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Research Article

TOXICITY PROFILE OF CINNAMON OIL BASED DRUG DELIVERY SYSTEM IN OREOCHROMIS MOSSAMBICUS (TILAPIA)

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ABSTRACT

Introduction: Certain aqueous insoluble drugs had shown remarkably good solubilization in lipophilic environment. Among such drugs: ramipril, azithromycin and fluconazole showed improved solubilization using essential oil based microemulsion drug delivery system. In this study, we focused on the toxicity profile of the drug delivery vehicle – cinnamon oil based system in *Oreochromis Mossambicus* (Tilapia) fishes.

Methods: Histopathology and biochemical parameters like proteins, carbohydrates, free amino acids, catalase, total reduced glutathione, glutathione s-transferase and superoxide dimutase were estimated in the drug delivery system with comparison to the control fishes.

Results: At higher concentrations of the vehicle, significant changes were observed in the system as compared to control, which, could be due to stress factors or severe pathologic infections.

Conclusions: The drug delivery vehicle showed drastic changes at extremely higher volumes of drug delivery vehicle when treated in Tilapia fishes. Hence, further studies have to be carried out in rodents to determine the safest limit by carrying out toxicity studies. Only then, this drug delivery system could be used for future applications in pharmaceutical medicine.

Keywords: Cinnamon oil; Microemulsion; Tilapia; Toxicity; Biochemical parameters.

INTRODUCTION

Drugs with poor solubility showed poor dissolution and low bioavailability problems *in-vivo*. We have worked on such drugs like azithromycin, ramipril and fluconazole that showed good solubility in both clove and cinnamon oil [1-6]. Based on its solubility potential, we have developed suitable microemulsion drug delivery system.

From our previous studies carried out in our lab, i.e., cinnamon oil microemulsion system [7], we tried to analyze the toxicity profile of blank counterparts of the drug delivery vehicle in Tilapia fishes. The cinnamon oil microemulsion system that could be used as a suitable drug delivery vehicle was composed of cinnamon oil, tween 20 and water respectively at a concentration of 6:24:70 v/v%. Certain biochemical parameters like carbohydrates, protein, SOD, GSH, GST, catalase and histopathological examination of gills and liver were examined.

MATERIALS AND METHODS

Collection and maintenance of Tilapia

Healthy fishes were collected for experimental purpose from different farms and were transported to living condition maintained in a 100-l fiberglass tanks with continuous aeration at room temperature (27–30 °C) in fresh water. The animals were fed twice a day with commercial fish feed [8].

Toxicity of cinnamon oil microemulsion

The toxicity of cinnamon oil microemulsion system (cinnamon oil: tween 20: water – 6:24:70 v/v) was tested in Tilapia fish by immersion method. Different volumes (µl) of the microemulsion were dispersed in about 2000 ml of water. The experiment was carried out for a period of 120 h.

The mortality was noted and the LC50 value was calculated using SPSS soft ware. The cinnamon oil microemulsion system used as the drug delivery vehicle for fluconazole was checked for some of the biochemical parameters and histopathological changes to overlook on the toxicity profile of *Oreochromis Mossambicus* (Tilapia).

Histopathological assays

Histopathological examination was carried out in 3 samples from each tanks were taken and the fish organs that was not treated were freshly killed, preserved in 10% formalin solution for 24 to 48 h, and then paraffin sections were cut to 5 µm thickness and stained with hematoxylin and eosin (Robert et al ., 1984). The slides were examined and photo micrographs were taken using phase contrast microscope for examination. This acts as the control. After treating fishes with different concentrations of the drug delivery vehicle (cinnamon oil microemulsion) for 120 h, 3 fishes from each aquarium of different concentrations were removed, killed by a sharp blow on the head and various organs such as gills, intestine, spleen, kidney and liver were obtained and processed as mentioned before to be examined histologically.

Biochemical assays

Various biochemical parameters such as proteins, carbohydrates, free amino acids, catalase (CAT), total reduced glutathione (GSH), glutathione s-transferase (GST) and superoxide dimutase (SOD) were assayed in different tissue organs such as gills, liver, intestine, spleen and kidneys of control fishes in comparison with cinnamon oil microemulsion (drug delivery vehicle) treated fishes. The experiments were carried out on those concentrations in which mortality was observed.

