13.8</td>
<td>0.9</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 -8.9</td>
<td>-5.1</td>
<td>4.1</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 -10.3</td>
<td>-8.9</td>
<td>4.6</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 -9.6</td>
<td>-8.2</td>
<td>-2.3</td>
<td>-3.3</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1 -9.2</td>
<td>-7.9</td>
<td>3.3</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 -10.0</td>
<td>-5.7</td>
<td>3.6</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 -11.2</td>
<td>-7.6</td>
<td>-3.7</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 -9.8</td>
<td>-3.8</td>
<td>-0.7</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 -9.5</td>
<td>-9.2</td>
<td>3.9</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 -2.8</td>
<td>-17.6</td>
<td>-3.9</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 -7.9</td>
<td>-4.0</td>
<td>2.5</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 -9.1</td>
<td>-5.3</td>
<td>8.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>
This persisted up to day 6 in disease control, standard, KC and KNC animals but disappeared on day 3 in KP treated animals. No mortality was observed throughout the study in any group of animals.
3.3.2. Hematology analysis
The results of hematology analysis are depicted in the figures below. The tabulated form of the data is given in the appendix of this chapter.

3.3.2.1. Erythrocyte and reticulocyte Population
There was a significant reduction in the population of erythrocytes (fig. 3.2) in disease control (4.53 million cells/µl) and KNC treated animals (4.31 million cells/µl) compared with normal control group.
Figure 3.2 Effect of käntam formulations on total number of erythrocytes of various groups (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control;$P>0.05, $$p<0.05, $$$p<0.01, $$$$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

Figure 3.3 Effect of käntam formulations on percentage of reticulocytes of various groups. (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control;$P>0.05, $$p<0.05, $$$p<0.01, $$$$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

A mild reduction of erythrocytes was observed in KC, KP and standard treated animals (6.4 – 6.6 million cells/µl) compared with normal control animals.

There was a significant increase in reticulocytes (Fig. 3.3) of all the APH treated
groups. Population of reticulocytes was very high in KNC treated group (>15%), moderately high (10-15 %) in disease control and slightly high (5-10%) in standard control, KC and KP treated groups compared with normal control.

3.3.2.2. Erythrocyte morphology

MCV (Mean Corpuscular Volume) (Fig. 3.4) values were significantly high (>70 fL/cell) in all APH treated groups compared with normal control. MCH (Mean Corpuscular Hemoglobin) (Fig. 3.5) value was very high in disease control (28.60 pg/cell) and KNC treated animals (30.10 pg/cell) whereas moderately high in standard (20.92 pg/cell), KC (21.14 pg/cell) and KP treated animals (21.16 pg/cell) compared with normal control.

![Figure 3.4](image)

**Figure 3.4** Effect of kāntam formulations on Mean Corpuscular Volume of various groups. (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $$p>0.05, $$$p<0.05, $$$$p<0.01,$$$$$$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

MCHC (Mean Corpuscular Hemoglobin Concentration) values (Fig. 3.6) are significantly high in disease control (39.40 g/dL) and KNC treated (36.57 g/dL) groups. But no significant difference is observed in all other groups compared with normal control.
Figure 3.5 Effect of *kāntam* formulations on Mean Corpuscular hemoglobin of various groups (*P*>0.05, **P**<0.05, ***P***<0.01, ****P***<0.001 vs. N.Control; $$$P$>0.05, $$P$<0.05, $$$P$<0.01, $$$$P$<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

Figure 3.6 Effect of *kāntam* formulations on Mean Corpuscular hemoglobin concentration of various groups (*P*>0.05, **P**<0.05, ***P***<0.01, ****P***<0.001 vs. N.Control; $$$P$>0.05, $$P$<0.05, $$$P$<0.01, $$$$P$<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

3.3.2.3. Total Hemoglobin levels:

Significant reduction of hematocrit value (Fig. 3.8) is noticed only in disease control
(32.86 %) and KNC treated group (35.45 %). But no significant variation was observed in all other groups compared with the normal.

Figure 3.7 Effect of kāntam formulations on hemoglobin % of various groups. (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $P>0.05, $$$p<0.05, $$$$p<0.01, $$$$$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

Figure 3.8 Effect of kāntam formulations on hematocrit value of various groups (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $P>0.05, $$$p<0.05, $$$$p<0.01, $$$$$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))
Despite the significant change in hematocrit value and erythrocyte count, no significant changes were observed in hemoglobin% (Fig. 3.7) of all other groups compared with normal control.

