

Original Research Article

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Ameliorative Potential of *Mentha piperita* against Arsenic Trioxide Induced Geno-Toxicological Alteration in Fish, *Channa punctatus* (Bloch.)

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ABSTRACT

Keywords

Mentha piperita, *Channa punctatus*, Arsenic dioxide, Amelioration, Micronuclei

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Present study aims to evaluate the ameliorative potential of *Mentha piperita* (MP) against arsenic trioxide (ATO) in freshwater fish *Channa punctatus* (Bloch). Fish were divided into six groups. G1 served as control. Fish of G2 and G3 were treated with 96 h-LC₅₀/10 of ATO and 8 mg/l of MP, respectively. In G4, G5 and G6, fish were given simultaneous treatment of 96 h-LC₅₀/10 of ATO along with 2 mg/l, 4 mg/l, and 8 mg/l concentrations of MP. Samples collected at the interval of 24 h till 96 h exposure period. Result reveals an increased frequency of micronuclei (MN) in G2 on comparing with the G1. However in groups G4, G5 and G6, where fish were exposed to ATO along with 2 mg/l, 4 mg/l, 8 mg/l of MP respectively were showing reduction in frequency of micronuclei (MN). This reduction in frequency of MN on introduction of MP must be due to phyto-chemicals present in the MP extract. This has been proved by the presence of well-known antioxidants viz., alkaloids, flavonoids, saponin and tannins in photo-chemical tests.

Introduction

Mentha piperita (MP) from Lamiaceae commonly known Peppermint as is well known for its flavouring and medicinal properties worldwide. In Ayurveda, this is a base ingredient of several compound formulations used in management of gastrointestinal and skin problems. The extract of MP possesses antimicrobials, antioxidant actions, antitumor and anti-inflammatory (Golestani *et al.*, 2015; Ramos *et al.*, 2017). In the present study MP extract was screened for its anti-oxidative property against arsenic trioxide (ATO). As concentration of arsenic in environment is continuously an increase due

to anthropogenic activities. Aquatic environment is the ultimate sink of pollutants which creates threat to aquatic biota particularly fishes. As fishes belong to higher trophic level, have greater chances to accumulate higher concentration of arsenic because of biomagnifications. On the other hand fishes are an important source of food, which multiplies risk of arsenic exposure and its toxicity to humans. Epidemiological studies showed that exposure of arsenic results in variety of adverse health issues such as hypertension, diabetes, cardiovascular disease, anemia, neurologic disorder, along with liver and kidney diseases (Morales *et al.*, 2000; Szymanska-Chabowska *et al.*, 2002). The

oxidative stress and increased reactive oxygen species by arsenic is one of the extensively studied mechanism of action for arsenic toxicity (Kitchin and Conolly, 2010).

Thus this study is an effort to find out a cost effective, safe and economical anti-genotoxic solution for genotoxic problems created by arsenic tri oxide exposure in freshwater teleostean fish *Channa punctatus* (Bloch.) in terms of micronuclei.

Materials and Methods

Test animal and test chemical

Healthy and live freshwater teleostian freshwater fish, *Channa punctatus* (Bloch.) of almost the same size (14.5 ± 1.0 cm and 30 ± 2.0 g) were obtained from local lentic habitats in the local fish market. They were given prophylactic dip in formalin (0.4%) for 15 min followed by KMnO_4 (1 mg l^{-1}) treatment for 1 h each to keep away dermal infections. Then prior to experiment, fishes were acclimatized for 10 days in large glass aquaria ($100 \times 40 \times 40 \text{ cm}^3$), during which they were fed minced goat liver and artificial fish food Tokyo. The faecal matter and other waste materials were siphoned out daily to reduce the ammonia content in water. Fishes were maintained by following standard fish maintenance procedure during acclimatization (Temperature of 14 to 22°C , dissolved oxygen- 6.62 to 6.76 mg l^{-1} , alkalinity- 62 to 68 mg l^{-1} , CO_2 Nil) as outlined by APHA *et al.*, (2012). Technical grade arsenic trioxide (ATO) was taken from Riedel-De Haenag, Seelze-Hannover, made in Germany.

