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POTENTIAL OF CURCUMA LONGA TO AMELIORATE PESTICIDE INDUCED GENOTOXICITY AND OXIDATIVE STRESS – STUDY ON CAPTAN, AN ORGANOCHLORINE FUNGICIDE

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ABSTRACT

Pesticides are some of the most frequently released toxic chemicals into the environment to enhance crop productivity and control insect-vector borne diseases. Eventhough and pesticides are in a way beneficial, their increased use has several drawbacks including health effects, loss of bio-diversity and irreversible changes in natural ecosystems. Around the world, approximately 3 million poisonings from pesticide exposure are reported annually. The present study report the genotoxic effect of a commercially available fungicide, Captan. DNA damage analysis by comet assay was performed in human peripheral blood lymphocytes, chromosomal aberration analysis was performed in onion (*Allium cepa*) root tips and assay of DNA damage and oxidative stress indicators was done in Guppy (*Poecilia Reticulata)*. The potential of a nutraceutical, *Curcuma longa* was evaluated for its ability to protect against Captan and induced genotoxicity and oxidative stress in the above experimental models.

KEYWORDS: *Allium cepa*, *Poecilia and Reticulata*,Pesticides, Captan, genotoxicity, oxidative stress.

INTRODUCTION

Pesticides are a group of chemicals used for better crop production and for vector-borne disease control. Subchronic exposure to pesticides adversely affects various organ systems and theses effects have been attributed to increased oxidative stress and cellular DNA damage (Abdollahi et al., 2004).

Captan (N-trichloromethylthio-4-cyclohexene-1, 2-dicarboximide) is a pesticide (organochlorine fungicide) of the dicarboximide chemical family (Mouchet et al., 2006) used to inhibit the growth of fungi on foodstuffs. The present study report the genotoxic effect of Captan in human peripheral blood lymphocytes, onion (*Allium cepa*) root tips and in *P. Reticulata* or Guppy (Family Poecilidae: Order Cyprinodontiformes). The effect of captan in inducing oxidative stress in gupyy gill tissue was also studied. The single cell gel electrophoresis, or comet assay, the widely used and an adequate methodfor genotoxicity assessment was performed in human peripheral blood lymphocytes for analysing DNA damage (Griffiths et al., 2002). The *Allium cepa* assay was used to monitor the chromosomal aberrations in root meristems of *A. cepa* (Asita and Matebesi, 2010). Guppy which belongs to larvivorous fish species and used as a biological indicator in ecotoxicological studies and chronic carcinogenicity bioassays (Williams et al., 2003) was used as a test species for screening DNA damage and oxidative stress (Sharbidre et al., 2011).

Efforts were also taken to explore the possibility of mitigating pesticide induced toxicity by means of a nutraceutical, *Curcuma longa* (Turmeric) which possess significant medicinal and antioxidant activities (Labban, 2014). Thus in the present study, the potential of alcoholic extract of Turmeric in ameliorating the genotoxicity and oxidative damage induced by captan was explored in the above experimental systems.

MATERIALS AND METHODS

Chemicals

The fungicide Captan was obtained commercially. Bovine serum albumin, propidium iodide, high melting point agarose and low melting point agarose were from Sigma Chemical Company Inc., St Louis, MO, USA. All other chemicals were of analytical grade procured from reputed Indian manufacturers.

Preparation of extracts

Rhizome of Turmeric (*Curcuma longa*) was dried and subjected for size reduction to coarse powder. The powder was extracted with ethanol at 70^{o}C using soxhlet apparatus for 48h. The ethanolic extract was concentrated by evaporation to get solid crude extract.

Phytochemical screening and HPLC analysis.
The alcoholic extract of Turmeric (TE) was subjected to various qualitative phytochemical screening (Khandelwal, 2004) to test for the presence of secondary metabolites such as alkaloids, saponins, phytostreols, tannins, flavonoids and terpenes. The HPLC profile of the extract was obtained from Kerala Forest Research Institute, Thrissur, Kerala, India. The instrument specifications are as follows. Shimadzu system controller: SCL-10Avp, Shimadzu UV/Vis Photodiode array detector: SPD-M10 Avp, Shimadzu solvent delivery pump: LC 10ATvp, Shimadzu column oven: CTO-10ASvp, HPLC Column Luna 5u C18: 250x4.6mm (Phenomenex), Hamilton microsyringe (25ul capacity) with Gradient pump and a sample volume of 20 µl was used.

