Effect of a polyherbal formulation, Ambrex, on butylated hydroxy toluene (BHT) induced toxicity in rats


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Effect of polyherbal formulation Ambrex was evaluated in butylated hydroxytoluene (BHT) induced toxicity of lungs and liver in rats. Toxicity was produced by administering BHT (500 mg/kg/day) for 3 days. Lung damage was evidenced by elevated levels of bronchoalveolar lavage fluid (BAL) parameters such as protein, lactate, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP) and glucose-6-phosphate dehydrogenase (G6PDH). Liver damage was proved by elevated levels of serum protein and markers such as LDH, ALT, aspartate aminotransferase (AST), alanine amino transferase (ALT), decreased level of lipid peroxides (LPO) in serum and glutathione (GSH) in liver. Administration of aqueous suspension of Ambrex (50 mg/kg orally) retained these elevated levels of BAL-protein, lactate, LDH, ALP, ACP, G6PDH and serum-protein, LDL, ALT and AST and ALT at near normal values. Decreased level of liver GSH was retained at near normalcy in Ambrex pretreated BHT-administered animals. There was no change in liver LPO in all the four groups.

Key words: Ambrex, BAL (Bronchoalveolar lavage), BHT (Butylated hydroxytoluene), Hepatotoxicity, Lung toxicity, Serum markers

BHT, a synthetic phenolic antioxidant, widely used as a food additive, confers substantial benefits to man by preserving and improving the palatability of food. Preservatives may inhibit growth and activity of microorganisms by interfering with their cell membranes, enzyme activity or genetic mechanisms. Other preservatives may be used as antioxidants to hinder the oxidation of unsaturated fats, as neutralizers of acidity, stabilizers to prevent physical changes, firming agents, and as coating or wrappers to keep out microorganisms, prevent loss of water, or hinder undesirable microbial, enzymatic and chemical reactions. BHT compound has been the subject of extensive toxicological investigation in various animals. Though generally considered to be safe at the concentrations present in foods (the acceptable daily intake of BHT for man 0.5mg/kg), high doses of BHT cause haemorrhagic death accompanied by inhibition of hepatic prothrombin synthesis in rats, pulmonary injury in mice. BHT has been shown to induce reversible mixed function oxidases and liver enlargement in rats and peliosis, hepatocellular vacuolation, degeneration and necrosis in the liver of mice and found to increase the mitotic activity of liver cells in rats. In some investigations on the mechanism of BHT-induced hepatotoxicity, plasma transaminase levels were used to indicate toxicity. BHT, if given at high doses to mice causes lung damage.

Traditional drugs have been the starting point for the discovery of many important drugs. This fact has led to chemical and pharmacological investigations of plants and the undertaking of general biological screening programmes of plants not only in India, but all over the world. Ambrex (trade name) is a polyherbal formulation (a coarse powder partly soluble in water) consisting of Aswagandha (Withania somnifera), Amber, Shalaminshiri (Orchis mascula), Roomi mastagi (Shorea robusta) and Madanakamappu (Ceyos cinguinalis). Shorea robusta is given in ulcers, haemorrhoids, bleeding, anaemia and weak digestion. Amber is an antiseptic, antispasmodic and also given in delirium and infectious diseases. In olden days, Amber-based medicine was used for preventing premature aging and for increasing longevity. The medicine is used by the sick to recover health and increase resistance to diseases, stamina, endurance of athletes and for tremendous boost to their performance by increasing the energy in the human body. This is due to the presence of succinic acid in Amber. Amber bracelet eases rheumatic pain...
and amber coral beads supposedly help in cases of thyroid illnesses. *Cycas circinalis* is good for hyperdipsia, burning sensation, sores and swellings. *Wittaniana somnifera* has antioxidant effect and reported to have antistress, immunomodulatory, antiinflammatory effects by free radical scavenging activity. *Oryxus muscula* has antidiarrhoeal and antidysenteric effects. Hence, an attempt has been made to evaluate the potency of Ambrex in modulating BHT-induced toxicity in rats.

### Materials and Methods

Male albino rats of Wistar strain (100-150g) were used as experimental animal model in the present study. Rats were fed with commercial pellet diet and water *ad libitum*. BHT was purchased from Sisco Research Laboratory, Chennai and Ambrex was gifted by Care and Cure Herbs Pvt. Ltd., Chennai. Animals were divided into four groups of six animals each. All animal experiments were carried out as per the guidelines provided by Institutional Animals Ethics Committee (IAEC).

