EVIDENCE OF OXIDATIVE STRESS, BIOCHEMICAL AND HISTOLOGICAL ALTERATIONS IN KIDNEY AND LIVER ON SHORT TERM INHALATION OF A SPECIFIC MIXTURE OF ORGANIC SOLVENTS.

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ABSTRACT:
The present study was carried out to investigate the possible toxicity in liver and kidney of adult mice exposed to a combination of three solvents, viz., benzene, toluene and xylene, in a 1:1:1 ratio, similar to that used in paint and thinner industries. The mice were divided into three groups viz., Control, Low Dose (450ppm) and High Dose (675ppm) treatment groups using randomization methods. The treated groups were exposed to vapours of a mixture of benzene, toluene and xylene at a low dose of 450ppm and a high dose of 675ppm, for 6 hours per day, for a short-term, 7 day exposure period. The study revealed that the exposure to the solvent mixture caused an increase in the weights of liver and kidney as compared to control. Following exposure significant histological alteration was observed in both liver and kidney. Biochemical analyses indicated a significant decline in the activities of free radical scavenging enzymes SOD and Catalase in both treated groups, with corresponding increase in lipid peroxidation. Solvent exposure also resulted in elevated activity of serum aminotransferases (ALT, AST) which are marker enzymes for liver function, with significant alterations in the levels of protein, creatinine and cholesterol in these tissues. In correlation serum thyroid hormones T\(_3\) and T\(_4\) were also significantly altered. This study therefore demonstrates that exposure to the solvent mixture resulted in significant dose dependent biochemical and histological changes in the vital tissues (liver and kidney) studied. The study has specific relevance since human beings are increasingly being exposed to such solvents due to their increased use in combinations in paint related industries.

Keywords: Inhalation, Solvent mixture, Oxidative stress, Liver and Kidney.

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INTRODUCTION:
In the past few decades organic solvents like benzene, toluene and xylene have become an increasing potential health hazard because of their widespread industrial use in the production of plastic, paints, glues, solvents and also as intermediates in the production of other chemical substances.

Solvent are a class of liquid organic chemicals of variable lipophilicity and volatility and these properties, as well as their small molecular size and lack of charge, make inhalation the major route of solvent exposure. Solvents are also easily absorbed by the lung, gastrointestinal (GI) tract and skin eventually effecting vital organs, like liver and kidney.

Human beings are exposed daily to solvents, although in minute amounts [1]. A large fraction of organic compounds volatilize when products containing them (e.g., aerosol propellants, paint thinners, cleaners, soil fumigants) are used as intended. High concentrations of certain solvents (e.g., 10 to 520 µg/m\(^3\), or 3 to 163 ppb of benzene) have been measured in urban areas around petrochemical plants and in the immediate vicinity of
hazardous waste sites [3-4]. This increased exposure to solvents has manifold effects on diverse tissues and organs.

Much of the research being carried out currently has been centered around investigating the effects of organic solvents individually. Exhaustive studies have been carried out demonstrating inhalation and absorption of Benzene [4]. Similarly, Rickert et al. [5] have reported high benzene concentrations in several tissues including fat, bone marrow, kidney, liver, spleen, suggesting vital organ toxicity. In separate studies toluene has also been shown to accumulate in vital tissues, with an age dependent oral toxicity [6]. Exposure to toluene at high doses was also reported to cause cancer, hypoactivity, ataxia, lacrimation and body tremors in rats. Various experiments have revealed that xylene metabolites affect blood, lung and intestine [7].

Earlier researchers have reported hormonal changes on exposure to industrial chemicals, vapours and solvents [8]. Zaidi et al. [9] have demonstrated sub-clinical hypothyroidism in spray painters, suggesting an implication of solvents in altering endocrine function.

Major health impacts have been reported by the National Institute of Occupational Safety and Health [10] due to organic solvent exposure including nervous, reproductive, skin, kidney and liver damage. Chen et al. [11] have described the toxic effects of solvent mixtures on the liver of paint workers. Wang and Chen [12] have also demonstrated the acute and neurological symptoms among those individuals occupationally exposed to mixtures of organic solvents. Xylene and formaldehyde inhalations are also known to have severe toxic effects on vital functions [13].