Free radical scavenging activity
The free radical scavenging potetntial of the ethanolic extract of Turmeric (TE) was studied in terms of DPPH free radical and Hydroxyl free radical scavenging assay (Von Gadow et al., 1997)

Antifungal activity
Antifungal activity of Captan (0.1 - 10 mg/ml) in combination with TE (0.1-10 mg/ml) was studied against *Aspergillus flavus, Aspergillus niger* and *Penicillium chrysogenum*.

Alkaline single cell gel electrophoresis or Comet assay - *in vitro*
Alkaline single cell gel electrophoresis was performed (Singh, 2000) with minor modifications (Chandrasekharan et al., 2011) under *in vitro* conditions on human peripheral blood leukocytes. Blood was collected by venipuncture from 2 healthy volunterrs and incubated with captan (10 µg/ml) for 30 minutes in presence or absence of TE(10 µg/ml). After incubation, comet assay was performed. Microscopic slides were coated with normal melting point agarose and 200 μl of 0.8% low melting point agarose containing 50μl of treated cells (containing 10^4–10^5 cells) were added onto the slide and the slides were kept at 4 ◦C. After solidification the slides were immersed in prechilled lysing solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10, 1% DMSO, 1% TritonX and kept for 1h at 4◦C. After lysis, slides were drained properly and placed in a horizontal electrophoretic apparatus filled with freshly prepared electrophoresis buffer containing 300mM NaOH, 1mM EDTA, 0.2% DMSO, pH≥13. The slides were equilibrated in buffer for 20 min and electrophoresis was carried out for 30 min at 20 V, 300 mA. After electrophoresis the slides were washed gently with 0.4mM Tris–HCl buffer, pH 7.4 followed by distilled water, dried and silver staining was carried out.

Extent of chromosomal aberrations in Onion root tips
Onion root tip chromosomal aberrations study was performed by the method given by Matsumoto et al. (2006). *A cepa* (onion) seeds were germinated in petri dishes (5 onion per plate and 3 plate per group) containing 10 μg/ml of Captan in presence or absence of 10 μg/ml TE. The growth of onion root tips in terms of average length of roots and the extent of chromosomal abberrations in terms of sticky, bridge, lagging and pulverised chromosomes were analysed after 24 hours of incubation.

DNA damage and oxidative stress in gill tissue of fish (Guppy)
Fish (Guppy) (*Poecilia reticulata*) was selected as the experimental model. Fish (3 fish per group) were kept in water containing different concentrations of the pesticide, Captan (0.01 - 1ppm). At 24 hours, fish were were euthanized in ice, gill tissue obtained, homogenized in ice cold phosphate buffered saline (pH 7.4) and homogenate (10 %) was prepared and levels of lipid peroxidation Buege etal, 1978), reduced glutathione (GSH) (Moron et al, 1979) and protein (Lowry et al, 1951) were determined. The dosage of captan for further *in vivo* studies was determined to be 0.1 ppm captan based on the results.

To study the effect of turmeric extract on captan induced tissue toxicity in terms of oxidative stress and cellular DNA damage, Guppy were kept in presence or absence of captan, and/or Turmeric extract as detailed below.
Group I 15 Guppy + Water
Group II 9 Guppy + 0.1 ppm captan
Group V 9 Guppy + 0.01 mg/ml Turmeric extract
Group VI 9 Guppy + 0.1 ppm captan + 0.01 mg/ml Turmeric extract

Extent of DNA damage in gill tissue of fish (Guppy)
Comet assay or alkaline single cell gel electrophoresis (Singh, 2000) was performed on the gill tissues of fish kept in Captan containing water in presence or absence of Turmeric extract for a period of 24 hours to study the extent of genotoxicity in terms of DNA strand breaks. After comet assay, the slides were stained using propidium iodide and observed under fluorescent microscope.