Group I was given sunflower oil intraperitoneally for 3 days (control), group II animals were injected (ip) with BHT (500 mg/kg/day in sunflower oil) for 3 days, group III animals were pretreated with aqueous suspension of Ambrex (50 mg/kg/day, po) for 18 days and the animals were treated with BHT for 3 days prior to sacrifice and Group IV — drug control rats were administered aqueous suspension of Ambrex alone (50 mg/kg/day, orally) for 18 days and sunflower oil was administered (ip) for 3 days prior to sacrifice (the total experimental period was 18 days).

The experimental animals were sacrificed after overnight fasting. Blood was collected by cutting the jugular vein and serum was separated by centrifugation. Liver was removed, homogenised with Tris-Cl buffer and the supernatant was used for the estimation of LPO and GSH. The lungs were removed and washed with ice-cold saline to remove blood.

### Isolation of bronchoalveolar lavage (BAL) fluid

The lungs were lavaged two times with 5 ml of lavage fluid (0.15M NaCl-0.01M Tris HCl, pH 7.4). The lungs were connected to a syringe via a tracheal tubing and allowed the lavage fluid to pass till all the alveoli adjacent to the visceral pleura were filled. The lavage fluid was withdrawn and again infused. The recovered fluid was stored in ice. The combined lavages were centrifuged at 1000 g for 10 min and the supernatant fluid was used for estimation of various marker enzymes.

Estimation of protein, assay of LDH and ALP in both serum and BAL were performed. Estimation of lactate, ACP and G6PDH were assayed in BAL fluid and AST, ALT and lipid peroxides were estimated in serum. Intergroup differences were compared using Student’s t-test.

### Results

Table 1 shows the levels of protein, lactate and activities of LDH, ALP, ACP and G6PDH in BAL fluid. A significant increase (*P*<0.001) in the levels of protein, lactate and the activities of LDH, ALP, ACP and G6PDH was observed in the BAL fluid of BHT-treated group II rats when compared to group I.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
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</thead>
<tbody>
<tr>
<td>Protein (mg/ml)</td>
<td>354.86 ± 33.01</td>
<td>469.75 ± 36.66&lt;sup&gt;****&lt;/sup&gt;</td>
<td>358.03 ± 35.39&lt;sup&gt;****&lt;/sup&gt;</td>
<td>365.98 ± 36.54&lt;sup&gt;****&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate (μmol/ml)</td>
<td>1.701 ± 0.18</td>
<td>3.19 ± 0.22&lt;sup&gt;****&lt;/sup&gt;</td>
<td>1.73 ± 0.08&lt;sup&gt;****&lt;/sup&gt;</td>
<td>1.91 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDH (IU/dl)</td>
<td>5.32 ± 0.38</td>
<td>11.6 ± 0.8&lt;sup&gt;****&lt;/sup&gt;</td>
<td>5.87 ± 0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.92 ± 0.45&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACP (μmol of phe-nol liberated/min/dl)</td>
<td>1.33 ± 0.44</td>
<td>2.28 ± 0.12&lt;sup&gt;****&lt;/sup&gt;</td>
<td>1.45 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.21 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G6PDH (IU/dl)</td>
<td>1.33 ± 0.28</td>
<td>7.47 ± 0.37&lt;sup&gt;****&lt;/sup&gt;</td>
<td>3.54 ± 0.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.31 ± 0.39&lt;sup&gt;c&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup> Group I vs group II; <sup>b</sup> Group I vs group III; <sup>c</sup> Group I vs group IV; <sup>d</sup> Group II vs group III.

<sup>****</sup> *P* < 0.001.
control rats whereas the levels of protein and lactate and the activities of marker enzymes were retained at near normal status in Ambrex pretreated BHT administered group III animals when compared to group I control animals and decreased significantly (P<0.001) when compared to group II BHT treated animals. No significant changes in these parameters were observed in the Ambrex alone treated group IV animals.

Table 2 shows the level of protein and activities of LDH, AST, ALT, ALP and LPO in serum. Level of serum protein, activities of LDH, AST, ALT and ALP increased significantly (P<0.001) in BHT treated group II when compared to group I control rats. These parameters were retained at near normalcy in Ambrex pretreated BHT administered group III when compared to group I control rats and significantly decreased (P<0.001) when compared to group II BHT treated rats. No significant changes were observed in Ambrex alone treated group IV animals. Lipid peroxide levels in BHT treated group II were significantly (P<0.001) decreased when compared to control group I, whereas Ambrex pretreated BHT administered group III animals showed significant decrease (P<0.001) in LPO levels when compared to group I control animals. In Ambrex pretreated BHT administered group III animals, LPO level was not changed significantly when compared to group I BHT treated rats. In Ambrex alone group IV animals, the LPO level showed no significant change when compared to control group I animals.

