Macrocytic Anemia and Liver Inflammation Induced by Eucalyptus Oil

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ABSTRACT

The toxic effects of a toxicant result in histo-pathological and hematological variations that may lead to hepatic inflammation. Plant extracts and essential oils have long been used in medicines and food additives despite of their detrimental effects. Eucalyptus oil (EO) is commonly used in pharmaceutical industry for its high contents of menthol. The current study was planned to study the hepatotoxicity and hemotoxicity in Wistar rats. A dose dependent study was executed with \( T_1 = \text{1.25ml/kg}, \ T_2 = \text{2.5ml/kg} \) and \( T_3 = \text{5ml/kg} \) (kg of body weight) of oral administration of EO. The three experimental groups were compared with control. The animals were dissected and blood extracted by direct heart puncture. Liver was excised and formalin fixed for H&E staining. A statistically significant increase was observed in MCV, percentage RDW and HCT that arguments for macrocytic anemia. With conserved morphology, the hepatic lobules have shown recruitment of inflammatory cells in perivascular areas with lower degree of hepatic cellular necrosis. Taken together these results we can conclude that EO can cause sterile inflammation to the liver with macrocytic anemia.

Keywords: Eucalyptus Oil, inflammation, Macrocytic anemia, Hematology, Liver histology

INTRODUCTION

Essential oil extracted from Eucalyptus, is commonly used in food and pharmacological industries (Batish et al., 2008; Poke et al., 2005) for its various biological properties such as its cognitive performance (Gobel et al., 1994; Lis-Balchin, 1997; Serafino et al., 2008) and antiseptic activity particularly against Streptococcus mutans (Takarada et al., 2004).

Hematological variations provide direct indices for diagnosis of many disorders and malfunctioning physiological parameter e.g. hemolytic anemia in hepatitis (Agrawal et al., 2011; KIVEL, 1961), and drug induced variations in blood structure (Rubran, 1989; Vandendries & Drews, 2006). Particularly red blood cell distribution width (RDW%) is routinely assessed as part of the complete blood count (CBC) to gather information on the variations in the size (volume more precisely) of circulating RBCs and therefore higher RDW% reflects greater variability in RBC size (anisocytosis), which is usually caused by perturbation in erythrocyte maturation or degradation (Evans & Jehle, 1991). The RDW is used as supporting index to help diagnose different types of anemia. It has also been serving as a marker for colon cancer and celiac disease because of its responsiveness to subtle nutrient deficiency (Patel et al., 2009; Sategna et al., 2002; Spell et al., 2004). Recent studies provide RDW% to be an efficient marker of cardiac disorders (Allen et al., 2010; Felker et al., 2007; Tonelli et al., 2008). Mean corpuscular volume (MCV) is a ready and direct indication for various kinds of anemia and macrocytosis (increased MCV) (Seward et al., 1990). Macrocytosis, a condition that refers to enlargement of RBC with nearly constant hemoglobin concentration, can be categorized in two types (Kaferle & Strzoda, 2009). Macrocytosis with megaloblastic features (with increased Reticulocytes, due to bone marrow dysfunction and folate or vitamin B12 deficiency) whereas the other types refers to non megaloblastic features (with normal Reticulocytes percentage, occurring by dint of iron deficit, pregnancy, alcoholic liver diseases, thyroid dysfunction, renal disorders, spleenotectomy and gammopathy (Horstman et al., 2005; Mukhopadhyay et al., 2007; Oosterhuis et al., 2000)).

Acute inflammation is first strategy in case of any immunological assault to body including changes in various chemical mediators level and metabolic pathways (Gabay & Kushner, 1999; Malik et al., 2010; Malik et al., 2011; Sheikh et al., 2007). The liver is most prominent organ in detoxifying unwanted agents; it is vulnerable for the acute inflammation and necrotic damage more than any other organ in body (Kuntz & Kuntz, 2006; Sheikh et al., 2006) so liver is most hot topic of toxicological or pharmacological studies of various plant extracts.

The point of conflict is that despite of popularity in common uses of essential oils, they do have some side effects (Bakkali et al., 2008). Eucalyptus oil is reported for met-hemoglobin formation and chronic liver failure (Craig, 1953; Eisen et al., 2004; Webb & Pitt, 1993). The aim of current study was to particularly describe acute phase response induced by Eucalyptus oil in comparison to previously reported chronic or sub chronic investigations.