Hepatotoxicity is a striking feature of exposure to such solvents. Moreover, it has been suspected that several solvents commonly used today may be hepatotoxic [14] and the liver toxicity of these solvents may be due to metabolic activation with formation of reactive metabolites. Many solvents like xylene when absorbed, about 95% is metabolized in the liver to methylhippuric acid (MHA) [14]. Hence it is imperative to evaluate changes in hepatic cells exposed to solvents. Hepatotoxic alterations in liver histology have been reported by Gotohda et al. [15] on exposure to toluene alone. It was also reported that 70-80% of metabolites excreted in the urine within 24h may play a role the glomerular disease as shown by Askergren [16] and solvent induced proximal tubular cell injury [17]. Ehrenreich [18] has demonstrated the development of glomerulonephritis and membranous nephropathy due to chronic organic solvent inhalation. In addition, Lauwerys et al. [19] have described several nephrotoxic effects of solvent that cause cellular injury within the tubules and glomeruli including epithelial enlargement, ballooning and hydrophilic necrosis. Toxic agents can also induce accumulation of lipid in the renal tubular epithelium, thus damaging kidney function [20] and there are several reports in this regard. However, most of these pioneering researches focus on the effects of benzene, toluene or xylene alone; very few investigations indicate the impact of combinations of such solvents.

Due to the rampant increase in the paint industry, workers in paint and thinner units are constantly being exposed to varied mixtures of benzene, xylene and toluene vapours. On the other hand, there are very few basic research reports documenting the hazardous impact of exposure to mixtures of these three noxious solvents. Hence, research directed towards the possible synergistic toxicity or augmented mode of these solvents in combinations andmixtures is now imperative.

Most solvents used in various organic industries are considered to be toxic and hence induce damage to the liver, resulting in fatty liver, hepatomegaly and cirrhosis. However, despite considerable efforts to elucidate the hepatotoxic effects of toluene and its derivatives, it still seems controversial whether the exposure to these substances may actually induce substantial damage to the liver in the factory workers [21]. Hence the present study was
aimed at determining the biochemical and histological changes in liver and kidney on exposure to a specific mixture of xylene, toluene and benzene.

MATERIALS AND METHODS:

Animals and chemicals:

In the present study healthy adult male albino mice, *Mus musculus* of Swiss strain, weighing between 25 to 35 gm were obtained from a recognised supplier. All the animals were acclimatized for 7 days prior to the commencement of treatment and were maintained under controlled conditions with 12 h light and 12 h dark cycles at a temperature of 26 ± 2°C and relative humidity of 30–70 %. Standard chow (obtained from Amrut Laboratory, Baroda, India) and water were given *ad libitum*. Experiments were conducted in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experimental protocols were approved by the Institutional Animal Ethics Committee (Certification No. 167/1999/CPCSEA).

Experimental design:

Standard inhalation procedures were followed according to the exposure protocol described by Valentine and Kennedy [22], with minor modifications based on the method employed by Uboh et al.[23]. Mice were subjected to whole body exposure of solvents. According to the design, a separate supply of test solvents was placed in each cage where the solvent quanta were calculated in relation to cage volume. To acclimatize the mice, they were housed in closed chambers/mice cages, made of stainless steel, and kept in sets of 6 animal per cage under standard laboratory conditions (light period 6am – 7pm h, 26 ± 2 °C, water and standard pellet diets were given *ad libitum*). Experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experimental protocols were approved by the Institutional Animal Ethics Committee (Certification No. 167/1999/CPCSEA).

Bone collection:

The experimental mice were anesthetized and sacrificed as per CPCSEA specifications. Body weight was measured using a standard animal balance. Liver and kidney were dissected out carefully, blotted free of blood and weighed on a torsion balance, to the nearest milligram. Tissue was processed for histological studies and a homogenate was prepared for various biochemical analyses. All the chemicals used in the experiment were of analytical grade.