Table 3 shows the levels of LPO and GSH in liver. The level of hepatic LPO was not changed significantly in all the four groups, whereas the level of hepatic GSH was decreased significantly (P<0.001) in BHT treated group II rats when compared to control group I rats. In Ambrex pretreated BHT administered group III rats, the GSH level was increased significantly (P<0.001) when compared to BHT treated group II animals and decreased significantly (P<0.001) when compared to control group I animals. Ambrex alone administered group IV animals showed a increase (P<0.001) in GSH content when compared to control group I rats.

**Discussion**

BHT is biotransformed into a reactive, electrophilic metabolite BHT-quinone methide by cytochrome P450 mediated metabolism and causes toxicity which can covalently bind to various cellular nucleophiles, especially those containing thiol groups such as glutathione and cysteine. BHT quinone methide has been detected in vivo in liver and lungs.

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**Table 2 — Activities of serum markers of normal and experimental groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/dl)</td>
<td>5.73 ± 0.61</td>
<td>9.15 ± 0.80^***</td>
<td>5.72 ± 0.44^***</td>
<td>5.88 ± 0.28^a</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>79.48 ± 7.41</td>
<td>114.2 ± 12.98^***</td>
<td>98.66 ± 9.39^***</td>
<td>76.33 ± 3.96^a</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>33.28 ± 3.58</td>
<td>60.77 ± 4.05^***</td>
<td>69.67 ± 3.09^***</td>
<td>31.73 ± 3.14^a</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>17.67 ± 1.25</td>
<td>25.67 ± 1.73^***</td>
<td>21.45 ± 2.25^***</td>
<td>18.67 ± 1.25^a</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>3.57 ± 0.48</td>
<td>5.73 ± 0.17^***</td>
<td>5.7 ± 0.16^***</td>
<td>3.86 ± 0.17^a</td>
</tr>
<tr>
<td>LPO (μmoles of MDA/hr/mg protein)</td>
<td>1.86 ± 0.14</td>
<td>1.18 ± 0.14^****</td>
<td>1.03 ± 0.15^a</td>
<td>1.62 ± 0.36^a</td>
</tr>
</tbody>
</table>

^aGroup I vs group II; ^bGroup I vs group III; ^cGroup I vs group IV; ^dGroup II vs group III.

^*** P < 0.001.

**Table 3 — Levels of LPO and GSH in liver of four different groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO (μmoles/g wet tissue)</td>
<td>342.50 ± 7.85</td>
<td>355.50 ± 6.55^a</td>
<td>339.66 ± 3.04^d</td>
<td>348.66 ± 5.65^a</td>
</tr>
<tr>
<td>GSH (μmoles/g wet tissue)</td>
<td>5.33 ± 0.38</td>
<td>1.79 ± 0.26^****</td>
<td>3.35 ± 0.32^****</td>
<td>8.08 ± 0.59^****</td>
</tr>
</tbody>
</table>

^aGroup I vs group II; ^bGroup I vs group III; ^cGroup I vs group IV; ^dGroup II vs group III.

^**** P < 0.001.
An increase in BAL protein and lactate contents in BHT-induced rats may indicate an increase in alveolar capillary permeability and metabolism respectively. BHT has been reported to produce acute lung injury. Lavage LDH is a marker for cell lysis and a statistically significant increase in BAL-LDH indicates lung cell damage. In the present study, oral pretreatment with Ambrex decreased the BHT-induced alterations.

G6PDH is a cytoplasmic enzyme which could occur extracellularly only in the presence of increased cell membrane permeability. G6PDH has been reported to occur in type I pneumocytes which are most vulnerable to injury. It is also a marker enzyme for hexose monophosphate shunt pathway of glucose metabolism which increases in activated macrophages and in tissues where synthetic process or repair is needed. G6PDH has been increased significantly in the present study which denotes the increased cell membrane permeability due to BHT-induced toxicity.

Acid phosphatase is a lysosomal enzyme released by polymorphonuclear leucocytes during phagocytosis and to some extent by macrophages. Damage or death of these cells would release this enzyme into the lavage fluid. Alkaline phosphatase is associated with plasma membrane of mammalian cells. In lungs, type II cells contain alkaline phosphatase but not type I or macrophages. An increase in lavage fluid ALP is a useful index for detecting type I pneumocyte hypertrophy. Type II cells are involved in the storage and synthesis of pulmonary surfactant and injury to these cells may result in loss of proper oxygen exchange function of the lung. Increase in ACP, ALP, and G6PDH is indicative of an inflammatory response which is due to BHT-induced toxicity. Activities of ACP, ALP, and G6PDH were retained by Ambrex pretreatment. The observed results may be due to the protective efficacy of ingredients present in Ambrex.

