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SCREENING OF FOOD ADDITIVES ON MODEL ORGANISM CAENORHABDITIS ELEGANS

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ABSTRACT

The usage of Food additives in high concentrations has deleterious effect on human health. Therefore a rapid method to determine the effect of these toxic chemicals is required. In the present study the toxicity of four food additives were evaluated on Caenorhabditis elegans model organism. The mortality after 24hr was adopted as the endpoint for toxicity testing. The LC50 data was calculated and dose responsive curves were obtained. The study revealed that the toxicity was in order of Tannic Acid>Propyl gallate>Thiourea >Monosodium glutamate. The LC50 values were then compared with published data of LD50 of rats and mice using Spearman Rank correlation method .The data obtained showed a high positive correlation of toxicity of food additives in C.elegans with rats and mice and hence we demonstrate C.elegans as a suitable model organism for toxicity testing.

INTRODUCTION

The use of flavoring, coloring agents and preservatives has been known in the food industry for a long time.Excess concentration of these are dangerous to human health(Bernard, 2008., Julia and Barett, 2007) . Evidence showed that excessive accumulation of monosodium glutamate is associated with neuronal injury due to hypoxia-ischemia, trauma and associated metabolic failures (Mallick, 2007).Reports suggest that alkyl gallates blocked mitochondrial electron flow, mainly at the NADH-CoQ segment, preventing ATP synthesis, which would lead to cellular death. These results indicate that alkyl gallates are selectively cytotoxic to tumor cells (Frey, 2007).Reports also reveal the toxic effects of unspent tannins on liver, kidney and heart of albino rats. Histopathological examination of the sections showed, major tissue damage with the highest concentration of tannins (1500mg/kg body weight) irrespective of their nature and source (Sudha *et al*. 2008). Therefore using an animal model would be a rapid method to determine the toxicity level. Previous studies used rats and mice that needed a longer incubation time, expensive, maintenance and so on.

The present study was based on the new model organism for toxicity testing Caenorhabditis elegans .The organism is inexpensive, well characterized and easy to grow on agar medium in Petri dish maintained on a diet of *Escherichia coli*. Among the invertebrates it is unique as it is used in toxicological studies for several reasons. The adult worm measures 1mm long, completes its life cycle in 3 days, consists of just 900+cells with the complete description of cell lineage (Sulston and Horvitz, 1977) and has a transparent body allowing observation of internal structures and gives birth to a large number of progeny (>300) (Sulston and Horvitz, 1988). It is the first multicellular organism to have its complete genome sequenced (Consortium., 1998). Every neuron in its

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simple nervous system has been identified (White *et al*. 1976) with the constancy of cell number and the cell position from individual to individual being the most unique advantage offered by this organism. Most studies focused on the effects of heavy metals, but little is known about the toxic effects of other environmental chemicals on C. elegans (Peredney *et al*. 2001; Dhawan *et al*. 2000).The current study aims at establishing efficient and faster method to detect toxicity of food additives. The main objectives of this study was to evaluate the effect of lethal concentration of food additives on the mortality of the nematodes and also to find the correlation of LC^{50} calculated for different food additives on *C. elegans* in comparison to LD^{50} of rats and mice.

MATERIALS AND METHODS

Srain

Caenorhabditis elegans were procured from the Department of Biological Science and Bioengineering , Indian Institute of Technology Kanpur, India.

Cultivation

A stock solution of the dauer larval stage of *C.elegans* in M9 buffer was kept in an incubator at 20°C and renewed monthly (Cox *et al*. 1981). All the developmental stages of *C.elegans* were grown in an enriched NNGM Agar medium (Williams and Dusenberry, 1988). The plates were seeded with a lawn of *Escherichia coli* strain OP50 and incubated at 37°C for 24 hrs (Sulston and Hodgkin, 1988). Several hundred dauer were placed onto 60mm petri dish with NNGM agar having *E.coli* OP50 lawn and allowed to grow for three days at 20°C (Brenner *et al*. 1979). Adult hermaphrodites were observed after 48 hrs and taken for synchronization.