Protein:

Protein levels in liver and kidney of control and treated groups of animals were estimated by the method of Lowry et al. [24]. Protein containing preparation when treated with phenol reagent of Folin-Ciocalteau results in a blue colouration which was read at 540 nm.
Cholesterol:  
The levels of cholesterol in the liver and kidney of control and treated groups of mice were estimated by the method of Zlatkis et al. [25]. In the presence of concentrated sulphuric acid and glacial acetic acid, cholesterol forms a coloured complex with ferric chloride (FeCl$_3$) which was measured on a Systronics Digital Spectrophotometer Visiscan 167, against blank.

Creatinine:  
Creatinine was estimated in the kidney of control and treated groups of animals according to the method given by Merck [26]. Creatinine present in the tissue homogenate reacts with Picric acid in an alkaline medium to form an orange red coloured complex which is measured spectrophotometrically at 520 nm.

Superoxide dismutase activity (SOD):  
Superoxide dismutase activity (SOD) activity in liver and kidney of mice was estimated using the technique of Kakkar et al. [27]. In this method, the formazan formed at the end of the reaction indicates presence of the enzyme. One unit of the enzyme activity is defined as the enzyme concentration required to inhibit 50% of the absorbance of chromogen formed in one minute at 560 nm under the assay condition. Results were expressed as unit of SOD/min/mg protein.

Determination of thiobarbituric acid-reactive substances (TBARS):  
Lipid peroxidation was assessed by determination of thiobarbituric acid reactive species (TBARS) levels in liver and kidney of control and treated animals were determined by the method of Okkawa et al. [28]. This method is based on the formation of a red chromophore and the absorption is read at 532 nm following the reaction of thiobarbituric acid (TBA) with malonyl dialdehyde (MDA) and other breakdown products of peroxidised lipids collectively termed thiobarbituric acid reactive substances (TBARS). Lipid peroxidation (LPO) was expressed in terms of nmoles MDA/mg tissue using an extinction coefficient of 1.56×10$^5$ M$^{-1}$ cm$^{-1}$.

Catalase (CAT):  
The catalase activity in the liver and kidney of control and treated animals was assayed by the modified method of Sinha [29].

Alanine aminotransferases (ALT) and Aspartate aminotransferases (AST):  
Serum ALT and AST levels are elevated in viral and other forms of liver diseases associated with hepatic necrosis. In serum ALT and AST activity was determined by the standard karmen unit assay. Alanine aminotransferases catalyse the transamination of L-Alanine and α-Ketoglutarate (α-KG) to form Pyruvate and L-Glutamate. Pyruvate so formed is coupled with 2, 4- Dinitrophenyl hydrazine (2,4-DNPH) to form a corresponding hydrazone, a brown coloured complex in alkaline medium and this can be measured spectrophotometrically at 505nm.

T$_3$ and T$_4$:  
Serum T$_3$ and T$_4$ levels were assayed by a direct competitive enzyme immunoassay using Horseradish Peroxidase- labelled Thyroxine and TMB (Tetramethyl Benzidine substrate). The test was carried out using specific ELISA kits from Labor diagnostics Nord GmbH and Co. K.G.
Histology:
The tissues were freshly obtained, blotted free of blood and fixed in Bouin’s fixative after which they were processed and stained by the standard Haematoxylene-eosine staining procedure. The slides were viewed on a Lawrence and Mayo binocular microscope at low and high power magnifications and images were captured using a Nikon digital camera.

Data analysis:
All the data are presented as Mean ± Standard Error. Statistical analysis was carried out using the SPSS software package version 16.0 (USA). Student’s t-test was carried out taking significance at the 5% confidence limit (**P < 0.001 and *P < 0.01).

RESULTS:

Terminal body weight and tissue weight:
While low dose and high dose of solvent exposure in mice showed an insignificant increase in terminal body weight as compared to control values (Table- I), an insignificant increase was also observed in organ weights after low dose solvent exposure. However after exposure to the high dose level of 675ppm, the organ weights of liver and kidney were significantly increased (P < 0.001) in the exposed group of mice as compared with control mice.

Biochemical Analysis:
The protein content in liver and kidney of solvent exposed and control group is depicted in Figures 1 and 2. Solvent treatment brought about an elevation in the protein content in the low dose and high dose (P < 0.01) groups. Protein levels in kidney showed a significant increase (P < 0.001) after high dose treatments. Creatinine and cholesterol levels significantly increased (P < 0.001) in both treatments studied (Figure 2).

