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Productive Effect of Garlic Extract and L-Ascorbic Acid (Vitamin C) Against *in vivo* Triclosan Induced Genotoxic Damage In Zebra Fish *Brachydanio rerio* (Ham).

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Abstract

Triclosan (TCS) is commonly used as an antibacterial and antifungal agent in household cleaning products. TCS can be bio-accumulated and create endocrine-disrupting and damaging the DNA in some exposed fish.In the present study aimed at verifying the productive effect of garlic extract (GRE) and Vitamin C (VTC) against in vivo tricolosan exposure in zebra fish *Brachydanio rerio* (Ham). The species were exposed to sub lethal concentration of triclosan (0.32mg/L) for 7 and 28 days.The results show that TCS exposure significantly induced genotoxic effects, as revealed by the significant increase in the mean frequencies (MN) of micro nucleated polychromatic erythrocytes and various structural chromosomal aberrations in liver cells of treated fish. GRE and VTC groups showed a significant ($P< 0.05$) reduction in the frequency of CA (57.14% and 40.66%) and MN (53.13% and 40.63%) then the treated group. On the other hand, this investigation clearly revealed the protective effect of GRE and VTC, either each alone or in combination, against the genotoxic potential of triclosan: the garlic extract was often more efficient in its protective action against the pesticide toxicity than vitamin C.

Keywords: Triclosan, zebra fish, garlic extract, Vitamin C, micronucleus, chromosomal aberrations.

Introduction

Fish are being used as useful genetic models for evaluation of pollution in aquatic ecosystems. Fish as bio-indicators of pollutant effects are very sensitive to the changes in their environment and play significant roles in assessing potential risk associated with contaminations of new chemicals in aquatic environment (Lakra and Nagpure, 2009). Moreover, pesticides have been noticed to interfere with fish health and reproduction(Mani and Konar, 1998).

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) is a widely used broad-spectrum bactericide. Triclosan can effectively prevent bacterial lipid biosynthesis by blocking enzyme enoyl-acyl carrier protein

reductase (McMurry et al., 1998). Due to its effectiveness and thermal stability, triclosan is widely incorporated into numerous consumer products, including soaps, toothpastes, disinfectants, cosmetics, and detergents (Dann and Hontela, 2011). Disposal and usage of the triclosan-containing products result in continuous release of triclosan from these products into wastewater. It was estimated that triclosan-containing products account for 96 % of triclosan in wastewater (Ciba Specialty Chemical, 1998). Triclosan and its metabolites are not only detected in rivers, lakes, soils, and sediments, but also in human urine, blood, and breast milk samples (Calafat et al., 2008). The molecular structure of triclosan has shown in **Fig.1**.

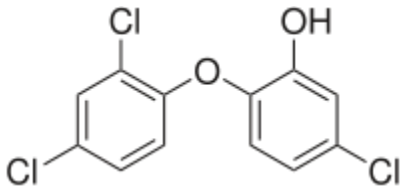


Fig.1. Molecular structure of Triclosan.

Garlic is probably one of the earliest known medicinal plants (Lewis et al., 2003). Its bulbs (cloves) had been used as a cure-all in ancient Egypt and are mentioned in the Ebers papyrus, one of the earliest treaties on medicinal plants. Garlic contains sulfur containing compounds. Alliin is converted to the anti-microbial active alliin when the bulb is cut or bruised. Ajoene which is secondary degradation product of alliin, is presumably the most active compound responsible for the anti-thrombotic activity of garlic (Wichtl, 2004). Garlic has also been shown to have a productive nature against gastrointestinal neoplasias, against blood clots (anti-platelet action) due to part to the compounds alliin and ajoene, which have fibrinolytic activity.

Antioxidants are known as potential scavengers of ROS, so they protect biological membranes from oxidants. However, the balance between free radicals and synthesis of antioxidant defenses can be broken by chemicals. Vitamin C is a water-soluble antioxidant found in the cytosol and extracellular fluid that can interact directly with free radicals, thus preventing oxidative and DNA damage (Padayatty et al., 2003). Vitamin C is a powerful antioxidant and free radical scavenger (Du et al., 2012). Furthermore, results show a strong relationship between low vitamin C levels and cardiovascular diseases (Deicher et al., 2005).

Most fish species cannot synthesize vitamin C, and have to depend on external sources to meet their needs (Chatterjee et al., 1975). The vitamin C requirement for normal growth and survival is quite low (Blom and Dabrowski, 2000), however, a higher level is required to improve the stress resistance of fish (Garcia et al., 2007).