Protein estimation

About 10 µl of the sample was added to 0.2 ml of 1× Bradford's reagent and incubated in dark conditions at room temperature for 5 min. The absorbance of the solution was taken at 595 nm against blank containing distilled water. The absorbance values are compared using the standard BSA solutions.

Carbohydrate estimation

Carbohydrate content of the samples was analyzed by the phenol sulphuric acid method, a protocol of Masuko et al [9]. About 50 µl of the sample was mixed with 150 µl of concentrated sulphuric acid and shaken for 15-30 min. Then, a 30 µl of 5% phenol was added to the mixture and heated at 90 °C for 5 min and the absorbance was measured at 490 nm and this was compared with the standard agarose solutions.

Free amino acid content

The estimation of free aminoacids was done using ninhydrin reagent. About 20 µl of the tissue sample was homogenized in 5 µl of 10% TCA and shaken well for precipitation to occur. The mixture was then centrifuged at 4000 rpm for 5 min. A 20 µl of the supernatant is then added to 200 µl of ninhydrin and the contents are transferred to the microtitre plate. The absorbance of the solution was measured at 570 nm.

Catalase activity

The activity of catalase was determined by the method of Sinha KA et al [10]. To 0.1 ml of cell suspension, 2ml of 50 mM phosphate buffer (pH-7.0) and 1ml of H_2O_2 were added and read immediately. The decrease in absorbance was measured at 240 nm for 3 min at regular intervals of 30 sec using spectrophotometer. The enzyme activity was expressed in units/mg protein; 1 unit corresponds to the amount of enzyme consuming 1 nM H_2O_2/min.

Total reduced glutathione (GSH)

The estimation of total reduced glutathione (GSH) was carried out as described by Moron et al [11]. About 0.5 ml of the cell suspension was homogenized with 0.1 ml of 5% TCA and mixed well for complete protein precipitation and centrifuged at 4000 rpm for 5 min. A 500 µl of the supernatant was then added to 2.5 ml of 0.2 M phosphate buffer and 50 µl of 0.6 mM DTNB (di-thio nitro-benzoic acid) in 0.2 M phosphate buffer. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar manner were also run to determine glutathione content and the amount of GSH was expressed as nmol of GSH/mg of protein.

Glutathione s-transferase (GST) assay

The Glutathione-S-Transferase activity (GST) was determined by the protocol of Habig et al [12]. To 0.1 ml of the homogenate, add 1 ml of 0.3 M phosphate buffer (pH-6.5); then 0.1 ml of 1-chloro, 2, 4-dinitrobenzene (CDNB) was mixed and made up to 2 ml with distilled water. About 0.1 ml of GSH was then added and the change in absorbance was measured at 340 nm for 3 min at every 30 sec interval respectively. The activity of GST was expressed in terms of Units/ml of enzyme using the formula: Activity = change in absorbance × total volume × dilution factor / (9.6 × volume of sample) where, 9.6 is the molar extinction coefficient of the conjugate formed.

Superoxide dismutase activity

The activity of superoxide dismutase (SOD) was determined by the method of Markuland and Markuland et al [13]. To 0.1 ml of the cell suspension, 50 µl of cold absolute ethanol and 30 µl of chloroform were added and centrifuged at 13,000 rpm for 15 min. About 0.1 ml of the supernatant was used for the assay. 2 ml of Tris buffer (pH-8.2 with 2 mM EDTA) and 0.5 ml of 2 mM pyrogallol (0.5 mM Tris HCl, pH-7.5 as the solvent) was added to 0.1 ml aliquots of the enzyme. Water was then added to make the total volume of 4.5 ml. The reaction mixture for auto-oxidation consisted of 2 ml buffer, 2 ml distilled water and 0.5 ml of 2 mM pyrogallol. The absorbance of the solution mixture was measured at 470 nm against a blank containing 2 ml buffer and 2.5 ml distilled water.

RESULTS AND DISCUSSION

Toxicity of the drug delivery vehicle

The cinnamon oil based microemulsion system was dispersed at varying volumes of 100, 200, 300, 400, 500, 600 µl in 2000 ml of water. The LC 50 was observed when treated with about 400 µl of the drug delivery vehicle. In the histopathological observation, we examined between the lowest concentration, LC 50 (dead animals) and LC 50 (live animals); and the highest concentration.

