



Amelioration of arsenic-induced oxidative stress in CHO cells by *Ixora coccinea* flower extract

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Abstract

Chronic exposure to inorganic arsenic creates various health problems. *Ixora coccinea* flower extract was investigated for its ability to protect against arsenic-induced cytotoxicity and genotoxicity in CHO cell line. MTT assay confirmed the efficacy of the extract in ameliorating arsenic-induced cytotoxicity. The value (48 mM) of 24 h inhibitory concentration (IC₅₀) of sodium arsenate for CHO cells was obtained by MTT assay. Various free radical scavenging assays like DPPH, ABTS and nitric oxide scavenging assay confirmed antioxidant activity of the *Ixora coccinea* flower extract. Pretreatment of the extract significantly inhibited the arsenic-induced DNA damage ($p < 0.01$) in CHO cells. The extract administration significantly ($p < 0.01$) inhibited the intracellular ROS and depolarization of mitochondrial membrane induced by sodium arsenate. *Ixora coccinea* flower extract reduced oxidative stress in cells. Antioxidant enzymes like catalase and SOD activity was restored significantly ($p < 0.01$) in pretreated CHO cells. *Ixora coccinea* flower extract also exhibited the anti-apoptotic potential by decreasing the percentage apoptotic index ($p < 0.01$). These results may expand the applications of *Ixora coccinea* flowers as an alternative food with antioxidant properties and protective functions against arsenic (iAs) induced toxicological effects.

Keywords *Ixora coccinea* · CHO cells · ROS · Cytotoxicity · Comet assay

Introduction

Arsenic is a metalloid ubiquitously present in the environment. Drinking arsenic contaminated water is the major source of human exposure to arsenic (Wang et al. 2011). According to WHO the permissible limit is 50 µg/l. Chronic exposure to high concentrations of inorganic arsenic leads to deleterious effects in living cells (Mazumder 2008). Arsenic induces cytotoxicity through the generation of intracellular ROS and oxidative stress which results in the altered enzyme activities (Aung et al. 2013). Increased ROS production causes oxidative damage to DNA and apoptosis in

cells. Arsenate also leads to lipid peroxidation in exposed cells (Singh et al. 2011).

Ixora coccinea has been used traditionally in folk medicine (John 1984) and in Ayurveda to treat several diseases. It is called as Bandhuka, Bandhujivaka, Ishwara, Parali, Paranti, Raktaka in Sanskrit, Kangan, Rajana, Rangan, Rookmini, Rugmini in Hindi, Rookmini, Kangan, Kannada-Gudde dosal, Kepala, Kisukare, Kempulagida in Hindi, Patkali, Podkali, Malayalam-Chethi, Thechi, Thetti in Konkani, Bakali, Bakora, Pankul, Patkalin, Pendgul, Oriya-Bondhuko, Romoniphulo in Marathi, Koran kullai, Sedaram, Sinduram rangan Vetchi in Tamil, Bandhujivakamu, Bandhujivamu, Bandhukamu, mankana, manmadabanam in Telugu (Baliga and Kurian 2012). In Ayurveda, flowers are used to treat diseases such as dysentery, leucorrhoea, dysmenorrhoea, hypertension, menstrual problems, sprains, bronchitis fever, chronic ulcers, sores, skin diseases and scabies (Saha et al. 2008; Sankaranarayanan et al. 2010). The flowers are used as a wound healing agent, to treat catarrhal bronchitis as well as dysentery (Sivarajan et al. 1994). Studies indicate that the *Ixora coccinea* has antioxidant and antibacterial properties. The shade dried, powdered flowers are used in treating eczema (Sivaperumal et al. 2009). The concoction prepared

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from the root is traditionally used in treating nausea, anorexia and hiccups. Sores and chronic ulcers are also treated with the use of finely pulverized dry roots (Vadivu et al. 2010). *Ixora coccinea* flowers and fruits are edible and used as functional food (Baliga and Kurian 2012). In the southern part of India they are used in different cuisines (raw) as a nutrient supplement.

In this paper, the interactions of CHO cells with sodium arsenate and the reversal of its toxic effects by *Ixora coccinea* aqueous extract has been studied by different assays including cell survival assay, ROS assay, comet assay, MMP and apoptosis. *Ixora coccinea* aqueous extract was evaluated for the ability to attenuate arsenic-induced cytotoxicity and genotoxicity in CHO cells. Phytochemical studies were performed to estimate the total phenols, total flavonoids. Antioxidant ability of aqueous extract was evaluated through DPPH, ABTS, Nitric oxide scavenging assay and total antioxidant capacity. MTT assay was carried out in CHO cells to evaluate cell viability. Altered Intracellular ROS, mitochondrial membrane potential leading to change in fluorescent intensity were read on FACS. The extent of DNA damage was estimated by comet assay in an alkaline medium. Cellular apoptosis was evaluated in CHO cells. Antioxidant enzymes like SOD and catalase levels were also estimated.