The frequency of micronucleus (MN) in the peripheral blood erythrocytes is one of the best established in vivo cytogenetic assays in the field of genetic toxicology, providing a convenient and reliable index of both chromosome breakage and chromosome loss(Fenech, 2000). Therefore, MN is recommended to be conducted as a part of the monitoring protocols in aquatic toxicological assessment programs(Udroiu, 2006).

Liver is the major site of xenobiotic accumulation and biotransformation, analyses of initial molecular lesions elicited by pollutants in this organ gives early-warning and sensitive indicator of chemical induced carcinogenic lesions(LeBlanc and Bain, 1997). So, it was reliable to use the liver cells as an indicator for the genotoxic effect of triclosan using comet assay.

The present work aimed at verifying the protective role of natural products garlic extract and L-ascorbic acid (Vitamin C) of zebra fish Brachydanio rerio (Ham) against the genotoxic effects of sub lethal dose of triclosan exposure.

Materials and Methods

Collection of fish

The healthy, zebra fish Brachydanio rerio (Hamilton) were purchased from the friend's aquarium, Kolathur, Chennai, having mean weight 3 to 5 g and length 4 to 6cm. Fishes were immediately transported to the fish laboratory in the Department of Zoology, Faculty of Science, Annamalai University. The experimental fishes were reared in glass tanks (100 L capacity) and acclimatized for one month before being used in the experimental study. They were given the treatment of 0.1%$KMNO_4$ solution for bacterial contamination. Then they were fed with tubifex warms regularly.

LC_{50} value of Triclosan (TCS)

Technical grade triclosan $C_{12}H_7Cl_3O_2$ [5-chloro-2-(2,4-dichlorophenoxy)phenol] from [The I.L.E.co., Chennai. India]. The LC_{50} value of triclosan was determined in the laboratory. Three hundred fishes were randomly distributed into six aquarium tanks (100L) filled with different concentration of triclosan (0.15,0.20,0.25,0.30,0.35mg/L). The mortality was recorded for 96h. The LC_{50} of triclosan calculated with the help of probit analysis using SPSS software. The 96h concentration (0.32mg/L) of calculated LC_{50} was selected.

Sub lethal study

LC_{50} value (0.32mg/L) were taken as sub lethal study. Sixty fishes were selected and divided into six groups for the experiments. The triclosan was used in this study and stock solutions were prepared. The experiment was carried out for a period of 7 and 28 days.

Group-I: Control fish
Group-II: Triclosan exposure (0.32mg/L)
Group-III: Triclosan exposure (0.32mg/L) + Garlic extract (1ml)
Group-IV: Triclosan exposure (0.32mg/L) + L-Ascorbic acid (Vitamin C-1g)
Group-V: Garlic extract alone (1g)
Group-VI: L-Ascorbic acid alone (Vitamin C -1g)

Garlic extract (GRE)

An aqueous extract of whole crude garlic was prepared as follows: freshly peeled cloves of garlic (A. sativum, purchased from local market) were sliced into small pieces and ground in a clean mortar using a mortar pestle to produce a fine paste. The working solution was then prepared by dissolving 5 g of the paste in 100 ml of distilled water, where 1 ml of the extract contains 50 mg of crude garlic. Fresh garlic extract was dissolved in the aquarium tank daily.

Vitamin C (VTC)

A pure form of L- ascorbic acid (Vitamin C) was supplied as pure crystals (I.L.E.Co.,) Kattangulathur, Chennai. A freshly prepared aqueous solution of L-ascorbic acid (1g) was dissolved in to aquarium tank daily throughout the experiments.

Chromosome aberrations (CA)

Fish was injected with yeast suspension at a dose of 1 ml/100 g BW. 24 h later; specimens were injected intramuscularly with freshly prepared colchicines at a dose of 0.01 ml of 0.03 mg/g BW. At least 50 metaphase spreads were examined per sample and the CA were detected using light microscope (X 100). CA was expressed as the percentage of aberrant cells and total aberrations per sample.

The reduction percentage in number of CA, MN or DNA fragment were calculated according to the following formula (Manoharan and Banerjee, 1985).

Reduction%

$$= \frac{\text{frequency of CA and MNin A} - \text{frequency of CA and MN in B}}{\text{frequency of CA and MN in A} - \text{frequency of CA and MN in C}} \text{X100}$$

Where A = treatment, B = anti-genotoxic mixed with treatment and C = control.