Figure 3.9 Effect of kāntam formulations on total number of platelets of various groups (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $^5$P>0.05, $$^5$$p<0.05, $$$^5$$p<0.01, $$$$$^5$$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

3.3.2.4 Platelets:
Though there is a statistically significant decrease in platelet count, (Fig. 3.9) it was found to be physiologically normal in all the animals compared with normal control.

3.3.2.5. Leukocytes:
A significant increase in total leukocytes (18.04 thousand cells/µL) (Fig. 3.10) with increased neutrophils (5.35 thousand cells/µL) (Fig. 3.11) and monocytes (1.13 thousand cells/µL) (Fig. 3.12) was observed in animals of the disease control group. However, lymphocyte count (Fig. 3.13) remained unaffected in this group.
Figure 3.10 Effect of kāntam formulations on total number of leukocytes of various groups (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $\gamma^2 P>0.05$, $$$p<0.05$, $\\sssp$p<0.01, $\\ssss$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

Figure 3.11 Effect of kāntam formulations on total number of neutrophils of various groups (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $\gamma^2 P>0.05$, $$$p<0.05$, $\\sssp$p<0.01, $\\ssss$p<0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))
In contrast to this, a significant decrease in population of leukocytes (7.6 - 9.3 thousand cells/µL) and lymphocytes were noted in KC, KP and standard treated groups. Both neutrophil and monocyte counts were unaffected in all these groups.

KNC treated group showed increase in neutrophil population (4.22 thousand cells/µL) and monocyte population (0.63 thousand cells/µL) without any significant change in leukocyte and lymphocyte population.

No significant changes were observed in the population of eosinophils and basophils in all other groups as compared to normal control.
3.3.3. Biochemical Analysis:

The results of biochemical analysis are depicted in the figures below. The tabulated form of the data is given in the appendix of this chapter.

No significant difference was noted in the glucose levels (Fig. 3.14), total and direct bilirubin levels (Fig. 3.15 & Fig. 3.16) in all the APH treated groups as compared with the normal control group. However, Bilirubin (total and direct) levels were significantly reduced in KC, KP and standard treated compared with disease control.
Figure 3.14 Effect of *kāntam* formulations on serum glucose levels of various groups (*P* > 0.05, **P** < 0.05, ***P*** < 0.01, ****P*** < 0.001 vs. N.Control; $^5$P > 0.05, $^6$P < 0.05, $^7$P < 0.01, $^8$P < 0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

Figure 3.15 Effect of *kāntam* formulations on serum direct bilirubin levels of various groups (*P* > 0.05, **P** < 0.05, ***P*** < 0.01, ****P*** < 0.001 vs. N.Control; $^5$P > 0.05, $^6$P < 0.05, $^7$P < 0.01, $^8$P < 0.001 vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))
Figure 3.16 Effect of kāntam formulations on serum total bilirubin levels of various groups (*P>0.05, **p<0.05, ***p<0.01, ****p<0.001 vs. N.Control; $P>0.05, $$$p<0.05, $$$$p<0.01, $$$$$p<0.001$ vs. D.Control (One way ANOVA was followed by Dunnett’s post hoc test))

3.3.4. Histopathology analysis:
3.3.4.1. Spleen
Tissue sections of spleen were observed under various magnifications (4X, 10X, 40X and 100X) and the scoring was done for various pathologies (Congestion (greater accumulation of erythrocytes in the red pulp of spleen as compared to that of the control), decreased lymphocytes and erythropoiesis) with 0 as minimum and 5 as maximum. The scores of individual animals in a group are added and shown in table.

3.3. Tissue section of spleen (Fig. 3.17) showed congestion, erythropoiesis, erythrophagocytosis and low lymphocyte population in all groups treated with APH. Congestion was relatively low in KC and standard treated groups, high in KNC and moderate in KP compared with disease control. Erythropoiesis was low in KC, moderate in disease control and high in standard and KP. Though there was a minimal decrease of lymphocytes in all APH treated groups significant decrease was observed only in KNC.
Table 3.3 Histopathological scorings of spleen in animal groups treated with different *kāntam* formulations. (Data are represented as cumulative value of scores given to each pathological features observed in the spleen of five animals from each group)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Congestion</th>
<th>Lymphocyte reduction</th>
<th>Erythropoiesis</th>
<th>Erythrophagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Disease control</td>
<td>11</td>
<td>8</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Standard Control</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>KNC treated</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>KC treated</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>KP treated</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