Solvent exposure caused decline in liver SOD activity, the first line of antioxidant defence (Figure 3). Exposure to mixtures of solvents appeared to diminish the antioxidant status by decreasing the activity of hepatic SOD in low dose (P < 0.001) and high dose (P < 0.001) groups as compared to control mice (Figure 3). Hepatic LPO, as indicated by TBARS estimation, was enhanced significantly after solvent exposure in animals exposed to low dose and high dose solvent mixture (P < 0.001; Figure 3). The liver Catalase (CAT) activity decreased significantly after high dose (P < 0.001) solvent exposure. Similar results for these parameters were obtained in kidney tissues after treatment (Figure 4).

Serum ALT and AST enzyme activity was also significantly increased (P < 0.001) in both treatment groups, as a compared to control group. An insignificant increase serum T₃ and T₄ levels was observed in both low dose and high dose groups of solvent exposed mice as compared to control mice (Figure 5).

Histological Studies:

Liver:
Histological observations of the Liver of control mice showed radially arranged hepatic cords around the central vein (Plate A. fig. 1, 2). After exposure to low dose solvent mixture the hepatocytes showed no alteration in nuclear morphology however, mild vacuolization and hyalinization of hepatocytes, with evidence of cellular necrosis around the central vein. In addition hepatic fatty infiltration was observed in the form of fat granules. Nuclear pycnosis was also seen (Plate B. fig. 3,4). Increased hyalinization and loss of radial arrangement of hepatocytes were observed in the liver, when animals were exposed to high
dose. Moreover, the high dose treated tissue showed increased vacuolization, with necrosis of hepatic cells and alteration of nuclear morphology (Plate C. fig. 5, 6).

**Kidney:**

The Haematoxylin and Eosin stained section of control mice kidney revealed normal structure of Bowman’s capsule with intact glomeruli and proximal and distal renal tubules with normal cell morphology (Plate D. fig. 7,8). Low dose solvent exposure resulted in increased periglomerular space, vacuolization between renal tubules and evidence of cellular necrosis in the proximal tubules (Plate E. fig. 9, 10). It was observed that high dose exposure led to disorganisation of proximal and distal tubules with cellular atrophy. Observations under higher magnification showed evidence of cellular necrosis, with infiltration of fat particles and dilation of blood vessels after exposure to the high dose solvent mixture (Plate F. fig. 11,12).

**DISCUSSION:**

The present investigation revealed that organic solvents in mixtures caused immense toxicity in vital tissues such as liver and kidney after solvent exposure. The increase obtained in the weights of liver and kidney after solvent exposure suggests hepatocyte swelling and inflammation with possible oedema a finding which was correlated with our histological observations. Kum et al. [13] have also reported increased organ weights on exposure to a mixture of other solvents viz., xylene and formaldehyde. In addition these authors have also shown that increase in liver and kidney weight was significantly correlated with the dose of toxic solvent administered at levels between 270 and 600ppm [13].

The data obtained indicated that exposure to mixtures of toluene, xylene and benzene also caused an increase in level of protein, creatinine and cholesterol in both liver and kidney tissues indicating possible accumulation of these metabolites, due to impaired metabolic turnover. Aiso et al. [30] have earlier reported elevated protein and cholesterol levels in liver of animals exposed to 600ppm of a benzene derivative. The significant increase observed in the activities of Alanine aminotransferases (ALT) and Aspartate aminotransferases (AST) gave further indication of the toxicity of the solvent mixture. These results suggest severe Hepatotoxicity, along with changes in liver metabolism. Moreover, reports of Uboh et al. [23] suggesting a 191% increase in ALT and 161% increase in AST activity on exposure to a crude solvent (Kerosene), lend support to our findings.