Synchronization

To minimize the variation in the age related effect on toxicity testing ,the worms were synchronized. The plates with gravid adults were washed with M9 buffer and centrifuged at 1000rpm for two minutes. The M9 buffer was aspirated out and the worm pellet was subjected to hypochlorite treatment for 10-15 min, to kill the adult worms and the eggs were isolated (Emmons *et al*. 1979). The eggs were transferred to a new Petri plate of NNGM with a lawn of OP50 bacteria and were allowed to hatch .The embryos hatched out to give an age synchronous population.

Chemicals tested- Monosodium Glutamate, Propyl Gallate, Thiourea, Tannic Acid (Grade : Merck chemicals, Mumbai, India).

Preparation of Test Chemicals

1M Stock solution of each food additives were made and from the stock solution different concentration range were prepared .All the dilution of the test food additives were prepared in K-medium . The concentrations of the food additives were made based on the Active ingredient of the compounds.

Experimental design and procedure

In lethality testing each test consisted of five - six xenobiotic concentration and control(Freeman *et al*., 1999). The toxicity testing was carried out in 24 Tissue culture plates (Tarsons). 20 worms of L3 stage were dispensed using platinum wire aseptically into 0.5ml of K-medium. For each replicate different concentration of the toxicants were added and the final volume was made upto 1ml in each well. Corresponding control were maintained for each xenobiotics. The worms were exposed to the xenobiotics for 24hrs at 20°C. No food was given during testing (Anderson and Williams, 2004). The endpoint was taken as lethality wherein the dead worms appears highly rigid compared to the live worms, which are motile when observed under dissection microscope using a gentle touch- provoke method (Anderson *et al*. 2001).

STATISTICAL ANALYSIS

The data was obtained after screening a wide range of concentration of the specific food additives for a period of six days. The respective control was kept for each test and 100% survival of worms were recorded in control. The graphs were plotted using SYSSTAT and was analyzed by student t-test. Concentrations response curves were generated for each compound. The toxicity values (LC_{50} or LD_{50}) for each test organism were ranked compared using Spearman Rank Correlation coefficient.

LD_{50} values for rats and mice were converted from milligram per kilogram to millimoles per liter body water using values from the Handbook of Biological Data for average water volume per kilogram body weight. These values were 660 and 766mL/kg for rats and mice, respectively (Spector, 1956).

RESULTS

Lethality observation(Table 1a)

In Tannic Acid > 90% mortality was found in 58.7

Table-1a. LC_{50} of different Food additives on *C.elegans*

Food additives	*Caenorhabditis elegans* LC_{50} (mM)
Tannic Acid	13±0.135
Propyl Gallate	50±1.00
Thiourea	105 ±0.76
Monosodium Glutamate	400±1.78

± Indicates S.E. of observations
Values are significant at (P<0.05)

mM , LC^{50} amounted to 13 mM whereas < 20% mortality was found below 5.8mM. In Thiourea (>95%) worms were found dead in 400mM, LC_{50} recorded was 105mM and < 10 % mortality was observed in (40mM). In Propyl gallate 90% mortality was found in 400mM, LC_{50} amounted to 50mM and all the worms were found to be alive in 20mM. In Monosodium glutamate >90% mortality was found in 800mM the LC_{50} was found as 400mM and less than 20% mortality was found in 200mM.The suitability of K-medium for toxicity testing was earlier reported for heavy metals (Donkins and Williams, 1995). In the current study the data had a similar finding that there is 100% survival in K- medium used as vehicle.