Extent of oxidative stress in gill tissue of fish (Guppy)
Guppy were kept in presence or absence of captan and/or Turmeric extract as detailed above and at 24 hours, 48 hours and on 72nd hour, fish (3 per group) were euthanized in ice, gill tissue obtained and extent of lipid peroxidation and levels of GSH and protein were determined.

Statistical analysis
The results are presented as mean± SD of the studied groups. Statistical analyses of the results were performed by ANOVA with Tukey-Kramer multiple comparisons test using Graph Pad InStat software.

RESULTS AND DISCUSSION

Phytochemical screening

The percentage yield of turmeric extract was found to be 43.5%. The Turmeric extract contained Coumarins, Quinones, Terpenoids, Phenols and Flavanoids. The HPLC profile is given below in Figure 1.

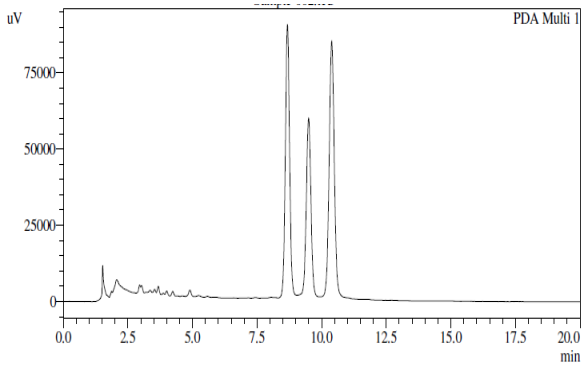

Figure 1: The HPLC profile of ethanolic extract of Turmeric.

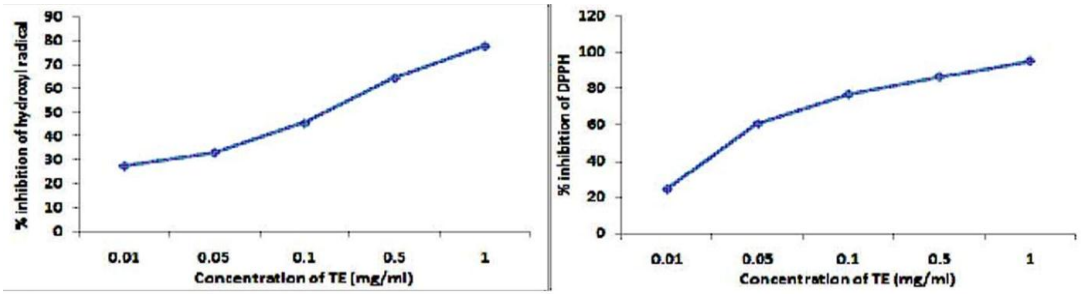

Figure 2: Percentage inhibition of DPPH free radical and hydroxyl radical by different concentrations of Turmeric Extract.

Free radical scavenging activity

The free radical scavenging activities of the extract are given in figure 2 and the results indicate that there is a significant free radical scavenging action by the extract.

Antifungal activity

The antifungal activity of Captan was found to be maintained or enhanced in combination with the extract (Table 1).Table 1 shows the antifungal activity of Captan in combination with TE against *Aspergillus flavus*, *Aspergillus niger* and *Penicillium chrysogenum*. The fungicide showed a significant level of antifungal activity against the fungal species tested. The combination of Captan with TE exerted an enhanced antifungal activity than that of Captan alone against the tested species. The results indicated that TE did not interfere with the antifungal activity of Captan.

Table 1: Antifungal activity of captan (CP) in combination with Turmeric extract (TE).