Collection and preparation of ethanolic extract of MP

The whole plant of MP was collected from Local market of Lucknow, India. The identification of samples was performed by

the experts in the Department of Botany, University of Lucknow, Lucknow.

Collected plant materials of MP were air-dried in room ($31^\circ\text{C} \pm 2^\circ\text{C}$) for minimum 20 days and grind it into powdered form. 200g of powdered whole plant material was extracted in soxhlet apparatus with 50% ethanol for about 72 h. After 72 h filtrate was collected and concentrated till semi solid state, by the rotary vacuum evaporator, below 60°C temperature. This concentrated semi solid ethanolic extract was stored in the refrigerator and used for the experiment.

Determination of sub lethal concentration of ATO, (LC_{50})

Stock solution of ATO was prepared by dissolving 1 g of ATO in appropriate amount of diluted acid water (pH 6.5). For determination of 96 h- LC_{50} different concentration of (54-144 mg/l) ATO in logarithmic ratio were prepared from the stock solution and added in different aquaria containing 80 L of water.

A set of 10 acclimatized fish of almost equal size and weight was randomly selected and transferred to each concentration. Experiment was conducted into triplicate to verify reproducibility and mortality of fish was recorded at a regular time intervals up to 96 h of exposure and the dead fish were removed, immediately. The value of 96h- LC_{50} of arsenic was calculated by using the Trimmed Spearman-Kärber Method (Hamilton *et al.*, 1977).

Experimental design

For the sub lethal studies acclimatized fish were divided into 6 groups having 10 fish in each group. The first group (G1) was used as a control. Fish in group 2 (G2) and 3 (G3) were exposed to 96 h- $\text{LC}_{50}/10$ of ATO and 8 mg/l

concentration of MP, respectively. While fish in group 4 (G4), 5 (G5) and 6 (G6) were exposed to different concentration of MP viz., 2 mg/l, 4 mg/l and 8 mg/l along with 96 h-LC₅₀/10 of ATO. Three replicate were used for each group. The samples were randomly collected from control as well as treated group for further analysis at regular interval of 24 h till 96 h exposure period.

Phytochemical screening of plant extract

Phytochemical analysis was carried out according to the standard methods outlined by Kokate (2000) for the test of flavonoid, alkaloids, saponin and tannin. For this 1 gm crude semisolid ethanolic extract was dissolved in its own mother solvent and this stock solution was used for phytochemical screening. Shinado's test was used for the presence of flavonoids. For this a few magnesium turnings and few drops of concentrated hydrochloric acid were added in stock solution and boiled for five minutes.

Appearance of magenta color confirms the presence of flavonoids. To test the presence of alkaloids Mayer's reagent was added in stock solution. Appearance of creamy white precipitate confirms the alkaloid presence. Mixing of basic lead acetate in stock solution forms white precipitate indicates tannin presence. Formation of white precipitate indicates the presence of Tannins. Saponins presence was tested by shaking stock solution with water by presence of foamy lather formation.

Micronuclei (MN) assay

Peripheral blood samples were smeared on pre cleaned microscopic slides and fixed with absolute methanol for 5 min. After fixation, slides were stained with May-Grunewald's solution 1 and 2 for 3 and 5 min, respectively followed by 5% Giemsa staining for 30 min.

After overnight drying DPX mounted slides were observed under oil immersion microscope (Nikon Corporation K 12432) using 40/100X objective lenses. Micronuclei (MN) were scored by following the criteria of Fenech *et al.*, (2012). A minimum 1000 erythrocytes for each specimen were examined.

Results and Discussion

In present study 96 h-LC₅₀ value of ATO for the *C. punctatus* was estimated 81.73 mg/l with 95% confidence limits viz., 98.94 upper confidence limit and 73.45 lower confidence limit with 10% Spearman Karber Trim. For the sub lethal exposure in present study 96 h-LC₅₀/10 *i.e.*, 8.173 mg/l was used for in vivo experiments. No mortality was noticed during sub lethal exposure period.

