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# Defining approaches to mitigate the toxicological impacts of pyrogallol on exposure to biological systems

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**ABSTRACT:**

From the insights gained by evaluating pyrogallol on both plants and animals, we have reported its toxicological effects in this research. The compound was exposed to zebrafish and the histopathological evaluations of gill and intestine were performed. Likewise, the impacts were assessed in the germinating *Pisum sativum* for a period of about 7 days. Finally, a 3% human RBC suspension incubated individually at pH 6, 7 and 8 were used to know pH-dependent hemolysis caused by pyrogallol. The gill parenchyma became dislodged and the lamellar epithelia were seen to be degenerated in the gills of zebrafish. In the intestine, inflammation was observed with accumulation of infiltrates in the mucosa layers. Further, *Pisum sativum* seeds showed stunted growth parameters during germination due to the application of pyrogallol. At acidic and neutral pH, destruction of human erythrocytes did not take place whereas, at alkaline condition (pH 8), hemolysis seemed to occur at a rate of about 13.2 ± 0.04%. Thus, these research findings suggest that pyrogallol could cause potential toxicity to many life forms damaging the environment and hence, the usage of this chemical compound must be limited.

To mitigate the impacts of pyrogallol, four different microorganisms were tested for their antagonistic activities out of which *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* could potentially work against it. Lastly, the immobilized bioenzyme allowed the zebrafish to sustain the vulnerability induced by the compound during the treatment course than did the immobilized citrus peel extract.

**KEYWORDS:** pyrogallol, toxicity, zebrafish, antagonistic activities, bioenzyme

## 1. INTRODUCTION:

Pyrogallol is a phenolic compound that acts as a free radical scavenger when exposed to aqueous or alkaline solutions.[1] This compound is naturally present in some plants and algae as well. [2,3] It is often used in photograph development and has also been extensively used in the manufacture of hair dyes and related cosmetic products.[4] At some concentrations, it is used as, metallic protectant in case of colloidal solution preparation, and staining of leathers.[5] For treatment purposes, it is widely being used as anti-psoriatic and antiseptic drugs.[6] Although, it has its own benefits and usage in daily life, the ill effects of pyrogallol clearly surpass its many benefits. Pyrogallol is a carcinogenic agent which will trigger various tumors and the production of ROS can lead to cell apoptosis when exposed for a prolonged period of time.[5] Living organisms may get in contact with pyrogallol either directly or indirectly when the contaminated effluents get mixed with the water bodies without proper treatment resulting in serious damages to the health and environment.[7]

The mechanism of pyrogallol action must be analyzed thoroughly in the first place for its use in the industries to avoid toxicity, genetic defects and environmental changes in the future. Hence, it has become important to analyze the serious alterations in biological systems caused due to the exposure of pyrogallol. For this matter, we have performed a series of experiments to understand the influence of this compound on zebrafish (*Danio rerio*), green peas (*Pisum sativum*) seeds and human erythrocytes. The experiments were conducted with pyrogallol administered in a dose-dependent manner to know their performances against this chemical entity during the course.

When considered collectively, the experiments conducted highlight the ill effects of pyrogallol on living species beyond a certain level. It was found out that pyrogallol drastically affects the cellular and organ systems in Red Blood Cells and zebrafish respectively and also has significant growth stunt during the germination of *Pisum sativum*. Thus, pyrogallol affects the biological entities to the core causing visible damage and reduction in metabolism in spite of its industrial and related chemical uses. Since, most of the

manufacturing industries and laboratories still utilize this compound for processing, there are several possibilities that their effluents may influence the destruction of both flora and fauna. Bioremediation processes are rarely being considered to detect and remove pyrogallol from the polluted environment till date. So, defining novel protocols using microorganisms and natural extracts can mitigate the harmfulness of pyrogallol and its derivatives.

## 2. MATERIALS AND METHODS:

### 2.1. Materials

The experiments were carried out with adult wildtype zebrafish (*Danio rerio*) which were allowed to acclimatize to 28-29°C, pH 7.0 and light: dark cycle was maintained as 14:10 hours respectively for about 7 days.