Micronucleus preparation (MN)

A drop of blood collected from the caudal vein was mixed with a drop of fetal calf serum and smeared directly on slide then air dried, fixed in absolute methanol for 5 min and stained with5%Giemsa for 7 min. 2000 cells per fish were analyzed for the frequency of MN in mature erythrocytes. The erythrocytes of zebra fish *Brachydanio rerio* were generally observed as round with a centrally located round nucleus and a considerable amount of cytoplasm. The diameter of the micronucleus (MN) was less than one-third of the main nucleus, separated from or marginally overlapped with main nucleus and had similar staining as the main nucleus. The number of MN was expressed per thousand erythrocytes (De Flora, 1993).

Statistical analysis

Statistical analysis was performed with SPSS (ver.16.0.) software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post hoc test for comparison between different treatments. Results were reported as mean ± S.E. and differences were considered as significant when $P<0.05$.

Results

Table.1. Productive effect of garlic extract and L-ascorbic acid (Vitamin C) against triclosan induced different types of chromosomal aberrations in zebra fish *Brachydanio rerio* (Ham) liver cells.

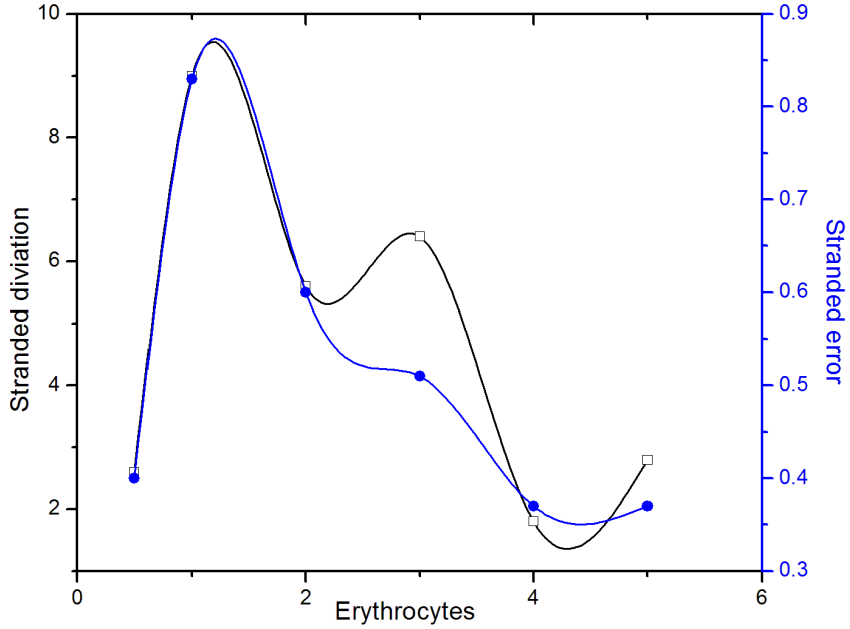
Group	Types of chromosomal aberrations			C.A	End	Aneuploidy	Reduction(%)
	Gap	Break	Delection				
Gr-I	0.80 ± 0.37^{c}	0.00 ± 0.00^{c}	0.40 ± 0.24^{c}	1.60 ± 0.68^{c}	0.00 ± 0.00^{b}	3.40 ± 0.40^{a}	
Gr-II	3.00 ± 0.55^{a}	3.00 ± 0.55^{a}	0.48 ± 0.58^{a}	5.20 ± 0.97^{a}	1.00 ± 0.44^{a}	4.00 ± 0.89^{a}	
Gr-III	2.80 ± 0.66^{ab}	1.60 ± 0.24^{b}	3.20 ± 0.48^{b}	2.80 ± 0.49^{cb}	0.20 ± 0.20^{b}	2.80 ± 0.37^{a}	
Gr-IV	3.00 ± 0.55^{a}	1.60 ± 0.24^{b}	4.00 ± 0.31^{ab}	3.00 ± 0.63^{cb}	0.80 ± 0.37^{ab}	3.00 ± 0.55^{a}	
Gr-V	1.20 ± 0.58^{cb}	0.20 ± 0.20^{c}	0.80 ± 0.37^{c}	2.60 ± 0.40^{cb}	0.00 ± 0.00^{b}	3.00 ± 0.63^{a}	57.14%
Gr-VI	1.00 ± 0.55^{c}	0.40 ± 0.24^{c}	0.80 ± 0.37^{c}	3.80 ± 0.66^{ab}	0.00 ± 0.00^{b}	2.80 ± 0.37^{a}	40.00%

Data were expressed as mean ± S.E (n=5 per each group). Values with different letters (a,b,c) within the coloumn were significantly different at ($P< 0.05$).