DISCUSSION

The LC_{50} of various food additives screened for acute toxicity were compared. Among the tested food additives *C. elegans* was most sensitive to Tannic Acid wherein 50% death was observed in (Fig.1) 13±0.135mM the most toxic compound in the group compared to Propyl gallate (Fig.2) 50±1.00mM and Thiourea (Fig. 3) 105±0.76mM respectively. The LC50 for flavour enhancer Monosodium glutamate was (Fig.4) 400±1.78mM which indicates that the Tannins are highly toxic compounds compared to other food additives (Table 1a).The LC_{50} values are significant at a=0.05 (P<0.05) for all the toxic compounds.

The toxicity values (LC_{50}) for each test organism were ranked compared using Spearman Rank Correlation coefficient(Table 1b). The results of these comparisons displayed a significant Correlation between the orders of toxicity for the tested compounds on *C. elegans*, rats and mice. In the comparison of *C. elegans* with rats and mice values for the food additives shows high positive correlation in mice (r=0.8) and rats (r=0.8).In case of food additives the data were tightly clustered which could not be distinguished between the three model except for Monosodium glutamate. Data points for *C.elegans* is tightly clustered for some food additives Tannic Acid, Thiourea, (Figure 6). In the current study Tannic Acid shows same order of toxicity in all the three species, ranks (1) and is highly toxic. Propyl Gallate showed lower toxicity in rats (3) & mice (3) and showed higher toxicity in *C.elegans* (2). Thiourea showed lower toxicity in *C.elegans* (3) and higher toxicity in mice (2) and rats(2). The rank order for Monosodium Glutamate is similar for rat (4) and *C.elegans* (4) and mice (4). The LC_{50} data from the toxicity testing section of the experiment were compared to acute oral LD_{50} values in rats and mice for the same compounds obtained from Registry of Toxic effects of chemical substances (RTECS) database. After comparison it was found that there is one log difference between LC_{50} values of *C.elegans*, rats and mice. The present study has similar finding for food additives tested for lethality.

In the previous studies it was observed that tannic acid fed to rats showed toxicity in liver (Suschetet, 1975). It was reported that Tannic acid and its hydrolysed product were tested for mutagenic studies using Ames test (Chen & Chung, 2001). In the current study out of all the food additives Tannic acid is the most toxic.

Reports suggest that gallic acid and its alkyl

Table 1b. Comparison of LC_{50} in rats and LD_{50} in mice

Food additives	*Caenorhabditis elegans* LC_{50} (mM)	Rats LD_{50} (mM)	Mice LD_{50} (mM)	*Caenorhabditis elegans* a Spearman rank order	Ratsb Spearman rank order	Mousec Spearman rank order
Tannic Acid	13±0.135	0.876	1.01	1	1	1
Propyl Gallate	50±1.00	1.18	5.29	2	3	3
Thiourea	105 ±0.76	1.08	1.25	3	2	2
Monosodium Glutamate	400±1.78	56.4	67.93	4	4	4

Number of comparisons ; Spearman Rank Correlation coefficient (P) ; Significant (P)(N=4, a=0.05)
a.Values represent C.elegans toxicity ranking from high (1) to low (4)
b. Values represent Rats toxicity ranking from high (1) to low (4)
c. Values represent mice toxicity ranking from high (1) to low (4)