The results obtained in the present study suggested that this specific solvent mixture could induce oxidative stress, with increased oxygen toxicity due to generation of reactive oxygen species coupled with impaired activity of protective, free-radical scavenging enzymes like SOD and Catalase. Lipid peroxidation was also found to be enhanced with increased malonyl dialdehyde (MDA) levels in these vital tissues. On the other hand, Kum et al. [31] have demonstrated that there was no statistically significant alteration in the SOD, CAT and GSH-Px activities in the xylene-formaldehyde exposed groups as compared to control.

The mixture of xylene, benzene and toluene (450ppm and 675ppm) used for the exposure regimen in this study however, caused a significant decline in SOD and CAT activities. Inadequate scavenging of oxygen free radicals and therefore increased oxygen toxicity, resulted in hepatocellular and renal damage. The data also revealed that the serum levels of T₃ and T₄ were increased as compared to control possibly due to transient hyperthyroidism which in turn could be correlated with increased activity of ALT and AST suggesting altered metabolic pathways. Hyperthyroidism is known to augment hepatic sensitivity to toxicants like chloroform as reported earlier by Paget [32]. Moreover, research under the National Toxicology Programme has indicated that thyroid gland follicular cell hyperplasia also occurred after the exposure to ethyl benzene [33]. Thyroid toxicity due to
subchronic exposure to a complex mixture of sixteen organo-chlorines has also been documented Wade et al. [34]. On the other hand, Zaidi et al. [9] have shown a condition of sub-clinical hypothyroidism in solvent exposed spray painters, an observation which is contrary to our findings, but never the less points to altered basal metabolism.

Similar to our histological findings in the liver after exposure to the low dose solvent mixture, Gotohda et al. [15] have also observed hepatic fibrosis and hepatic damage on inhalation of toluene vapour alone. These authors have emphasised that liver damage due to toluene exposure was because of a direct action. Hepatic necrosis, as observed in this study was also reported by Reid et al. [35] due to Bromobenzene exposure. Moreover, Casini et al. [36] have also explained the mechanism of cell injury in the rapid destruction of hepatocytes by benzene exposure.

Exposure of the experimental animals to high doses of solvent mixtures in this study caused disorganisation, vacuolization and fatty infiltration. Research carried out by Lundquist et al. [37] confirms the role of organic solvents in the induction of liver steatosis. Chang et al. [38] have also demonstrated fatty infiltration in rat liver after inhalation of a combination of xylene and formaldehyde.

Histological observation of the kidney of the animals exposed to the low dose revealed alteration in the glomerular organisation with cellular necrosis and vacuolization in the collecting tubules. Similar renal histopathological changes have also been described by Kum et al. [13] on exposure to a combination of formaldehyde and xylene. Nephrotoxicity has also been described by AL-Ghamdi et al. [17] on exposure to solvents like formaldehyde with evidence of progressive renal damage and solvent induced proximal tubular cell injury, as observed in the present study. Jones et al. [20] have demonstrated accumulation of fat in the renal tubular epithelium with glomerular fatty inhalation, due to xylene or formaldehyde exposure. Sporadic fatty infiltration was observed in the kidney of the animals exposed to the solvent mixture used in the investigation.

Exposure to the specific mixture of xylene, toluene and benzene caused significant biochemical and histological alteration in liver and kidney. Moreover, these toxic changes were manifest in a dose dependent manner, since it was evident that the toxicity induced alterations were more significant in the higher dose (675ppm) exposed group as compared to the group of animals exposed to a lower dose (450ppm).

CONCLUSION:
The results of this work suggest that exposure to a specific solvent mixture at low and high dose levels caused significant toxic changes on the organs studied viz., liver and kidney of the exposed mice and brought about alteration in the protein, cholesterol, creatinine, T3, T4, ALT, AST. Moreover, solvent exposure resulted in oxidative stress even at a lower dose of 450ppm, thereby impairing the normal liver and kidney function. These biochemical changes could further be correlated with the significant histological changes observed in these vital tissues, confirming toxic injury to both tissues studied. It was also noted that the changes observed in both tissues appeared to occur in a dose dependent manner.

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PLATE A

Transverse section (T.S) of liver of control mice  (Haematoxylin eosin stain, 5μ sections)

Figure 1 : T.S of control mice liver showing radially arranged hepatic cord around the central vein.10X.