3.3.4.2. Liver

Representative histopathology images of liver of normal and disease control animals are shown in Fig. 3.18. Activated kupffer cells were present in liver sections of animals in all groups treated with APH. Scorings of kupffer cell activation is shown in table 3.4. The activated kupffer cells were low in KC, moderate in standard and KP and high in disease control and KNC.
Figure 3.17 Representative images of section of spleen of A) Normal control (4X magnification) B) Disease control (4X magnification) C) Disease control showing erythrophagocytosis (40X magnification)

Table 3.4 Histopathological scorings of activated kupffer cells seen in liver of animal groups treated with different kāntam formulations

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal Control</th>
<th>Disease Control</th>
<th>Standard Control</th>
<th>KNC treated</th>
<th>KC treated</th>
<th>KP treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scores</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>
3.3.4.3. Bone marrow

Bone marrow of APH treated animals (shown in Fig. 3.19) showed mild diffuse erythroid hyperplasia, which was also significant in all groups treated with APH. Apart from that there is a significant difference in erythropoiesis of all groups compared with normal control and is tabulated in Table 3.5. No other difference in morphology was observed between disease control and drug treated groups.

Table 3.5 Histopathological scorings of erythropoiesis observed in the bone marrow of animal groups treated with different kāntam formulations

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal Control</th>
<th>Disease Control</th>
<th>Standard Control</th>
<th>KNC treated</th>
<th>KC treated</th>
<th>KP treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scores</td>
<td>0</td>
<td>13</td>
<td>8</td>
<td>10</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 3.19 Representative images of section of bone marrow of A) Normal control B) Disease control (images are shown at 4X magnification)

3.3.4.5. Pancreas

Figure 3.20 Representative images of section of pancreas of A) Normal control B) Disease control (images are shown at 40X magnification)

No notable pathological features were present in pancreas (fig 3.20) of any of the
APH treated groups compared with normal control.

3.3.4.6. Blood smears:

Figure 3.21 Representative images of blood smears of pancreas of A) Normal control B) Disease control C) Standard control D) KNC treated E) KC treated and F) KP treated (images are shown at 100X magnification)
Blood smears of the disease control and drug treated groups showed the presence of reticulocytes and erythrocytes of different surface morphology (shown in fig 3.21).

3.4. DISCUSSION:
In the study described in the previous chapter, the absence of the herbal extracts added during the preparation of KC and KP was demonstrated through various techniques. So, the pharmacological activities of both the formulations (KP and KC) should be purely derived from the inorganic substances present in the formulations.

In the present work, the effect of KC and KP in the management of hemolytic complications induced by APH in adult wistar rats was studied in detail. APH is an oxidant chemical that primarily destroys the mature erythrocytes by its effect on enzymes involved in energy metabolism, which, in turn, induces erythropenia leading to an accelerated erythropoiesis resulting in reticulocytosis

The destruction of erythrocytes stimulates the release of the immature reticulocytes into circulation from bone marrow, which further matures to erythrocytes in 2 days. Under normal conditions, the reticulocytes are released into the circulation with the loss of fibronectin receptors – a protein with adhesive nature. In hemolytic conditions, reticulocytes are released into the circulation without losing their fibronectin receptors. In such conditions spleen helps in clearing the fibronectin-adhesive molecules and promotes the process of reticulocyte maturation. But massive hemolysis can overwhelm the capacity of the spleen to deal with the immature reticulocytes resulting in delayed maturation of the reticulocytes. Hence promoting the maturation of reticulocytes to erythrocytes will be the primary task of any drug used in the treatment of hemolysis. Significant reduction of reticulocytes in standard drug, KC and KP treated animals compared to disease control groups indicate the positive role of KC and KP in maturation of reticulocytes. But, in KNC treated animals the reticulocyte population was significantly more compared to disease
control group. Moreover, erythropoiesis in bone marrow of KNC treated subjects was also moderately low compared with disease control (shown in table 3.5). This indicates that KNC has negative effect in maturation of reticulocytes in circulation. These results indicate that KC and KP were very effective in promoting the maturation of reticulocytes whereas KNC induce negative effect in reticulocyte maturation.