Author's Contribution: N.S., Developed concept and supervised the project; M.S., Designed and performed experiments; N.Z., Wrote manuscript. *Corresponding author: s_nadeem77@yahoo.com.
MATERIALS AND METHODS

The Wistar rats (*Rattus norvegicus*) were segregated in three experimental groups (n=5) and one group as negative control (treated with 0.9% pyrogen free saline solution orally). The experimental groups were designated as T1 (1.25ml/kg), T2 (2.5ml/kg) and T3 (5ml/kg of body weight). The animals were anesthetized, dissected and blood was collected by direct heart puncture in EDTA vials. CBC indices were obtained using automated hematology analyzer (Model MEK-6318; Power input 190V A; 220-240 Volts; Nihon Kohden Corp.). Livers were excised, washed with physiological saline solution and fixed in 10% formalin which was later used for H&E histological staining. The data were analyzed using one way ANOVA and the significance level was accepted where P<0.05 using Megastat Add-in for Microsoft Excel 2010.

**RESULTS**

**Hematology**

Statistically significant elevations were observed in MCV, RDW% and HCT as summarized in Table 1. Mean count values explain the increasing trend in the RBCs number. (R²-value =0.959). While the p-value from the one factor ANOVA of RBCs count is 0.2791 which represent the non-significant differences among the groups (control and test groups) suggesting a lesser significant effects of EO doses over the RBCs number of test groups’ animals (Table 1).

Comparison for MCV exhibited a trend in increase (R²-value=0.8064). One factor analysis of variance proves the significant difference between the treatments (p= 0.0306). There was significant change in MCV level in control and experimental groups by applying one factor ANOVA and Tukey’s post hoc test. Significant increase in values of T2 (2.5ml/kg EO) and T3 (5ml/kg EO) was observed as compared to control group (Table 1).

Table I: Mean values with standard error of mean (S.E.M.) of complete blood count parameters in different treatments against control animals.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>T1 (1.25ml/kg)</th>
<th>T2 (2.5ml/kg)</th>
<th>T3 (5ml/kg)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBCs count (10¹²/l)</td>
<td>8.49±0.26</td>
<td>8.85±0.35</td>
<td>9.2±0.4</td>
<td>9.35±0.05</td>
<td>0.27</td>
</tr>
<tr>
<td>Mean corpuscular volume (fl)</td>
<td>41.1±0.3</td>
<td>46.95±0.45</td>
<td>48.25±1.75</td>
<td>1.55</td>
<td>0.03</td>
</tr>
<tr>
<td>Red blood cell distribution width</td>
<td>14.85±0.25</td>
<td>17.45±0.55</td>
<td>17.3±0.2</td>
<td>17.1±0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>34.9±1.3</td>
<td>41.9±1.2</td>
<td>44.6±0.3</td>
<td>45.95±1.55</td>
<td>0.009</td>
</tr>
<tr>
<td>Platelets (10⁹/l)</td>
<td>712±5</td>
<td>943±0.133</td>
<td>687.5±279.5</td>
<td>794±0.20</td>
<td>0.673</td>
</tr>
<tr>
<td>Mean platelets volume (fl)</td>
<td>7.25±0.05</td>
<td>65±0</td>
<td>7.05±0.35</td>
<td>6.55±0.05</td>
<td>0.096</td>
</tr>
<tr>
<td>WBCs count (10⁹/l)</td>
<td>8.25±1.45</td>
<td>6.7±0.8</td>
<td>6.5±0.35</td>
<td>3.5±1.1</td>
<td>0.111</td>
</tr>
<tr>
<td>Hemoglobin content (g/dl)</td>
<td>15.75±0.35</td>
<td>16.3±0.2</td>
<td>16.3±0.85</td>
<td>17.4±0.1</td>
<td>0.069</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (pg)</td>
<td>17.3±0.1</td>
<td>18.3±0.5</td>
<td>17.6±0.15</td>
<td>18.5±0.15</td>
<td>0.947</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (g/dl)</td>
<td>42.2±0.6</td>
<td>39±0.6</td>
<td>36.7±1.6</td>
<td>2.05</td>
<td>0.102</td>
</tr>
</tbody>
</table>

A mild increasing trend in RDW% can be inferred from the graphical representation. RDW % increased from control in first dose T1, while, from T1 to T2 and T3, the index has not shown variation (R² values= 0.4837). Significant change in RDW% in control and experimental groups was found by one factor ANOVA (p=0.0178) and Tukey’s post hoc test. There was significant increase in values in T1 (p=0.0064) T2 (p= 0.007) and T3 (p= 0.0106) when compared to control group (Table I).