Green peas (*Pisum sativum*) seeds were purchased from a local grocery store at Coimbatore, India. Calcium chloride, potassium phosphate monobasic, potassium phosphate dibasic, pyrogallol, sodium alginate and sodium hydroxide were of analytical grades supplied by HiMedia.

### 2.2. Methods

#### 2.2.1. Pyrogallol treatment to zebrafish

Zebrafish were maintained as five groups irrespective of the gender with a population of N=4 in each group. Four test groups were treated with pyrogallol at increasing concentrations (2.5 µg/mL, 5.0 µg/mL, 7.5 µg/mL & 10.0 µg/mL respectively) for about 7 days and one group remained as control in which the fishes were maintained in normal fresh water. The water was replaced daily in all the groups and by adding appropriate amount of pyrogallol stock solution to final concentrations in the test groups.

#### 2.2.2. Histopathological evaluation

After the treatment period, one fish from each group including the control were selected and sacrificed by cervical section after anesthetizing them in ice-cold water. The gill and intestinal specimens were collected from each zebrafish and fixed in buffered formalin. All the tissue samples were dehydrated by means of ascending concentrations of ethanol accordingly and embedded in paraffin.[8] Thin sections of about 5-7 µm thickness were made and the tissues were stained using hematoxylin and eosin. The tissue slides were observed under the microscope and compared with the control tissue samples of zebrafish.

#### 2.2.3. Effect of pyrogallol on green peas seeds

Green peas (*Pisum sativum*) seeds were germinated by wet towel method.[9] Each group contained five seeds (N=5) selected at random. The control group seeds were watered with 5 mL of distilled water and the test groups with 5 mL of pyrogallol solution with increasing concentrations of 2.5 µg/mL, 5.0 µg/mL, 7.5 µg/mL and 10.0 µg/mL into the respective petri dishes. The seeds were watered once daily for seven consecutive days. The effect of pyrogallol treatment on green peas seeds were analyzed by calculating the germination percentage and seed vigor index according to Abdul-Baki and Anderson, 1973 for the seed samples from each test group and compared with that of the control group values after the seventh day.[10]

### 2.2.4. pH dependent hemolysis by pyrogallol

Human RBCs were used to assess pH dependent hemolysis induced by pyrogallol. A 3% RBC suspension was prepared using venous blood drawn from a healthy donor.[11] The test solutions contain 10 mL of 10 mM pyrogallol dissolved in phosphate buffer of pH 6, 7 and 8 taken in three different tubes respectively. In this experiment, 10 mL of distilled water in a tube served as a positive control and 10 mL of individual buffers at varying pH without RBC suspension were maintained as negative controls to zero the blank as the intensity of the solution containing pyrogallol increased with increasing pH. 200 µL of 3% RBC suspension was pipetted into the remaining tubes including positive control and the mixtures were incubated at 37℃ for 1 hour. After incubation, the samples were centrifuged at 10,000 rpm for 15 minutes followed by measuring the optical densities of the supernatants at 540 nm in order to determine the hemolysis percentage at varying pH.[12] Hemolysis percentage is defined as the degree of a test compound to cause destruction of RBCs relative to the positive control in terms of percentage.

### 2.2.5. Antagonistic effect of microorganisms against pyrogallol

To determine the antagonistic effect against the compound, different microorganisms were inoculated into nutrient broth containing 0.05% of pyrogallol. The microbes used were *Bacillus tropicus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Saccharomyces boulardii* CNCM I-745 strain. Briefly, 1% of fresh inoculum of each microorganism was inoculated into the broth containing pyrogallol and incubated at 37℃ for 24 hours with agitation maintained at 110 rpm. Broth without any inoculum remained as control. The experiment was performed in triplicates. After incubation, the broth from each sample was centrifuged at 5000 rpm for 5 mins and the supernatants were retained. To 2 mL of the supernatants, 1 mL of 0.25 N NaOH solution was added and the tubes were left undisturbed in dark for 30 mins. Finally, the optical densities of the broths were measured at 420 nm to estimate the remaining pyrogallol that was not utilized by the microorganisms during their growth in the media containing the compound. Pyrogallol becomes pyrogallol quinone in alkaline conditions on reacting with oxygen molecule which turns into brown color that can be measured colorimetrically.[13] This is the basic principle of this assay.