High levels of MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin) and MCHC (Mean Corpuscular Hemoglobin Concentration) in disease control and KNC indicates that erythrocytes in animals of these groups were macrocytic (Large cell size) and hyperchromic (More hemoglobin content) in nature. Usually, the reticulocytes and young erythrocytes are macrocytic and hyperchromic\(^3\),\(^25\), which later become normocytic and normochromic as they go through the maturation process in the blood\(^25\). Though MCH values were comparatively better with normal MCHC values in KC and KP treated groups, MCV remains the same in all drug treated groups. The blood smears also showed the presence of more reticulocytes in disease control and KNC treated animals compared with KC, KP and standard treated groups. Hence it is clear that KC and KP have a strong role in the maturation of macrocytic, hyperchromic erythrocytes to macrocytic, normochromic erythrocytes.

Low hematocrit value and decreased erythrocyte count reassert the case of severe hemolysis in animals of disease control. Despite the reduced hematocrit value and erythrocytes, total hemoglobin content of animals in this group was found to be normal. This can be particularly due to the contribution of extracellular hemoglobin (EcHb) formed due to hemolysis. Generally when the hemoglobin is present inside the erythrocytes, it is always accompanied with anti-oxidant protectants that prevent
the erythrocytes from getting destroyed by hemoglobin. But EcHb is devoid of the antioxidant protectants and can cause oxidative devastation in the vasculature. Usually, a protective physiology will be initiated after hemolysis with the help of specialized plasma scavenger proteins to neutralize and eliminate this EcHb. However, when this protective mechanism is overwhelmed by high degree of hemolysis, EcHb remains in circulation and promotes inflammatory reactions.

Significant leukocytosis with high level of neutrophils and monocytes observed in disease control corroborate the presence of EcHb, as EcHb is pro-inflammatory in nature and initiates inflammatory reactions resulting in leukocytosis. Moreover, regulation of heme-induced neutrophil survival and death is critical to resolve inflammation efficiently in any hemolysis. Completely normal hematocrit value and partially recovered erythrocytes with normal total hemoglobin levels observed in standard, KC and KP treated groups demonstrate the protective effect of these kāntam formulations by promoting the elimination of EcHb. The normal level of neutrophils and monocytes in KC and KP groups that demonstrates the complete absence of inflammatory reactions in these animals further reasserting the absence of EcHb in KC and KP treated animals. However, KNC did not show any effect, neither in hematocrit value and erythrocytes nor in monocytes and neutrophils. Thus it is very clear while KC and KP were helpful in clearing the EcHb formed due to hemolysis from the circulation and thus preventing inflammatory reactions, KNC had no impact on EcHb clearance.

Erythrophagocytosis, the primary mechanism involved in the removal of destroyed erythrocytes by macrophages is significantly active in disease control, standard control, KNC, KC and KP treated animals compared with normal control animals (as inferred from histopathology of spleen shown in fig. 3.17 and table 3.2). However,
erythrophagocytosis activity is very high in KC treated animals indicating the effect of KC in promoting erythrophagocytosis. Whereas KNC showed very low erythrophagocytosis activity (50% less than disease control) indicating that KNC is producing negative effect in erythrophagocytosis.

Stress erythropoiesis in spleen is a common compensatory mechanism activated by tissue hypoxia produced during hemolysis. Persistent stress erythropoiesis indicates the defect in the management of tissue hypoxia due to hemolysis. Moderate erythropoiesis with notable liver congestion observed in spleen of disease control, standard and KP treated groups, signifies that KP has no effect in regulating the erythropoiesis and maintaining the tissue oxygen level. But KC treated animals showed only very mild erythropoiesis with markedly reduced liver congestion demonstrating the role of KC in maintaining tissue oxygen levels.

Presence of kupffer cells was noted in liver tissues of all APH treated animals indicating active inflammatory reactions in liver. High degree of prolonged kupffer cell activation leads to destruction of liver tissues. High reduction of kupffer cell activity in KP treated groups demonstrate the effect of KP in preventing liver toxicity during hemolysis through the destruction of liver tissues (table 3.4 and fig 3.18). Though KC and standard groups showed moderate reduction in kupffer cell activity, no reduction was noted in KNC (inferred from table 3.3 and fig. 3.18). Hence it is clearly perceived that KP plays an important role in rapid immune response and tissue repair after hemolysis.