Concentration	Inhibition zone in cm		
	Aspergillus flavus	***Aspergillus niger***	***Penicillium chrysogenum***
10 mg/ml CP	1.425±0.05	1.8±0.081	2.125±0.095
1mg/ml CP	1.275±0.095	1.6±0.081	1.9±0.081
0.1mg/ml CP	0.925±0.05	0.95±0.057	1.075±0.049
10 mg/ml TE	1.2±0.081	1.225±0.049	0±0
1mg/ml TE	0±0	0.925±0.050	0±0
0.1mg/ml TE	0±0	0.775±0.050	0±0
10 mg/ml CP + 10 mg/ml TE	1.825±0.05	1.625±0.095	2.45±0.057
1mg/ml CP + 1 mg/ml TE	1.35±0.057	1.2±0.081	1.8±0.081
0.1mg/ml CP+ 0.1 mg/ml TE	0.95±0.057	0.9±0	0.825±0.050

Alkaline single cell gel electrophoresis or Comet assay - *in vitro*

Alkaline single cell gel electrophoresis or comet assay was performed on human peripheral blood lymphocytes to ascertain the genotoxicity of the fungicide, Captan (Figure 3). The untreated cells after comet assay and silver staining remained as circular homogenous discs while the cells treated with Captan (10 µg/ml) showed a comet like appearance with tail portion comprising the broken fragments of the cellular DNA which migrated out of the nucleus during the electrophoresis. When the cells were treated with Turmeric Extract alone, the cells remained circular indicating that the extract by itself was not genotoxic. The presence of TE along with the Captan treatment protected the cells from Captan induced DNA damage as can be evidenced from the lack of comet tail in these cells. Thus the result indicated that the presence of the extract along with Captan protected against captan induced DNA strandbreaks (figure 3).

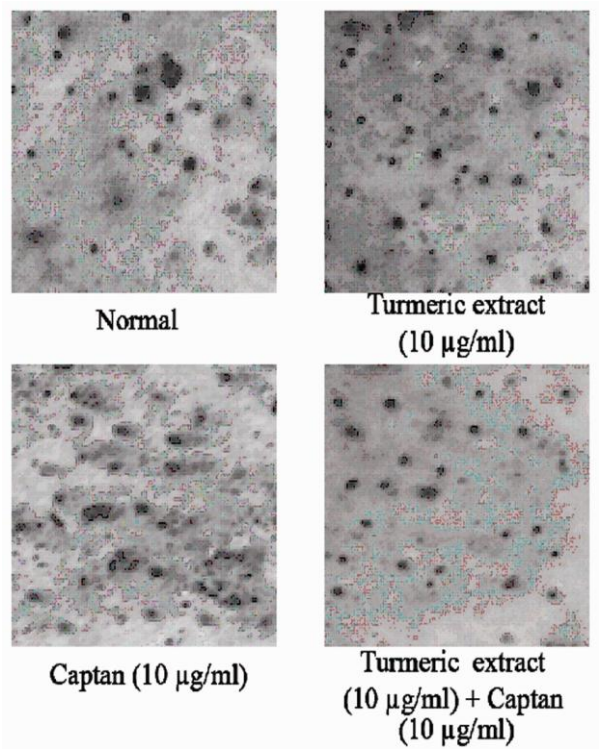

Figure 3: Effect of TE on cellular DNA damage induced by exposure to Captan in human peripheral blood leukocytes (*in vitro*) as analyzed by comet assay and silver staining.

Extent of chromosomal aberrations in Onion root tips

Table 2 shows the effect of Captan and TE on the extent of growth of onion root tips. The length of onion roots were measured in growing onion bulbs after 5 days of seeding. It was observed that the average length of roots were significantly less in Captan treated group of onions when compared to those grown in the absence of pesticide. The presence of TE along with Captan significantly enhanced the growth of roots.

Table 2: Effect of Captan and TE (10 µg/ml) on growth of onion root tips. a - p<0.001 when compared to Control and b - p<0.01 when compared to Captan (10 µg/ml). (5 onion per plate and 3 plate per group).

Treatments	Onion Root length (cm)
Control	2.00±0.91
Captan	0.46±0.11[a]
Turmeric	1.55±0.49[b]
Captan+ Turmeric	1.45±0.65[b]

Table 3: Effect of TE (10 µg/ml) on Captan (10 µg/ml) induced chromosomal aberrations in growing onion root tips. (5 onion per plate and 3 plate per group). a - p<0.001 when compared to Control, c - p<0.05 when compared to Control, d - p<0.001 when compared to Captan, g - p<0.001 when compared to respective TE.