$$\text{Antagonistic activity (\%)} = [(\text{O.D of Control} - \text{O.D of Sample})/\text{O.D of Control}] \times 100$$

### 2.2.6. Preparation of bioenzyme and extract from citrus peels

The bioenzyme mixture contained jaggery, citrus fruit peels (lemon, orange and tangerine) and distilled water in the ratio 1:4:10 which was fermented for about 28-30 days at room temperature. After complete fermentation, the mixture was filtered and collected for further use. Similarly, aqueous citrus peel extract was prepared by homogenizing the fresh peels in distilled water in the ratio 3:10.

### 2.2.7. Immobilization of bioenzyme and citrus peel extract

Calcium alginate immobilization method was adopted for this experiment. For this, bioenzyme and citrus peel extract were mixed separately with 4% sodium alginate solution to yield a final concentration of about 2% sodium alginate. The beads were prepared by carefully dropping into 0.2 M $CaCl_2$ solution.[11]

For control, the beads were prepared using only 2% sodium alginate solution diluted with distilled water. All of the immobilized beads were then washed thrice by rinsing them in distilled water.

### 2.2.8. Determining the inhibition of pyrogallol induced toxicity in zebrafish using the immobilized bioenzyme and citrus peel extract

Zebrafish were maintained in three groups and acclimatized to optimal conditions as in the above-mentioned protocol. 2% of the immobilized beads were added into the zebrafish groups maintained separately. The control group received control calcium alginate beads, one test group received the immobilized bioenzyme and the other test group received the immobilized citrus peel extract. Finally, all the groups were treated with 10.0 µg/mL of pyrogallol concentration and incubated for 7 days. Both pyrogallol aliquot solution and beads were changed once in 2 days. Similar histopathological evaluations of zebrafish's gills and intestines were made from each group.

### 2.2.9. Statistical analysis

All statistical analyses were performed using Anova: Single Factor tool through Microsoft Excel software. The obtained results of the triplicate samples are represented in the form of "mean ± standard error". The data were statistically significant and in compliance with the null hypothesis ($P<0.05$).

## 3. RESULTS AND DISCUSSION:

### 3.1. Histopathological evaluation of gill samples

The gill sections from the untreated zebrafish showed normal appearances with intact lamellar epithelium and gill arch as well. In pyrogallol treated specimen, changes in gill parenchyma were observed with degeneration of the lamellar epithelia. Figures 1 and 2 represent the histopathology of gill samples obtained from control and zebrafish treated with the highest concentration of pyrogallol (10.0 µg/mL) respectively. It is discussed that chronic exposure of cyclophosphamide led to the degeneration of gill tissues in zebrafish.[14] In another article, epithelial lifting, lamellar fusion and hyperplasia were observed in the histopathological findings of zebrafish gills treated with fluorene-9-bisphenol at a concentration of about 0.125 mg/L.[15]