Alterations in serum constituents derived from the liver may occur as a result of any one of the several morphological changes. The appearance of cellular constituents in the blood stream is probably the most accurate reflection of tissue damage. Determination of the activity of hepatic enzymes released into the blood by the damaged liver is one of the most useful tools in the study of hepatotoxicity.

Enzymes that are most commonly employed as indicators of liver damage are AST and ALT. Both enzymes are present in high concentrations in the liver. Aminotransferases are released from hepatic cells as a consequence of increased membrane permeability and hepatocellular reactions to toxic agents. The increase in aminotransferase is generally more striking with chemicals producing necrosis, for example carbon tetrachloride. An elevated activity of ALT in BHT-injected rats were reported earlier and the above data coincide with the results of the present study. ALP is mostly used as an index of liver function and increased levels were found in serum of most patients with liver damage. Many chemical carcinogens have been reported to increase the activity of serum ALP. BHT produces puzzling data. BHT, if given (in diet or by injection) before a chemical carcinogen would afford protection against chemical carcinogenesis. On the other hand, exposure to BHT after exposure to a carcinogen would sometimes enhance tumour development. The capability of BHT to produce cell hyperplasia in the target organ was thought to be instrumental in the tumour-enhancing or "promoting" activity of BHT. Present study showed an elevated serum ALP activity, an index of liver damage and it may be due to the possible carcinogenic effect of BHT. Pretreatment with Ambrex modulates the changes produced by BHT toxicity.

The lipid peroxidation of cellular membrane has been proposed as one of the mechanisms by which a number of foreign compounds produce structural tissue injury. However, for certain chemicals such as acetaminophen and bromobenzene, the importance of peroxidative damage is a matter of continued discussion. Lipid peroxidation which occurred during chemically induced hepatocellular necrosis was due to the depletion of GSH. The level of lipid peroxides in serum decreased after BHT induction. Similar observations have been recorded in the present investigations. The hepatic damage produced by BHT was associated with a consequence of GSH depletion rather than with lipid peroxidation. The results of the present study also imply the same. In Group III Ambrex pretreated BHT administered rats, the level of serum LPO decreased when compared to BHT treated Group II, this may be due to BHT itself being an antioxidant and hepatic damage produced by BHT was associated with GSH depletion rather than with LPO. The decrease in serum LPO in group III when compared to group I might be due to the antioxidant property of Ambrex. There was an increase in liver GSH in Group III Ambrex pretreated BHT administered rats when compared to BHT treated group II. There was no change in liver LPO in
all the four groups. This may be due to the antioxidant property of the whole formulation, particularly it may be due to Withania somnifera\textsuperscript{17}, Shorea robusta\textsuperscript{48} and also be due to the synergistic activity of the components present in Ambrex. The decrease in liver GSH in group III Ambrex pretreated BHT-administered animals when compared to control group I might be due to the utilization of GSH by damaged cells. GSH plays an important role in the detoxification of activated metabolites of BHT\textsuperscript{47}. When the hepatic GSH level is low for a long time or when excessive quantities of the activated metabolites are produced, the metabolites bind to essential cellular macromolecules causing hepatic damage\textsuperscript{45}. Therefore, the alterations by BHT is prevented by Ambrex which is evident from the results.

The augmented activities of some of the diagnostic marker enzymes in serum, such as AST, ALT, ALP and LDH may be related to the hepatotoxicity induced by BHT and/or its metabolites. Therefore, the result suggests that the hepatic damage produced by BHT was decreased by Ambrex. One of the constituents of Ambrex, Withania somnifera has free radical scavenging activity\textsuperscript{11} and contains a bitter alkaloid 'Somiferin'. It also contains reducing sugar, phytosterol, ipuranol and mixture of saturated and unsaturated acids. The various active principles in Withania somnifera show, neuroprotective, anxiolytic antidepressant (putative antistress) and adaptogenic activity\textsuperscript{46}. The other constituent, Shorea robusta contains flavonoids\textsuperscript{49}. Flavanoids inhibit cytochrome P450\textsuperscript{10} and are potent antioxidants and free radical scavengers\textsuperscript{51}. It can be concluded that, the ingredients present in the Ambrex might be responsible for the observed protective effect against BHT-induced toxicity. However, further study is needed in some more models of experimental hepatic and lung damage to elucidate the exact molecular and biochemical mechanisms involved to establish its therapeutic role as a hepatoprotective and pulmonary preventive agent.

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