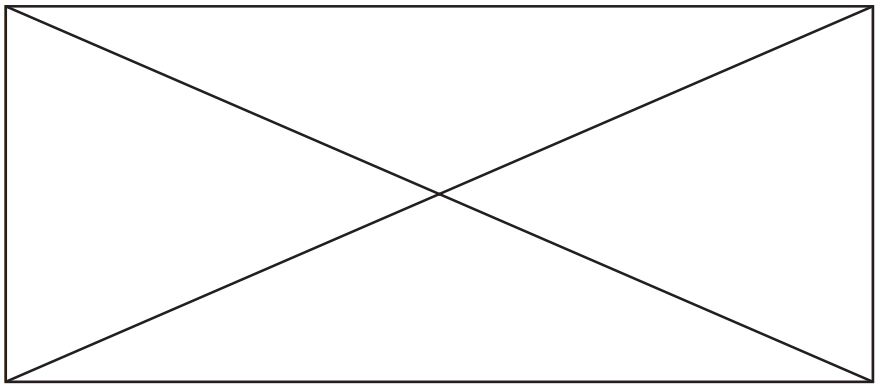

Fig. 1 Effect of Tannic acid in 24hr toxicity test on *C.elegans*

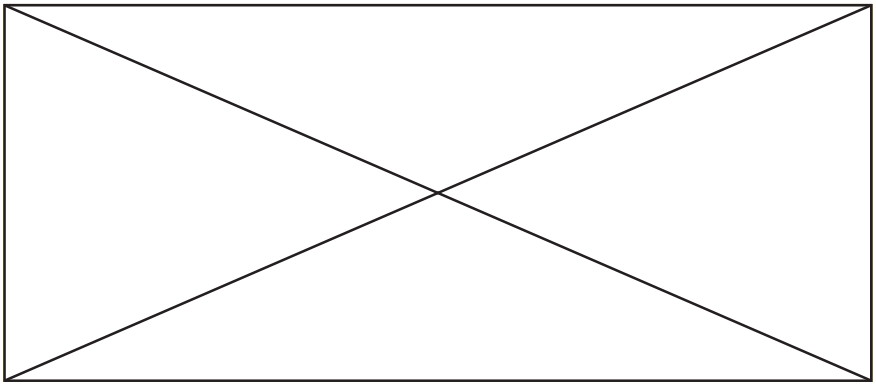

Fig. 2 Effect of Propyl Gallate in 24hr toxicity test on *C.elegans*

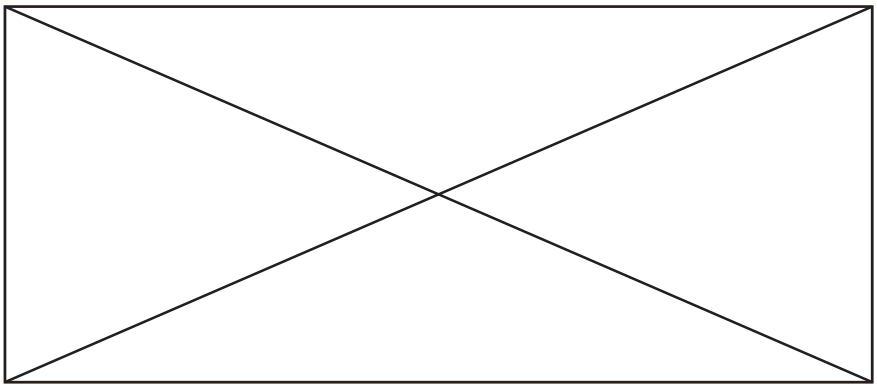

Fig. 3 Effect of Thiourea in 24hr toxicity test on *C.elegans*

esters were known to cause tumor cell line and inhibited lymphocyte proliferation (Serrano *et al*. 1998). It was reported that Propyl gallate causes growth retardation, anemia, kidney and liver changes and hyperplasia of stomach (10000mg/kg). At 5000mg/kg liver enzyme induction was observed (Vander *et al*. 1986).Studies showed that Propyl gallate is toxic to aquatic organisms with the loss of cells and the induction of cell death mainly by necrosis and apoptosis (Zurita *et al*. 2007).It was reported that PG induced sister chromatid exchange and chromosomal aberration (CHO-K1) cells and also showing cycle delay (Tayama *et al*. 2005).In the current study Propyl gallate showed more toxicity than thiourea and monosodium glutamate.