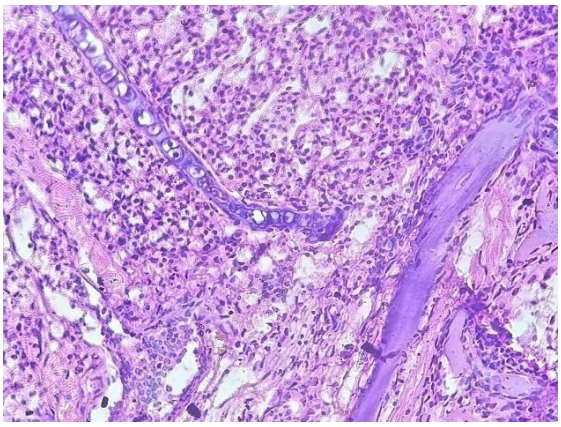

**Figure 1. Gill sample of control zebrafish with normal appearance**

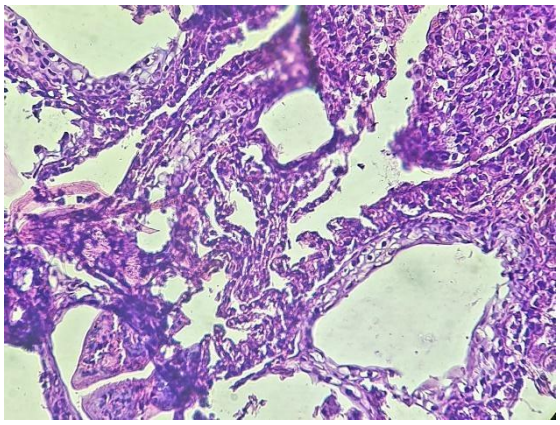

**Figure 2. Gill from 10.0 µg/mL treated zebrafish showing degenerated epithelia**

### 3.2. Histopathological evaluation of the intestines

The intestinal section of pyrogallol treated zebrafish (10.0 µg/mL) exhibited normal intestinal epithelium but an increased amount of infiltrates were present in the *Lamina propria* and *Muscularis mucosa* due to inflammation (Figure 4). On comparison, the tissue section of control specimen appeared with normal intestinal architecture as depicted in Figure 3. However, the effects were relatively less in the remaining tissue samples collected from the zebrafish treated with 2.5 µg/mL, 5.0 µg/mL and 7.5 µg/mL of pyrogallol (Data not shown). In a literature, it has been reported that pyrogallol induced white blood cell infiltrates to get accumulated in the liver tissue of rats similar to the results obtained in our experiment.[16] Likewise, zebrafish exposed to triclosan also caused inflammatory infiltrates to be present in the gut along with thinning of the epithelium.[17]