Though pancreatitis during hemolysis has been reported by other researchers, in our study, no significant pathological change was observed in the pancreas across the groups investigated (inferred from fig 3.20). Since histopathology analysis was carried out after 20 days of hemolysis, we surmise that the pancreas would have
recovered from the inflammatory tissue damage. Presence of mouth ulcers in all APH treated groups could be due to anemia induced by hemolysis as mouth sores are common in all types of anemia. Rapid recovery of mouth sores in KP treated group indicate the positive effect of KP in treating mouth sores formed as a result of hemolysis. The pharmacological significance of KC and KP is represented in table 3.6

From the above discussions it is clear that KC and KP have a protective role in hemolytic complications while KNC lacks the protective effect when administered for hemolytic complications. This highlights the significance/importance of the herbs in preparation and potency of the kāntam formulations. Interestingly, this study also gave us a clear picture that vehicle through which the formulation is administered (honey in our case) as such is not the only reason for the activity of the drugs. Because, if that had been the case, KNC should have resulted in some activity as it was administered with the same amount of honey that was used to administer other kāntam formulations.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>KNC</th>
<th>KC</th>
<th>KP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of reticulocyte maturation</td>
<td>✗</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Therapeutic action on oral ulcers</td>
<td>✗</td>
<td>✗</td>
<td>✔</td>
</tr>
<tr>
<td>Regulation of erythrophagocytosis</td>
<td>✗</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Removal of EcHb</td>
<td>✗</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Regulation of kupffer cell</td>
<td>✗</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Regulation of stress erythropoiesis</td>
<td>✗</td>
<td>✔</td>
<td>✗</td>
</tr>
</tbody>
</table>

✔ - Positive effect; ✗ - No effect

The current treatment strategy for hemolytic complications varies with the type and stage of the hemolysis. But folic acid supplements and corticosteroid are primarily used in managing of most forms of hemolytic complications at early stages\textsuperscript{13}, while
blood transfusions and splenectomy are reserved for severe form of hemolysis. In case
of chronic hemolytic conditions such as autoimmune anemia many people will
become steroid-dependent over a period of time\textsuperscript{13}, which force them to adopt second-
line treatment such as blood transfusion and splenectomy. These \textit{kāntam} formulations
can be a better alternative to the steroid dependent in the management of chronic
hemolytic disorders. However, detailed studies on effect of \textit{kāntam} formulations in
chronic hemolysis are warranted to determine its treatment efficacy.

3.5. CONCLUSION:
The pharmacological activity of KC and KP along with a negative control (KNC)
were studied for its effect in preventing the hemolytic complications and following
conclusions are drawn:
KC and KP are significantly effective in treating hemolytic complications by
promoting erythrophagocytosis, removing EcHb from circulation expeditiously and
preventing persistent inflammatory activities.
As KNC, which is prepared without any plant extracts showed no effect in the
recovery of any pathology caused due to hemolysis, it is clear that KNC is not
qualified to be a drug. So we unambiguously infer that the herbal materials added
during the preparation of \textit{kāntam} formulations indeed play a crucial role in modifying
the raw \textit{kāntam} to a final effective drug formulation. Moreover, significantly high
counts of reticulocytes in KNC treated groups compared to disease control groups
indicates that besides (obviously) not showing any positive effect, if the drug
formulation is not processed unscrupulously, herbo-metallic preparations can produce
toxic effects upon administration (as inferred from KNC effect). Furthermore, studies
described in chapter 2, we found that the plant extracts produce physico-chemical
modification of the raw \textit{kāntam} leaving no trace of organic contents in the final
formulation. Hence it is clear that the physico-chemical modification of the raw material is responsible for the drug activity. However, it is difficult to pinpoint the precise mechanism behind the activity of the kāntam formulations with the present analysis. Further controlled experiments with various vehicles such as ghee, butter, milk etc., are needed for better and effective use of these drugs besides working on the mechanistic pathways through which these kāntam formulations produce their therapeutic activity. Overall, this study has demonstrated the therapeutic effect of kāntam formulations in the management of hemolytic complications.

3.6. REFERENCES:


Shahab Abid and Haleem Khan, 'Severe Hemolysis and Renal Failure in Glucose-6-Phosphate Dehydrogenase Deficient Patients with Hepatitis E ', Am. J. Gastroenterol, 97, 2002.


Suh Yee Goh Sae-Kyung Lee, Yuan QiWong, Jeak Ling Ding, 'Response of Neutrophils to Extracellular Haemoglobin and Lta in Human Blood System', EBioMedicine, 2, 225-33, 2015.