Eco-toxicological manifestations of metals in aquatic habitat directly affect the behavioural and morphological parameters of fishes. Fishes being aquatic animals are more sensitive to these toxicological manifestations as their habitat is confined and an escape from such toxicant infested micro-habitats is not possible. Toxicological manifestations of metals in fishes are reflected both externally and internally. External response includes abrupt behavioural responses which find ample support in assessing the sub lethal impacts of toxicants (Weis and Candelmo, 2012). In fact, morphological and behavioural responses of fishes are the reflection of biochemical, physiological and molecular alterations in their body, on account of xenobiotic stress. In this study behavioural and morphological anomalies were recorded in different experimental groups and represented in table 1 and 2. In this study fish of control group (G1) were behaving normally without conspicuous behavioural alterations, as observed in ATO exposed groups.

Table.1 Behavioural anomalies recorded in different experimental groups

| S. No. | Nature of Behaviour | G1 | G2 | G3 | G4 | G5 | G6 |
|--------|------------------------|----|------|----|-----|-----|----|
| 1. | Hyper excitability | - | ++++ | - | +++ | ++ | ++ |
| 2. | Jumping | - | +++ | - | +++ | +++ | ++ |
| 3. | Restlessness | - | ++++ | - | +++ | +++ | + |
| 4. | Schooling | - | +++ | - | ++ | ++ | + |
| 5. | Imbalanced swimming | - | +++ | - | +++ | + | + |
| 6. | Fin movement | - | +++ | - | ++ | ++ | + |
| 7. | Loss of Equilibrium | - | +++ | - | ++ | + | + |
| 8. | Opercular Movement | - | ++++ | - | ++ | ++ | - |
| 9. | Gulping air at surface | - | ++++ | - | ++ | ++ | + |

Note: (-) = Normal response, (+) = Abnormal response, (++) = Mild increase response, (+++) = Moderate increase response, (++++) = Maximum increase response.

Table.2 Morphological anomalies recorded in different experimental groups

| S. No. | Morphological Changes | G1 | G2 | G3 | G4 | G5 | G6 |
|--------|-----------------------|----|------|----|-----|-----|----|
| 1. | Discoloration of skin | - | ++++ | - | ++ | ++ | ++ |
| 2. | Lesions on skin | - | ++++ | - | +++ | +++ | ++ |
| 3. | Shedding of scale | - | ++++ | - | +++ | ++ | + |
| 4. | Mucus secretion | - | ++++ | - | ++ | ++ | + |
| 5. | Muscular bleeding | - | ++++ | - | ++ | + | + |

Note: (-) = Normal response, (+) = Abnormal response, (++) = Mild increase response, (+++) = Moderate increase response, (++++) = Maximum increase response

Table.3 Frequencies of Micronuclei (MN) per 1000 erythrocytic cells in *Channa punctatus*

| | G1 | G2 | G3 | G4 | G5 | G6 |
|------|-----------|-----------|-----------|-----------|-----------|-----------|
| 24 h | 0.13±0.05 | 0.64±0.09 | 0.10±0.03 | 0.63±0.10 | 0.52±0.02 | 0.32±0.02 |
| 48 h | 0.12±0.03 | 1.07±0.06 | 0.11±0.04 | 0.55±0.04 | 0.37±0.13 | 0.25±0.04 |
| 72 h | 0.11±0.04 | 1.21±0.07 | 0.10±0.02 | 0.50±0.06 | 0.35±0.07 | 0.23±0.02 |
| 96 h | 0.12±0.05 | 1.38±0.06 | 0.12±0.05 | 0.37±0.04 | 0.27±0.10 | 0.20±0.03 |

G1: Control; G2: (96 h LC₅₀/10 of ATO); G3 (8 mg/l of MP); G4 (96 h LC₅₀/10 of ATO+2 mg/l of MP); G5 (96 h LC₅₀/10 of ATO+4 mg/l of MP) and G6 (96 h LC₅₀/10 of ATO+8 mg/l of MP).