	Sticky	Bridge	Lagging	Pulverised
Control	0±0	0±0	0±0	0±0
Captan	27.179± 16.836[a]	5.525± 3.465[a]	33.626± 7.552[a]	4.026± 4.309[a]
Turmeric	0±0[d]	0±0[d]	0±0[d]	0±0[d]
Captan+ Turmeric	14.536± 15.4[c,f,i]	2.38± 4.123[f]	5.639± 7.602[d]	0±0[d]

Table 3 shows the effect of Captan and TE on growing onion root tips as measured as the extent of Chromosomal aberrations. Onion root tip cells were observed for chromosomal aberrations such as sticky, bridge, lagging chromosomes and pulverized cells. From the result, it can be seen that a significant decrease in the number of normal and dividing cells (Mitotic index) were observed in Captan treatment when compared to control. The control set showed the maximum number of normal and dividing cells than others. The Captan treatment showed a significantly higher number of various chromosomal damages. There was a significant decrease in the number of chromosomal aberrations in the group of onion bulbs treated with the TE along with Captan. No chromosomal aberrations were observed in TE alone treated group.

The presence of TE prevented Captan induced growth retardation in onion root tips (Table 2) and significanlty reduced the extent of chromosomal aberrations (Table 3).

Toxicity by captan on guppy

Captan was analysed for its toxicity by growing fish (guppy) in the presence of it. After 24 hours the fish were euthanized and their gill tissue was obtained and analyzed for extent of lipid peroxidation and levels of tissue GSH. Lipids that contain unsaturated fatty acids with more than one double bond are particularly susceptible to action of free radicals. The resulting reaction, known as lipid peroxidation was found to increase with increased concentrations of Captan. When cells are exposed to increased levels of oxidative stress, GSSG (Glutathione) will accumulate and the ratio of GSH (reduced glutathione) to GSSG will decrease. The

increased Captan concentration was found to increase the oxidative stress in the fish as evidenced by the decreased levels of the GSH in gill tissues. From the toxicity studies, the dosage of captan for further studies was selected to be 0.1 ppm. The results are given in table 4.

Table 4: Levels of lipid peroxidation and tissue GSH levels in the gill tissue of guppy kept in water containing different concentrations of captan for 24 hours. (n=3 per group).

	MDA (nanomoles/mg Protein)	GSH (nanomoles/ mg Protein)
Control	12.55±5.00	337.00±12.70
0.01 ppm Captan	18.66±6.46	290.75±63.27
0.1 ppm Captan	22.49±8.87	263.54±24.31
1 ppm Captan	27.36±3.17	240.91±9.60

The ability of turmeric extract to minimize the oxidative stress and genotoxicity induced by Captan was studied *in vivo* by analyzing the levels of lipid peroxidation and GSH and the extent of cellular DNA damage in the gill tissue of fish (guppy) grown in water containing Captan in presence or absence of turmeric extract.

Extent of DNA damage in gill tissue of fish (Guppy)

Comet assay was performed in cells of gill tissues of fish kept in Captan containing water (0.1ppm) in presence or absence of Turmeric extract for a period of 24 hours. The results are shown in figure 4. When the slides were observed by fluorescence microscopy after the assay by propidium iodide staining, the cells obtained from fish treated with Captan alone showed a comet like appearance indicating cellular DNA damage while the gill cells of fish treated with Turmeric extract alone remained circular indicating the non-genotoxic nature of the extract. The cells of fish treated with Turmeric extract along with Captan were found to be protected from captan induced genomic DNA damage as indicted by the comet assay results in those cells since they remained circular indicating no DNA damage.