Hematocrit percentage exhibited an overall increasing trend in the experimental groups. Percentages tended to vary between control and test groups (R² value=0.884). There resided a statistically significant increase in HCT in treated groups in comparison to control (p-value=0.0094). Post-hoc analysis maximum difference (increase) was found between the control and treatment groups. There was significant increase in values of T1 (p=0.0140) T2 (p= 0.0044) and T3 (p= 0.0027) as compared to control groups (Table I).

Platelets percentages tended to vary least between control and test groups (R² value = 0.0001). PLT increased in the T1 then platelets
count decreased abruptly. No significant difference was observed between test groups (p-value = 0.6732) (Table I).

MPV tended to vary between control and test groups. With the (R^2-value = 0.2917) non-significant intergroup variations were observed for mean platelets volume (p-value = 0.0961) (Table I).

WBCs count (10^9/l) showed marked decrease (R^2-value = 0.8746) While the p-value = 0.1117 narrated non-significant intergroup variations for WBCs counts the experimental groups (Table I).

Hemoglobin contents were noticed to have an increasing trend in the test groups as compared to the control (R^2 = 0.8951). p-value = 0.0699 from one factor ANOVA suggests non-significant difference in HGB content between the groups (Table I).

MCH (mean corpuscular hemoglobin) level decreased in treated groups in comparison to control animals (R^2 = 0.4829) While p-value = 0.0947 from one factor ANOVA shows that there is non-significant variation among the groups (Table 1).

MCHC (mean corpuscular hemoglobin concentration) exhibited minimum variations between control and experimental groups (R^2 = 0.6811). Whereas p-value = 0.1025 from one factor ANOVA represented non-significant intergroup variations (Table 1).

**Histopathology**

**Control:** H&E stained sections of the control liver showed no infiltration of leucocytes to the neighboring surrounding sinusoid spaces. Hepatic lobules are not fragmented or distorted suggesting no disorientation in the microtubules architecture of liver tissue (Fig., 1a).

**T1 (1.25ml/kg) test group:** In the 1st test group with T1 the infiltration of leucocytes from the hepatic arteries to the sinusoids has been marked showing

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**Fig., 1:** Histological staining of the liver sections to demonstrate the tissue architecture integrity and effect of the eucalyptus oil. Normal hepatic lobular architecture is visible in control (a), with infiltration of leucocytes in T1 group (b), infiltration of leucocyte and mild degeneration with diffused necrosis in T2 (c) and high degree of the tissue damage along with recruitment of lymphocytes in T3 (d).
the immune response of the organisms. The portal vein and artery are not disrupted or distorted. Cellular degeneration is least observed in T1 animals (Fig., 1b).

**T2 (2.5ml/kg) test group:** In the 2nd test group with T2 the infiltration of leucocytes to sinusoids has been noticed representing that body has fired immune response. The portal vein and hepatic artery are evident with thinning of walls and damage. Lower level of cellular degeneration and diffused necrosis has been observed (Fig., 1c).

**T3 (5ml/kg) test group:** Livers of the animals with the 5 ml/kg EO administration revealed to have a maximum degree of the tissue damage by dint of inflammation and recruitment of lymphocytes to the surroundings and the vasculature of the hepatic tissues is deformed at its maximum relatively. Higher degree of coagulative necrosis and cellular degeneration has been observed in liver sections belonging T3 animals (Fig., 1d).

**DISCUSSION**

Taken together the variations in hematological parameters, it can be inferred that Eucalyptus oil high dose is able to result in macrocytosis with non-megaloblastic features (increased MCV, RDW% and HCT) which were in agreement with the studies reported by Breedveld et al. (1981) and acute inflammation in the liver: infiltration of lymphocytes to sites of hepatocellular damage has also been reported by Gruys et al. (2005).