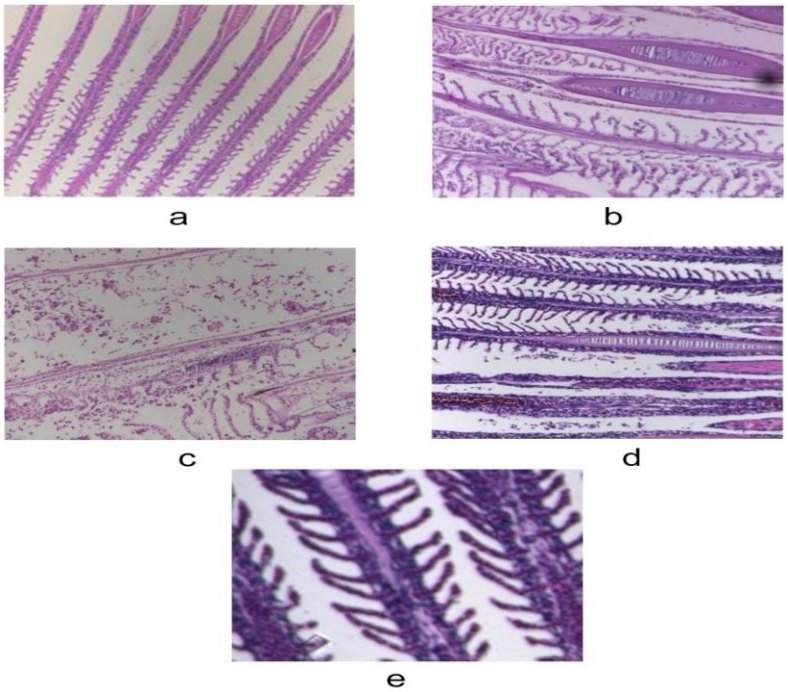

Fig. 1. (a) Gills from control (b) treated with maximum (dead animal) concentration (c) LC 50 (dead animal) concentration (d) LC 50 (live animal) concentration (e) minimum (live animal) concentration.

Liver

The liver of control fish (Fig. 2a) shows clear hepatic lobules that are formed from hepatocytes. These are arranged around the blood sinusoid called the hepatic cell cord and bile ducts are seen in between these cords. This organ is completely absent in the cinnamon oil microemulsion treated higher concentration and LC 50 dead animal. The LC 50 live animal showed slight vacuolations of hepatic cells (Fig. 2b). The minimum concentration (Fig. 2c)

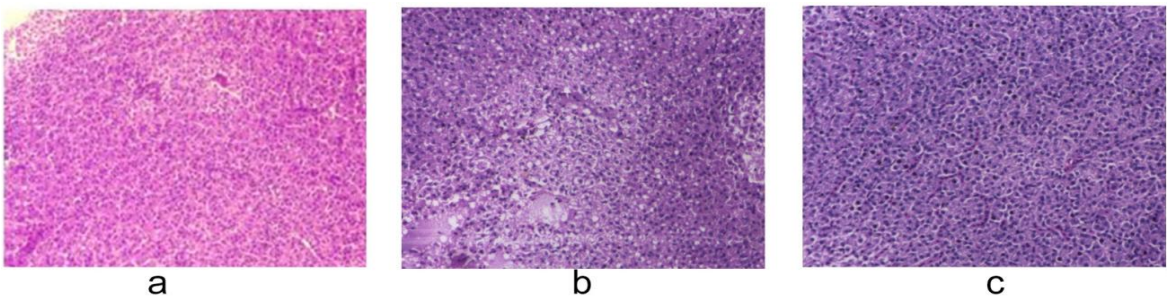

Fig. 2: (a) liver from control showing normal (b) slight vacuolation of hepatic cells in liver from the treated LC50 (live) concentration (c) minimum concentration of treated fish showing normal.

The treated animal exhibited no morphologic variations in comparison with that of control.

Histopathological examination

Gills

The tissues of gills from control (Fig. 1a) consist of two sets of four holobranches, which inturn has two hemibranches. From the hemibranch, a row of long thin filaments called the primary lamellae is present. This region of the primary lamellae shows semi-lunar folds across the dorsal and ventral surface called as secondary lamellae. These are covered with squamous epithelium which is further intercepted by a space consisting of macrophages, piller cells, RBCs and mucous cells. The primary lamella has a core of cartilage that utilizes supply and exchange blood vessels and this region is also surrounded by epithelium that extends to the secondary lamellae. Both the maximum (Fig. 1b) and LC 50 (Fig. 1c) concentration (dead animals) shows gill lesions.