Studies showed that Monosodium glutamate is

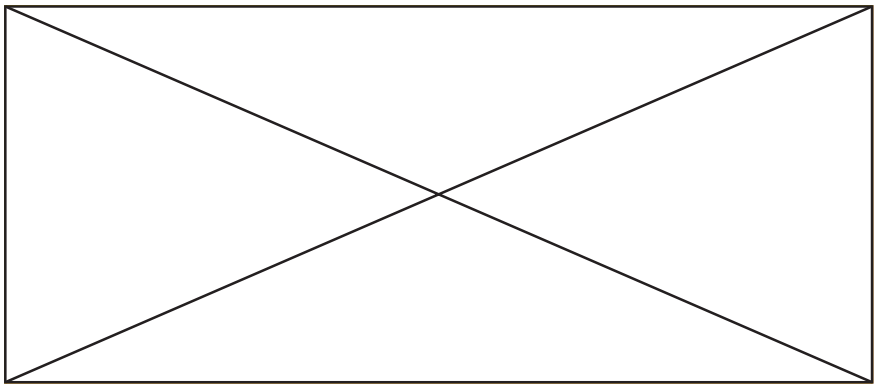

Fig. 4 Effect of Monosodium Glutamate in 24hr toxicity test on *C.elegans*

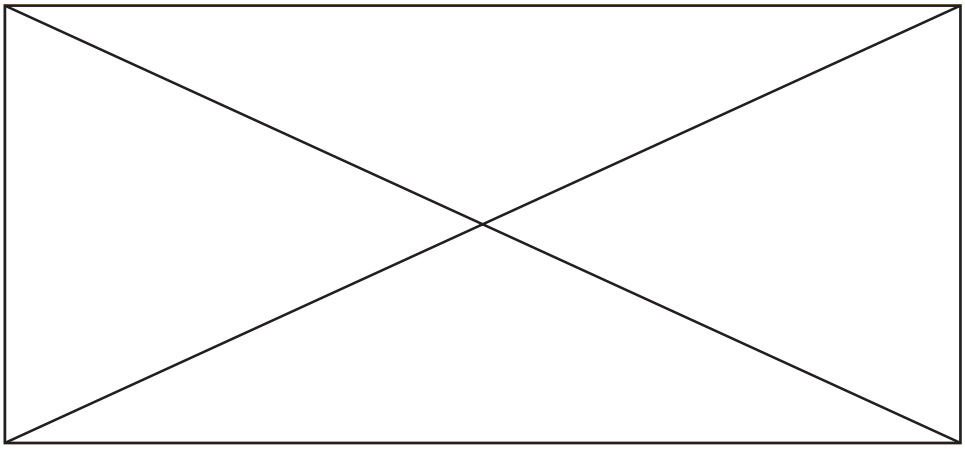

Fig. 5 Correlation study of Food additives on *C.elegans* , rats and mice.

known to cause reproductive dysunction in male rats (Pizzi *et al*. 1979). On administration MSG to neonatal rats reproductive deficits along with stunted growth and reduced testis were observed. Treatment of neonatal mice with large repeated dose of (MSG) induced obesity and endocronological dysfunction (Lourden and Caudle *et al.* 1986). The MSG treated showed increased body weight & decreased pituitary, thyroid, ovary and testis (Pizzi *et al*. 1977). Reports suggest that MSG treatment of mice induces obesity and diabetes with steatosis and steatohepatitis resembling human NAFLD and NASH with pre-neoplastic lesions. It was concluded that MSG should have its safety profile re-examined and be potentially withdrawn from the food chain (Nakanishi *et al*. 2008). In *C.elegans* MSG showed the least toxicity.

(Ugazio *et al*. 1985) reported hapatoxicity and neurotoxicity by ethylethiourea in mice whereas in the present study thiourea showed lower toxicity than Monosodium glutamate.

In the current study, the toxicity of food additives widely used in food industry has been evaluated using the simple model organism . Further studies are required to assess various other molecular effect of these toxic chemicals. Therefore using *C.elegans* could be a faster method for toxicity testing and other endpoints could be used for prediction of mammalian toxicity.

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