Table.2. Productive effect of garlic extract and L-ascorbic acid (Vitamin C) against triclosan induced (MN) micronuclei in zebra fish *Brachydanio rerio* (Ham).

Group	Erythrocytes (MN)	Reduction(%)
Gr-I	2.60 ± 0.40^{c}	
Gr-II	9.00 ± 0.83^{a}	
Gr-III	5.60 ± 0.60^{b}	
Gr-IV	6.40 ± 0.51^{b}	
Gr-V	1.80 ± 0.37^{c}	53.13%
Gr-VI	2.80 ± 0.37^{c}	40.63%

Data were expressed as mean ± S.E (n=5 per each group). Values with different letters (a,b,c) within the coloumn were significantly different at ($P< 0.05$).



Chromosomal aberrations

The typical metaphase complements of zebra fish Brachydanio rerio (Ham.) were found to consist of 44 chromosome of different types as submetacentric, subtelocentric and telocentric. Besides, various forms of chromosome abnormalities as chromatid gaps, breaks, deletions, centromeric attenuation (C.A), endomitosis and aneuploidy were recorded (n ± 1 or 2).

The incorporation of garlic extract –GE (Gr5) and L-Ascorbic acid (Vitamin C) (Gr6) in fish diet at the given concentration (1ml) significantly ($P<0.05$) reduce the frequency of CA induced after sub lethal concentration of triclosan exposure by 57.14% and 40.66% respectively **(Table.1).**

Break chromosomal (BCA) and centromeric attenuations (C.A) were significantly ($P<0.05$) decreased in (Gr3) and (Gr4) groups (Producted) then the control group. In the same time triclosan exposure significantly ($P<0.05$) increased gap chromosomal aberrations (GCA) in treated (Gr2) as well as (Gr3) and (Gr4) groups compare to (Gr1, Gr5 and Gr6). (Gr3) showed a significant ($P<0.05$) decreased in deletion chromosomal (DCA) and endomitosis aberrations in comparison with control group (Gr1).

Micronucleus assay

The size and position of micronuclei in the cytoplasm showed slight variation and normally one micronucleus per cell was observed. Triclosan induced a significant ($P<0.05$) increase in the frequency of MN in (Gr2) (9.00±8.3 vs 2.60±0.40). Confirming its genotoxic potential to fish. Supplementation of garlic extract and/or vitamin C significantly reduces the frequency of MN compare with control by 53.13% and 40.63% respectively **(Table.2).**

Discussion

The aquatic environment plays a vital role for functioning of ecosystem and is intimately related to human health. A majority of contaminants contain potentially genotoxic effects. These chemicals are responsible for DNA damage in variety of aquatic organisms and fish causing malignancies, reduced survival of embryos, larvae and adults, eventually affecting the economy of fish production significantly. The present study supposed that natural products garlic extract and vitamin C are able to provide genoprotection of zebra fish Brachydanio rerio (Ham) sub lethal dose of triclosan exposure.

Fish have been used as models for genotoxicity monitoring in aquatic environments and MN and CA tests have been showing to be sensitive indicators of chromosome damage (Hooftman and Raat, 1982; Carrasco et al., 1990; AlSabti and Metcalfe, 1995; Cavas and Ergene-Gozukara, 2005).Toxicity of TCS to adult Brachydanio rerio was assessed using lethal, genotoxic parameters .The 96h-LC50 value calculated in this study (0.32 mg/L) is very similar to the values found by (Orvos et al., 2002) for Lepomis macrochirus (0.37 mg/L) and Pimephales promelas (0.26 mg/L).The erythrocyte micronucleus test has demonstrated efficiency and sensitivity in different fish species to monitor aquatic pollutants displaying mutagenic features (Al-Sabti and Metcalfe 1995; Ali et al. 2008; Grisolia and Cordeiro 2000; Kligerman 1982).