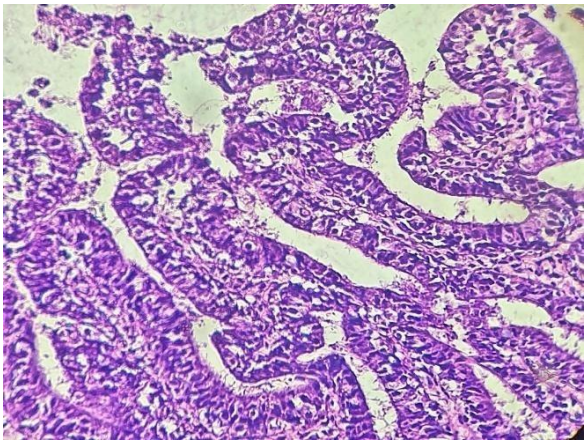

**Figure 3. Normal intestine from control zebrafish**

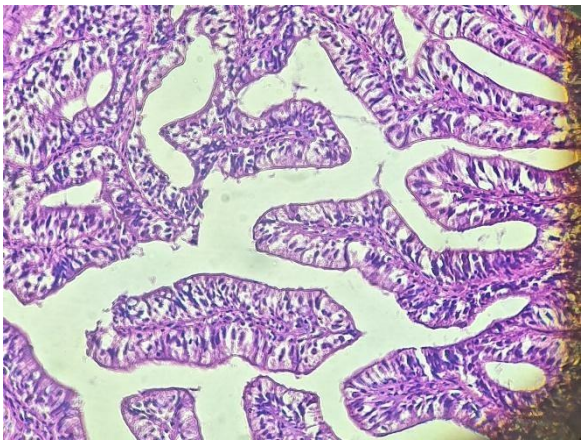

**Figure 4. Intestine from 10.0 µg/mL treated zebrafish**

### 3.3. Effect of pyrogallol on green peas seeds

Morphologically, the developing embryo from the germinated seeds showed bioaccumulation of pyrogallol. The growth of *Pisum sativum* seeds in the presence of pyrogallol showed stressed growth parameters until the seventh day when compared with the control seeds. A 20% decrease in the germination percentage was observed in the seed group treated with 10.0 µg/mL of pyrogallol while rest of the groups exhibited 100% efficiency to germinate. Likewise, the difference between the vigor indices of control and 10.0 µg/mL samples were estimated to be around 306 ± 19.92. This decreased growth may be due to the oxygen scavenging activity of pyrogallol making it unavailable for the growth of *Pisum sativum* seeds.[1] Also, pyrogallol inhibits some of the major enzymes like α-glucosidase and tyrosinase thus, affecting the primary metabolism and growth of the seedlings.[18,19] It is reported in an article that arsenic toxicity in green gram HUM-16 cultivars after 7 days of treatment showed a seedling vigor index of only about 298.99 at 20 µM concentration.[20] Similarly, lead exposure decreased the vigor index of Sorghum seeds from 1695.56 ± 176.68 (Control) to 605 ± 38.18 (800 mg/L).[9]

**Table 1. Germination percentage and vigor index of *Pisum sativum* seeds after exposure to pyrogallol**

| Concentration of pyrogallol | Germination percentage (%) | Seed Vigor Index (SVI) |
|---|---|---|
| Control | 100% | 820 ± 7.07 |
| 2.5 µg/mL | 100% | 800 ± 8.36 |
| 5.0 µg/mL | 100% | 750 ± 10.48 |
| 7.5 µg/mL | 100% | 718 ± 8.60 |
| 10.0 µg/mL | 80% | 514 ± 12.85 |

### 3.4. pH dependent hemolysis by pyrogallol

The hemolysis percentage of pyrogallol at pH 6 and 7 was zero. At pH 8, pyrogallol was able to lyse the human RBCs with an estimate of around 13.2 ± 0.04%. The results suggest that the capability of this compound to impart negative impacts on biological systems occurs mainly at pH above 7. It has been previously reported that pyrogallol is a potent oxygen scavenger at alkaline conditions due to which hemolysis would have taken place.[1] Human blood being slightly alkaline (7.4), can allow pyrogallol to

exhibit its effect. Walter et al. used pyrogallol at 0.17 mg/mL concentration as a positive control but unfortunately, they could not succeed inducing hemolysis in horse erythrocytes.[21] Similarly, pyrogallol was tested and reported that it did not cause hemolysis in sheep erythrocytes as well.[22] But our research has succeeded to show its effectiveness in alkaline environment to damage the erythrocytes. However, the percentage of hemolysis shall vary among different ABO blood groups.

### 3.5. Antagonistic activities of microorganisms against pyrogallol

The growth of certain microorganisms in the environment containing pyrogallol can utilize the compound in some manner which must be extensively studied. However, we are reporting the antagonistic effects of some microorganisms that we used in our experiment. Table 2 shows the quantitative potential of each microorganism to use or degrade the pyrogallol. During the course, we also observed that the microorganisms die and only a few amounts of pellet can be retained in the broth obtained from each sample upon centrifugation. But it was sufficient to antagonistically affect the compound by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. This efficiency of both the bacteria can remove the same compound from any sample or environment either through *in situ* or *ex situ* bioremediation. Unfortunately, *Bacillus tropicus* and *Saccharomyces boulardii* could not grow against the compound in the media. A few bacteria naturally possess a gene namely, gallate decarboxylase that can degrade gallic acid into pyrogallol and other intermediate compounds.[23] Some Enterobacters can utilize or degrade pyrogallol for their primary metabolism.[24] Usually, many bacteria are capable of degrading phenols through the expression of various enzymes and related proteins.