There was a deformation of lamella, majorily sloughing off the secondary lamellae. This correlated with the reports of Kirk and Lewis (1993), who observed deformation of lamellae in the gills of rainbow trout when exposed to certain concentration of ammonia. Further, Smith and Piper (1975) also reported that gill lesions may lead to less oxygen diffusion across the membranes, and therefore, the fishes might be more prone to infections.

The LC 50-live animal (Fig. 1d) showed very slight morphological changes in lamellae. The least (Fig. 1e) concentration (live animals) demonstrated clear morphology that could be compared well with the control fishes

Biochemical assays

The biochemical assays are performed on those concentrations in which mortality were observed.

Protein estimation

The proteins level in gills and intestine of control fish was found to be 444.28 and 1379.98 µg/ml respectively. Whereas, at a volume of 400 µl that is dispersed in 2 litres of water, the proteins level of dead fishes were found to be 45.39 and 42.71 µg/ml in gills and intestine respectively. At a higher volume of 600 µl that is dispersed in 2 litres of water, the proteins level of dead fishes were found to be 37.00 and 40.61 µg/ml in gills and intestine respectively as shown in Fig. 3).

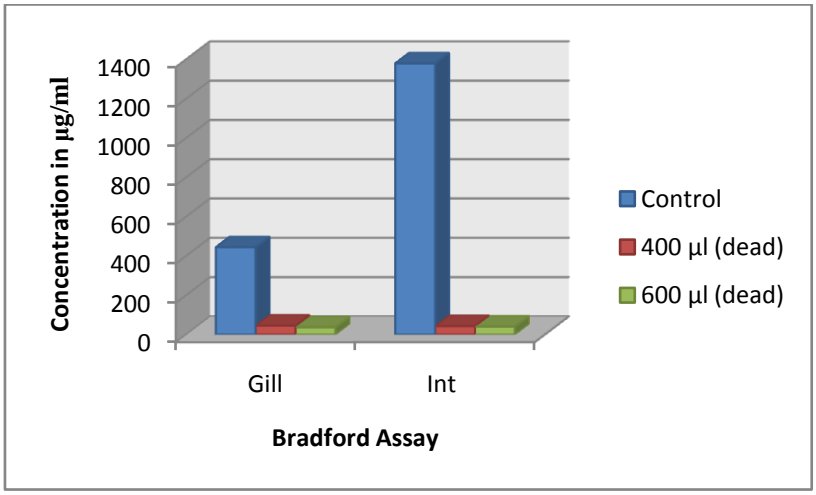

Fig. 3: Estimation of proteins at higher volumes of microemulsion system by Bradford assay.

Carbohydrate estimation

The carbohydrates level in gills and intestine of control fish was found to be 71.39 and 306.38 µg/ml respectively. Whereas, at a volume of 400 µl that is dispersed in 2 litres of water, the carbohydrates in dead fishes were found to be 144.91 and 90.20 µg/ml in gills and intestine respectively. At a maximum volume of 600 µl that is dispersed in 2 litres of water, the carbohydrates of dead fishes were found to be 244.31 and 170.88 µg/ml in gills and intestine respectively as shown in Fig. 4.

Free amino acids

The free amino acids level in gills and intestine of control fish was found to be 0.048 and 0.166 µg/ml respectively. Whereas, at a volume of 400 µl that is dispersed in 2 litres of water, the free amino acids of dead fishes were found to be 0.056 and 0.061 µg/ml in gills and intestine respectively. At a higher volume of 600 µl that is dispersed in 2 litres of water, the free amino acids level of dead fishes were found to be 0.079 and 0.075 µg/ml in gills and intestine respectively as shown in Fig. 5.