Table.4 Phytochemical analysis of ethanolic extracts of MP

| Phytochemical Constituents | Ethanolic extracts of MP (Whole plant) |
|----------------------------|--|
| Alkaloids | + |
| Flavonoid | + |
| Saponin | + |
| Tannins | + |
| Steroids | - |

(+) Present in MP extract; (-) Absent in MP extract

Abrupt fish behaviour *viz.*, hyperactivity, bust swimming, haphazard and s-jerk movements coupled with rapid opercular movements in arsenic exposed groups (G2) were observed within a few minutes of arsenic exposure. These behavioural responses persisted for a shorter period till fish became lethargic, settled at the bottom or aggregated in one corner at the bottom of the glass aquarium. Along with the altered behavioural parameters, certain prominent morphological alterations *viz.*, discoloration of skin, lesions in skin, shedding of scale, mucous secretion, and muscular bleeding were also recorded in G2 with increased redness of skin and mucous secretion. However, fish of control group were behaving normally without visible morphological alterations, which were observed in fish exposed with 96 h-LC_{50/10} of ATO exposed groups. However, fish of G4, G5 and G6 were exposed to 96 h-LC_{50/10} of ATO along with different concentrations *viz.*, 2, 4 and 8 ppm of MP showed lesser behavioural and morphological alterations in comparison to group 2.

Fish are in continuous exposure of several toxicological manifestations in aquatic environment. Thus, fish blood can be used a very sensitive indicator for risk assessment for these ecotoxicological manifestations. Recently, morphological changes in erythrocytes have been used as an importance bio-indicator for the interpretation of toxicological manifestations. Among several morphological alterations in erythrocyte micronucleus test has been widely used for toxicological assessments because of its non-invasiveness and sensitivity. MN is a additional nuclei less than one-third diameter of the main nucleus with similar staining and without overlapping with the main nucleus. Erythrocytes of G1 (Control) fish are elongated ellipsoid bodies with centrally located oval nucleus. However, MN was well visible in erythrocytes of fish exposed to ATO

(G2). In the present study table 3 represents recorded MN frequency per 1000 cells in different experimental groups at different exposure periods *viz.*, 24 h, 48 h, 72 h and 96 h. Results clearly showed an increase in the frequencies of MN in fish of G2 (96 h LC_{50/10} of ATO) than G1 (Control) at exposure periods of 24 h, 48 h, 72 h and 96 h exposure periods. The highest frequencies of MN were recorded after 96 h exposure in G2.

Enough documentation is already there which supports arsenic induced genotoxicity, on account of overproduction of reactive oxygen species (Kitchin and Conolly, 2010) that leads to oxidative DNA damage and then formation of MN. The observations recorded for G2 are in agreement with the findings of earlier studies in fishes exposed to pesticides and heavy metals (Anbumani and Mohankumar, 2014; Omar, *et al.*, 2012; Polard, *et al.*, 2011). Such increased expression of MN in G2 (96 h LC_{50/10} of ATO exposed fish clearly establishes the genotoxic nature of ATO.

Moreover, G4, G5 and G6, showed reduction in the frequencies of MN on comparing with G2 at the exposure periods of 24 h, 48 h, 72 h and 96 h. As G4, G5, G6 were groups where fish were exposed with 96 h LC_{50/10} of ATO along with different concentration of MP *viz.*, 2 mg/l, 4 mg/l and 8 mg/l. The decrease in the frequencies of MN was found to be concentration dependent for the tested concentrations of MP in the exposure periods of 24 h, 48 h, 72 h and 96 h. For all the three tested concentrations of MP extract, the maximum reduction was recorded in G6, having 8 mg/l concentration of MP.

This signifies the anti-oxidative potential of MP (Kitchin and Ahmad, 2003). This anti-oxidative property of MP must be a cumulative effect of phyto-chemicals present in the MP extract (Deepa, *et al.*, 2009). The present study also reveals the presence of

well-known anti-oxidative phyto-chemicals viz., alkaloid, flavonoids, Saponin and tannins in the MP extract (Table 4).

Our findings reveal the excellent ameliorative potential of ethanolic extract of MP against 96 h LC₅₀/10 of ATO induced genotoxicity in *C. punctatus*. We found that MP at all the tested concentration is safe to use as ameliorative agent. Among all tested concentrations, 8 mg/l concentration of MO was found more effective. This finding finds an ample scope in aquaculture as by using natural plant product as an ameliorative agent over other chemical drugs, we are reducing the threat of exposing environment to unknown toxic effects of other chemicals.

Conflict of interests

The author declares that there is no conflict of interest.

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