**Table 2. Antagonistic activities of different microorganisms in nutrient broth containing pyrogallol**

| Microorganism | Antagonistic effect (%) |
|---|---|
| *Bacillus tropicus* | 0% |
| *Klebsiella pneumoniae* | 34.11 ± 0.03% |
| *Pseudomonas aeruginosa* | 35.29 ± 0.05% |
| *Saccharomyces boulardii* | 0% |

### 3.6. Determining the inhibition of pyrogallol induced toxicity in zebrafish using the immobilized bioenzyme and citrus peel extract

The histopathological assessments of gill and intestine of control zebrafish were similar to the zebrafish group treated with 10.0 µg/mL in toxicity evaluation. The test group that received 2% of immobilized bioenzyme could dynamically inhibit the toxic condition induced by pyrogallol. Only minute changes in the histology of gill and intestine were observed under the microscope (Figures 5 and 6). However, the other test group that received the immobilized citrus peel extract could help improve the condition only to some extent. To discuss, the reason may be that the pH of both bioenzyme and citrus peel extract was around 3-5 and bioenzyme mixture has several enzymatic performances as well.[25] Hence, these can prevent the oxygen scavenging and other toxic impacts of pyrogallol to zebrafish. In addition,

immobilized bioenzyme and citrus peel extract would consistently perform their activities than being added in liquid form against pyrogallol.[26]

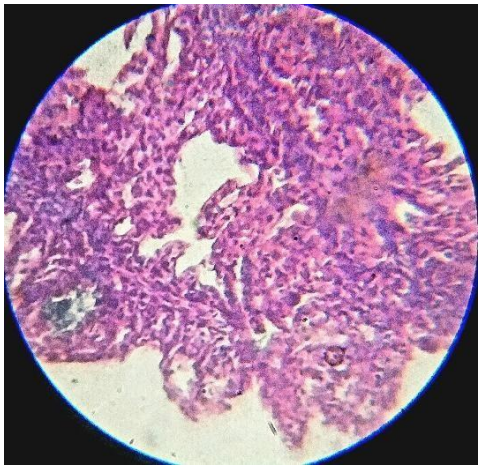

**Figure 5. Less degenerated lamellar epithelium of zebrafish gills on treatment with the immobilized bioenzyme against 10.0 µg/mL pyrogallol concentration**

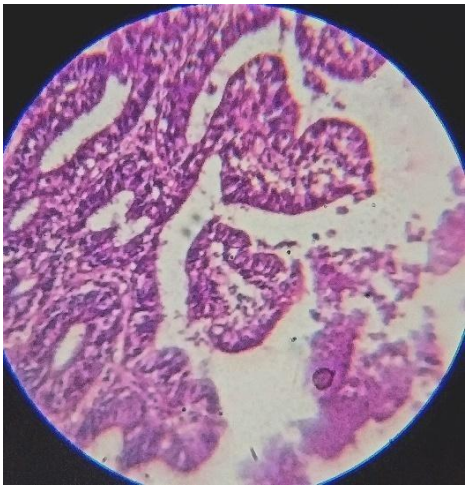

**Figure 6. Partial inflammation in the zebrafish intestine on treatment with the immobilized bioenzyme against 10.0 µg/mL pyrogallol concentration**

## 4. CONCLUSION:

The toxicological effects of pyrogallol on both plant and animals have been discussed in this research. The impacts of this compound on living matter exhibited dose-dependent negative performances. The pyrogallol treatment in zebrafish caused the degeneration of gill structures and inflammation in the intestinal tissues eventually affecting the respiratory and digestive systems respectively. Also, it could lyse the human erythrocytes potentially at alkaline pH but not in acidic and neutral environments. Further studies using *Pisum sativum* seeds indicate that pyrogallol induced the growth of seedlings with stressed parameters at increasing concentrations. The antagonistic effects of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and the immobilized natural products were able to overcome the toxicity induced by pyrogallol at a fixed concentration. Although this chemical entity is widely used in many industries and in our day-to-day life, it is important to find an alternative source in order to prevent pyrogallol toxicity in the environment.[27]

**CONFLICT OF INTEREST:**

The authors declare no competing interests.

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