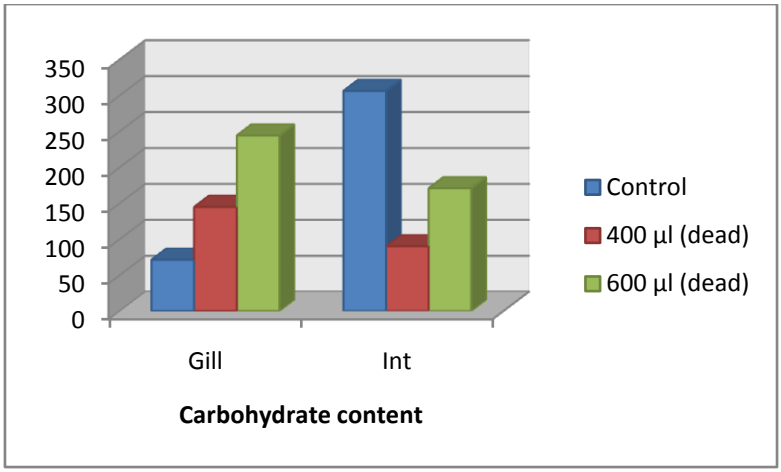

Fig. 4: Estimation of carbohydrates contents at higher volumes of microemulsion system.

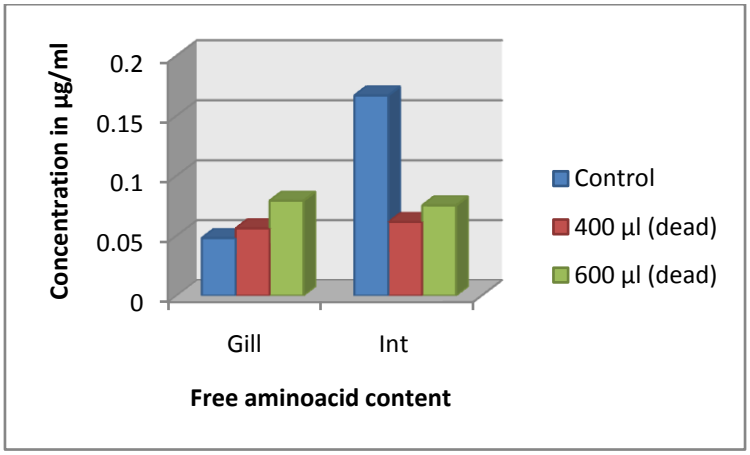

Fig. 5: Estimation of free amino acid contents at higher volumes of microemulsion system.

Catalase activity

The catalase activity in gills and intestine of control fish was found to be 1.39 and 3.475 nanomoles/mg of protein respectively. Whereas, at a volume of 400 µl that is dispersed in 2 litres of water, the catalase level of dead fishes were found to be 0.695 and 2.085 nanomoles/mg of protein in gills and intestine respectively. At a higher volume of 600 µl that is dispersed in 2 litres of water, the catalase activity of dead fishes were found to be 0.695 and 1.39 nanomoles/mg of protein in gills and intestine respectively as shown in Fig. 6.

Total reduced glutathione assay

The GSH levels in gills and intestine of control fish was found to be 5.80 and 7.91 nM of GSH/mg of protein respectively. Whereas, at a volume of 400 µl that is dispersed in 2 litres of water, the GSH level of dead fishes were found to be 5.02 and 7.48 nM of GSH/mg of protein in gills and intestine respectively. At a higher volume of 600 µl that is dispersed in 2 litres of water, the GSH level of dead fishes were found to be 3.76 and 5.36 nM of GSH/mg of protein in gills and intestine respectively as shown in Fig. 7.

Glutathione-s-transferase assay

The GST levels in gills and intestine of control fish was both found to be 0.0031 and 0.0031 respectively. The values were found to be similar for gills and intestine at both volumes of 400 µl and 600 µl repectively that is dispersed in 2 litres of water as shown in Fig. 8.

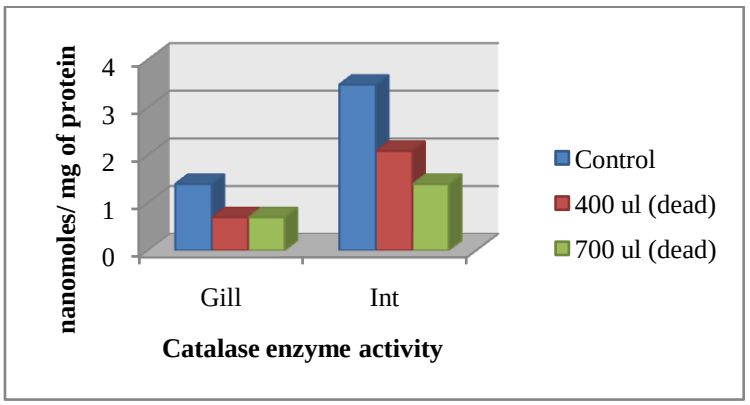

Fig. 6: Estimation of catalase enzyme assay at higher volumes of microemulsion system