Figure 2 : A magnified view of T.S of control mice liver with well preserved histoarchitecture 40X.
PLATE B

Transverse section (T.S) of liver of mice exposed to solvent mixture (Low dose) (Haematoxylin eosin stain, 5μ sections)

Figure 3 : T.S of liver after Solvent Exposure (low dose). Hyalinization of hepatocytes with dilated central vein with evidence of cellular necrosis around the central vein 10X.

Figure 4 : A magnified view of Figure 3. Note hepatocytes degeneration 40X.
PLATE C

Transverse section (T.S) of liver of mice exposed to high dose of solvent mixture (Haematoxylin eosin stain, 5μ sections)

Figure 5 : T.S of liver of mice after solvent exposure (high dose treatment) showed increased hyalinization and loss of radial arrangement of hepatocytes. There was increased vacuolization, with necrosis of hepatic cells. 10X.

Figure 6 : Magnified view of figure 5 Observe degeneration of hepatocytes and increased vacuolization 40X.
PLATE D

Transverse section (T.S) of kidney of control mice (Haematoxylin eosin stain, 5μ sections)

Figure 7: T.S of kidney of control mice showing normal glomerulus with organised proximal and distal renal tubules. 10X.

Figure 8: Magnified view of figure 7. Control kidney, exhibiting complete intact glomerulus and prominent nuclei of cells 40X.
PLATE E

Transverse section (T.S) of mouse kidney exposed to low dose of solvent mixture (Haematoxylin eosin stain, 5μ sections)

Figure 9 : T.S of kidney after low dose solvent exposure showing increased peri-glomerular space with vacuolization between renal tubules and evidence of cellular necrosis in the proximal tubules 10X.

Figure 10 : Magnified view of figure 9. 40X.
PLATE F

Transverse section (T.S) of kidney of mice exposed to high dose of solvent mixture (Haematoxylin eosin stain, 5μ sections)

Figure 11 : T.S of kidney after treatment with high dose solvent with evidence of cellular necrosis and infiltration of fat particles. Blood vessel dilation was also seen 10X.

Figure 12 : A magnified view of figure 11, Note the atrophic changes. 40X.
RESULTS:

TABLE-I: Body weight and organ weight of control and exposed animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Body weight</th>
<th>Organ weight</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>I</td>
<td>Control</td>
<td>39.00±0.570</td>
<td>2.24±0.015</td>
</tr>
<tr>
<td>II</td>
<td>450ppm</td>
<td>39.80±0.057&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>2.77±0.225&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>675ppm</td>
<td>40.23±0.145&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>2.83±0.010&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are Mean±S.E. * P < 0.01. ** P < 0.001. NS Non-Significant.

FIGURE 1: Levels of Protein, cholesterol, in control and treated animals.

Values are Mean ± S.E. * P < 0.01. ** P < 0.001. NS Non-Significant.

Protein expressed mg/100mg tissue weight. Cholesterol: mg/100 mg tissue weight.
**FIGURE 2:** Levels of Protein, Creatinine and Cholesterol in kidney of control and treated animals.

Values are Mean ± S.E. **P < 0.001. NS Non-Significant.

Protein, Creatinine and Cholesterol expressed as mg/100 mg tissue weight.

**FIGURE 3:** Activities of SOD, CAT and TBARS in liver of control and treated animals.

Values are Mean ± S.E. **P < 0.001. NS Non-Significant.

SOD activity expressed units/mg protein. TBARS expressed nM of MDA/100mg tissue weight. CAT μ moles H₂O₂ consumed/min/mg/protein
**FIGURE 4:** Activities of SOD, CAT and TBARS in kidney of control and treated animals.

Values are Mean ± S.E. ** P < 0.001.

SOD activity expressed units/mg protein. TBARS expressed nM of MDA/100mg tissue weight. CAT μ moles H₂O₂ consumed/min/mg/protein.

**FIGURE 5:** Serum Alanine transaminase (ALT), Aspartate transaminase (AST), T₃ and T₄ levels in control and treated animals.

Values are Mean ± S.E. ** P < 0.001. **NS** Non-Significant

ALT and AST enzyme activity IU/L.; T₃ and T₄ level of serum μm/l.