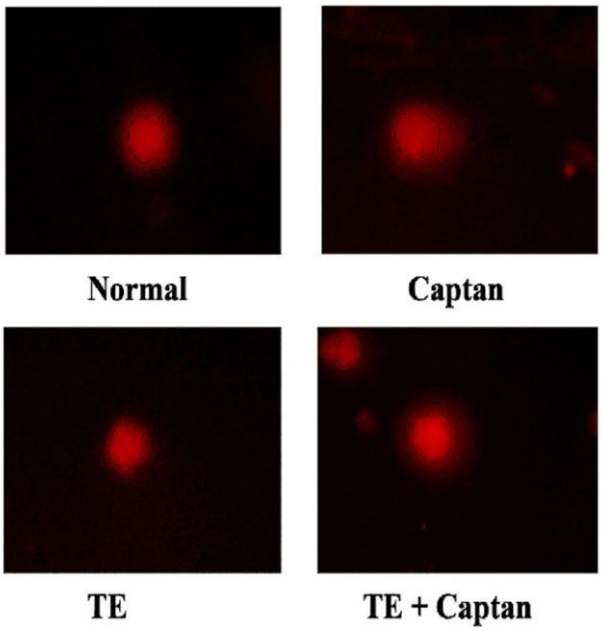

Figure 4: Effect of turmeric extract against captan induced cellular DNA damage in fish gill tissue of Guppy as assayed by comet assay.

Extent of oxidative stress in fish gill tissue

The effect of turmeric extract on Captan induced oxidative stress was analyzed by measuring the levels of GSH and lipid peroxidation on gill tissue of fish kept in presence of captan and/ or turmeric extract for different time durations. The results are given in table 5 and 6.

Table 5: Effect of turmeric extract against Captan induced oxidative stress as measured in terms of levels of GSH in gill tissue of Guppy kept in water containing captan and/or turmeric extract for different time durations. (n=3 per group).

GSH (nanomoles/mg Protein)	24th hour	48th hour	72nd hour
Control	337.00±12.70	337.00±12.70	337.00±12.70
0.1 ppm Captan	257.97±29.74	200.65±30.56	81.85±15.98
0.01 mg/ml TE	339.97±75.94	333.47±24.45	325.72±35.22
0.1 ppm Captan+0.01 mg/ml TE	274.30±22.72	265.29±36.70	123.58±24.31

Captan treated Guppy showed a significantly reduced level of GSH while Guppy kept in presence of captan along with TE showed near normal levels of GSH which indicate the protective ability of these extracts against captan induced oxidative stress.

The levels of lipid peroxidation was significantly higher in Guppy treated with Captan. The turmeric extract possess antioxidant activity which helped them to scavenge the oxygen radicals responsible for the lipid peroxidation therefore the extent of captan induced lipid peroxidation decreased when guppy were kept in the presence of GE.

Table 6: Effect of turmeric extract against Captan induced oxidative stress as measured in terms of levels of lipid peroxidation in gill tissue of Guppy kept in water containing Captan and/ or turmeric extract for different time durations. (n=3 per group).

MDA (nanomoles/mg Protein)	24th hour	48th hour	72nd hour
Control	12.55±5.00	12.55±5.00	12.55±5.00
0.1 ppm Captan	23.34±2.26	36.67±3.73	44.32±1.64
0.01 mg/ml TE	6.49±0.77	10.41±3.76	3.74±1.89
0.1 ppm Captan+0.01 mg/ml TE	14.16±3.53	25.26±3.64	24.18±8.11

The results clearly indicated that the extract could mitigate captan induced oxidative stress and genotoxicity which could be due to the efficient free radical scavenging activity by the extract.

CONCLUSIONS

Sustained use of pesticides is indispensible to maintain crop productivity by destroying pests and vectors and is very much essential to keep up the dietary requirements of a population. In the current scenario when the use of a toxic pesticide is restricted, it is getting replaced by another pesticide which may still be toxic. In the present work, a commonly and extensively used contact fungicide, Captan was analyzed for its genotoxicity and was found to be harmful to the genomic integrity of the crop. Its toxicity towards human peripheral blood leukocytes under *in vitro* condition implies its risk under prolonged exposure. It was also observed that Captan induced genotoxicity and oxidative stress under *in vivo* conditions in Guppy. Turmeric extract possessed significant antioxidant activity and significantly minimized the captan induced genotoxicity and oxidative stress while not interfering with its antifungal activity.

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