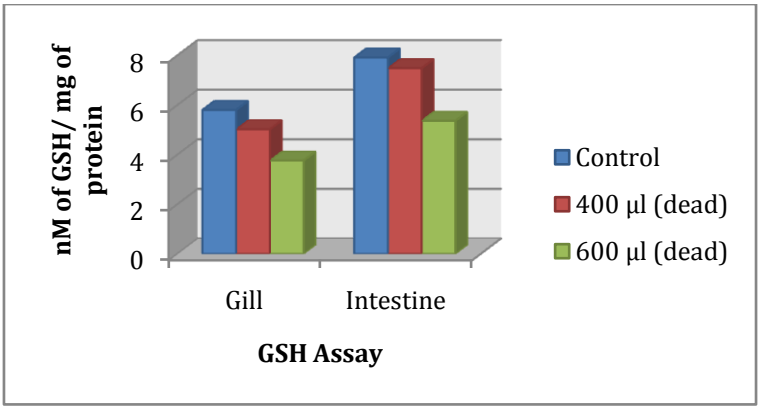

Fig. 7: Estimation of GSH assay at higher volumes of microemulsion system.

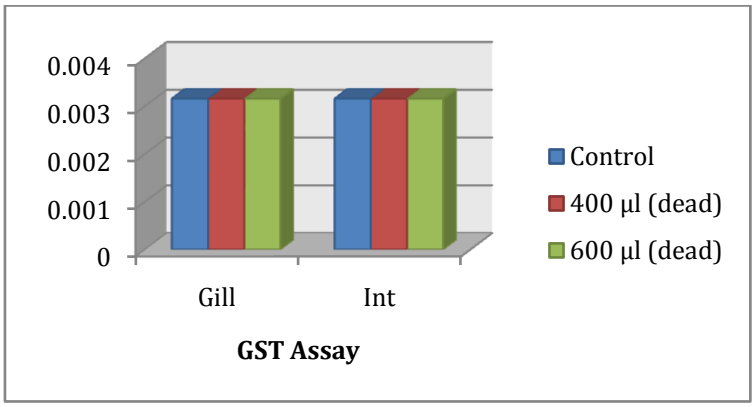

Fig. 8: Estimation of GST assay at higher volumes of microemulsion system

Superoxide dimutase activity

The SOD level in gills and intestine of control fish was found to be 0.059 and 0.062 Units/mg of protein respectively. Whereas, at a volume of 400 µl that is dispersed in 2 litres of water, the SOD level of dead fishes were found to be 0.043 and 0.052 Units/mg of protein in gills and intestine respectively. At a higher volume of 600 µl that is dispersed in 2 litres of water, the SOD level of dead fishes were found to be 0.04 and 0.05 Units/mg of protein in gills and intestine respectively as shown in Fig. 9.

All biochemical assays that were performed on dead fishes clearly showed a significant deviation from those of the untreated or control fishes.

The exemption being glutathione-s-transferase assay, in which there was no observation of deviation from the control groups. The assays clearly imply that the death of the fishes was mainly due to stress factors and severe pathologic infections of certain organs (as observed through histopathology) when treated at high volumes of the vehicle or system.

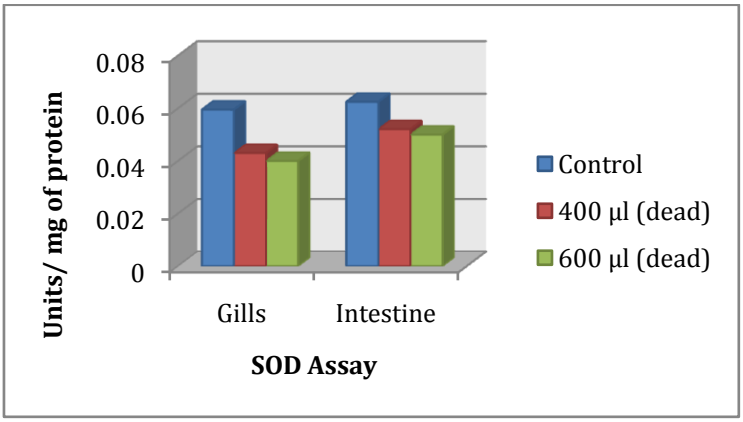

Fig. 9: Estimation of SOD assay at higher volumes of microemulsion system.

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