Acute hemotoxicity can be correlated to the occurrence of acute inflammation in the living system, as there is variation in serum levels of many chemical mediators having distinct characteristic functions. Their variations result in variability of the physiology of the body as well (Ceciliani et al., 2002; Gitlin & Colten, 1987; Gruys et al., 2005; Liz et al., 2010).

Increase in HCT level is evident in severe cases of dehydration (Tannheimer et al., 2010), and dehydration in acute phase response, on the other hand is caused due to the down regulation of serum albumin level i.e. a negative acute phase protein, dropped systematically in serum to facilitate synthesis of other immune active positive acute phase proteins (Ceciliani et al., 2002; Gitlin & Colten, 1987; Gruys et al., 2005; Liz et al., 2010; Ritchie et al., 1999). Serum albumin level along with immunoglobulins is responsible for osmotic balance and fluidity of blood, a decrease in the serum albumin level makes the blood impotent to leech tissue water (Zetterstrom & Hedstrand, 1981).

Another possible reason of increase in Hematocrit and mild increase in RBCs can be, increase in the Erythropoietin (EPO) as it is a positive acute phase protein and it is evident to be synthesized from liver cells during inflammation (Ramadori et al., 2010).

Erythropoietin is the principal hormone involved in the regulation of erythrocyte differentiation and the maintenance of a physiological level of circulating erythrocyte mass. It performs its function by activating the EPO- receptors particularly in Erythroid lineage of stem cells upon stimulation; EPOR dimerizes and activate JAK2/STAT5 signaling cascade (STAT1 and STAT3 are rarely initiated) that serves to switching on the transcription of various proteins. Erythroid transcription factor (Eryf1) (GATA-binding factor 1) also gets activated by action of EPO on stem cells. Transcriptional activator which probably serves as a general switch factor for erythroid development works by binding to DNA sites with the consensus sequence of [AT]GATA[AG] within regulatory regions of globin genes and that of other genes up regulated in erythroid cells.

To our knowledge EPO also regulates the body iron by causing an up-regulation of NRAMP 2 (Natural resistance-associated macrophage protein 2), DMT-1 (Divalent metal transporter 1). These proteins are involved in the iron uptake into duodenal enterocytes; in iron transport from acidified endosomes into the cytoplasm of erythroid precursor cells and also play an important role in hepatic iron accumulation and tissue iron distribution (Aird et al., 1994; Digicaylioglu & Lipton, 2001; Kapur & Zhang, 2001). All these biochemical changes may result in the increase of packed cell volume.

Macrocytosis is evident to occur under the effect of various toxicants and reason can be the oxidative stress and direct membrane damage to the cells in particular RBCs (Horstman et al., 2005; Kaferle & Strzoda, 2009; Mukhopadhyay et al., 2007; Tonnesen et al., 1986).

Hepatic histopathological variations are most obvious to occur under the influence of toxicants due to its detoxification potential and changes in Acute phase proteins, and various other hormones (Sundaram & Shaikh, 2011). Hepatocellular damage has its roots in mitochondrial dysfunction leading to initial diffused hepatocellular necrosis.
Mechanism for drug-induced liver injury is the formation of reactive metabolites, direct toxicity of liver cells or immune reactions resulting in mitochondrial membrane disruption. Organic toxicant itself can trigger mitochondrial membrane disruption or inhibits mitochondrial function through different mechanisms, such as it can seize coenzyme A or can inhibit mitochondrial β-oxidation enzymes, the transfer of electrons along the respiratory chain, or adenosine triphosphate (ATP) synthase.

Mitochondrial central dogma might also be impeded due to the organic molecules. A severe impairment of oxidative phosphorylation decreases hepatic ATP, leading to cell dysfunction or necrosis; inhibition of pyruvate catabolism, leading to lactic acidosis is also observed. Susceptibility to drug-mediated mitochondrial dysfunction can be increased by factors impairing the removal of the toxic parent compound or by the presence of other medical condition impairing mitochondrial function (Pessayre et al., 2012). Conclusively it can be said that EO can cause macrocytic anemia and inflammation of liver in Rattus norvegicus.

REFERENCES


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