The micronucleus (Mn) is composed of small chromatin fragments, which arise from chromosome breaks after clastogenic action, or whole chromosomes that do not migrate during anaphaseas a result of aneugenic effects. The efficacy of the micronucleus test as an indicator of structural genomic damage has already been proven and the test has been successfully used as a measure of genotoxic stress, under both laboratory and field conditions (avas and Ergene-Gözükara, 2003). The evaluation of chromosome aberrations is a fully accepted method to reveal genotoxicity, as it is indicative of real genetic effects (Tompa et al., 1992). The production of chromosomal aberrations (CA) is a complex cellular process, with mechanisms of chromosome breakage and re-joining that are not yet completely understood. According to the prevailing theories, structural CA result from: (i) direct DNA breakage, (ii) replication on a damaged DNA template, and (iii) inhibition of DNA synthesis. Underin vivoconditions, the genotoxicity and in particular the clastogenic potential of an agent is often evaluated by use of the CA assay (Preston et al., 1987). The present data of our investigation and those of similar previous studies clearly indicate that triclosan possesses the potential to interact with and to cause alterations in the DNA damage in zebra fish.Our results in Tables 1and 2 shows that sublethal doses of triclosan toxicity (0.32mg/L) would be more effective in producing chromosomal damage in liver cells, as such dose would overwhelm the capacity of repair mechanisms within a short time interval.

Considerable emphasis is put on the use of natural dietary constituents as a chemoprotective measure to control genetic diseases.The data obtained in this investigation revealed the anti-genotoxic potential of garlic extract against chromosomal damage induced by triclosan. Studies on anti-genotoxic effects of garlic extract against pesticides are not available in the literature.Earlier studies conducted with garlic, however, showed that sulphhydryl compounds and other organo-sulphur compounds (such as diallyl sulphide (DAS), diallyl disulphide (DADS), ajoene, allixin, allyl mercaptans and allyl methyl sulphides) are implicated in its anti-mutagenic and anticarcinogenic effects (Soni et al., 1997). Anti-genotoxic agents, especially those present in natural substances, act through different cellular pathways involving endogenous sequestration of mutagens by various enzymes.The mechanism for protection of garlic involves scavenging potentially toxic and mutagenic electrophiles and free radicals and modification of phase-II enzymes and the phase-I profile, which enhances detoxification pathways. The allyl group of garlic constituents enhances the level of glutathione-S-transferase (GST) thereby accelerating the detoxification of mutagens and carcinogens (Dion et al., 1997). Interference of the thiol moiety of sulphur compounds in garlic, particularly diallyl sulphide (DAS), with microsomal enzymes that induce inactivation of genotoxic metabolites has been suggested (Shukla and Taneja, 2002).DNA damage caused by reactive oxygen

species such as hydroxyl radicals, hydrogen peroxides and singlet oxygen has been implicated in mutagenesis, oncogenesis and ageing (Emerit, 1994).

The presence of garlic extract in endothelial cell cultures subject to oxidant stress generated increased levels of SOD, catalase, and glutathione peroxidase and suppressed in a doseand time-related fashion – the production of superoxide radical and hydrogen peroxide (Wei and Lau, 1998). Additionally, it has become obvious from the in vivo studies with rodents that some of the chemical constituents of garlic can enhance the activity of detoxification enzymes such as glutathione-S-transferase, glutathione peroxidase and glutathione reductase (Yang et al., 1994). It can also induce a number of drugmetabolizing enzymes in liver tissue such as the phase-II enzymes GST and the conjugating enzyme, gamma-glutamyltranspeptidase (ɣGT)(Manson et al., 1997).

On the other hand, this study demonstrates that oral administration of L-ascorbic acid (vitamin C) had the ability to reduce the mutagenic effect of triclosan, as indicated by the significant reduction in micronucleated polychromatic erythrocytes and structural chromosomal aberrations.Our results are consistent with those of (Geetanjali et al., 1993) who demonstrated that mice given the organophosphorous insecticide dimethoate and ascorbic acid simultaneously, showed a very low frequency of micronuclei in bone-marrow cellsin vivo, which did not differ significantly from control values in comparison with corresponding values in mice given the pesticide alone. Recently, some authors suggested that high doses of ascorbic acid increased the total antioxidant status (TAS) in human plasma, and ascorbic acid could be useful as a free-radical scavenger for paraquat-poisoned patients (Sae-Yong et al., 2002). In the present study supposed that natural products of garlic extract and vitamin C are able to provide genoprotection of triclosan exposure in zebra fish Brachydanio rerio (Ham).

Conclusion

The multifunctional roles of garlic extract and Vitamin-C in minimizing the health hazardous of triclosan (genotoxicity) besides its high nutritional value acclaims the integration of garlic extract and vit-C to the aquaculture feed specially in integrated fish farming or Agri-based systems e.g. rice-fish integration and cage culture, in which fish are susceptible for pesticides exposure.

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