Materials and methods

Plant material

The plant was authenticated by Botanist, Professor and Head (Ret.) Aravinda Hebbar, A voucher specimen (PP 619) has been deposited in the herbarium of our University, Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, MAHE, and Manipal for future reference.

Preparation of samples

Shade dried *Ixora coccinea* flower powder was soaked in distilled water with 1% chloroform for 7 days. The extract thus obtained was filtered to remove fiber, then concentrated with controlled temperature in a rotary evaporator. The powder thus obtained was dissolved in distilled water to desired concentrations for experimental purpose.

Determinations of total phenols

Total phenols were measured in aqueous extract of *Ixora coccinea* with the Folin–Ciocalteu reagent. The total concentrations of phenolics in the extracts were measured using gallic acid as standard. 1 ml of extract or standard solution was mixed thoroughly with 5 ml of FC reagent with ten

times dilution in water and 4 ml of 700 mM sodium carbonate, incubated for 2 h and then absorbance was recorded at 765 nm using UV-spectrophotometer (Slinkard and Singleton 1977). The total content of phenolic compound expressed as gallic acid equivalents mg/g of flower extract.

Determinations of total flavonoid content

Total flavonoids in *Ixora coccinea* aqueous extract were measured with the aluminium chloride colorimetric method. Quercetin was kept as standard, 500 µl of standard or extract was mixed with methanol (1.5 ml), aluminium chloride (0.1 ml, 10%), potassium acetate (0.1 ml, 1M) and distilled water (2.8 ml), after 30 min absorbance was recorded at 415 nm with the help of UV-spectrophotometer (Chang et al. 2002). The total flavonoid content was represented as quercetin equivalents mg/g of the dry flower extract.

DPPH free radical scavenging assay

The free radical scavenging activity of the aqueous extract of *Ixora coccinea* was measured in the presence of DPPH. 1 ml of DPPH (0.1 mM) solution in methanol was added to 1.0 ml of extract with various concentrations, after 20 min absorbance was recorded at 517 nm using spectrophotometer (Bansal et al. 2011).

ABTS free radical scavenging assay

ABTS free radical assay was performed using 7 mM ABTS solution with potassium persulphate (2.4 mM), mixed and incubated for 15 h in the dark at room temperature. Various concentrations of extract were prepared in methanol and 20 ml of test solutions were mixed with 180 ml of ABTS free radical solution, incubated for 20 min and absorption was measured at 750 nm (Tachakittirungrod et al. 2007).

Nitric oxide scavenging assay

Nitric oxide scavenging assay was performed using the Griess method. 2 ml of Sodium nitroprusside (10 mM) was mixed with 0.5 ml of PBS, 0.5 ml of standard or extract solutions at various concentrations and incubated at 25 °C for 1 h 30 min. 0.5 ml of the reaction mixture was mixed with 1 ml sulphanilic acid reagent (0.33% sulphanilic acid in 20% glacial acetic acid) and allowed to stand for 5 min, followed by mixing of 1 ml 0.1% naphthyl ethylene diamine dihydrochloride. After 30 min absorbance was recorded at 540 nm (Gouthamchandra et al. 2010).

Total antioxidant capacity

The phosphomolybdenum method was used to measure the total antioxidant capacity of the extract. 0.1 ml of standard/extract solution was mixed with 0.3 ml of reagent (4 mM ammonium molybdate, 0.6 M sulfuric acid with 28 mM sodium phosphate) and kept for 90 min at 95 °C. The mixture was allowed to attain room temperature, then absorbance was measured at 695 nm. Ascorbic acid was used to obtain the calibration curve, and represented as ascorbic acid equivalents.

Cell culture

CHO cells were purchased from National Centre for Cell Sciences (Pune), India. The cells were cultured in T-25 culture flask (Falcon, Becton Dickinson, USA), containing DMEM supplied with 10% FBS, 1% penicillin–streptomycin, at 37 °C in a CO₂ incubator (NuAire, Plymouth, MN, USA) with 5% CO₂ in 95% air. For different assays, cells were maintained at 80–85% confluency in culture flasks and used. Trypan blue dye exclusion method was used to check the viability of cells.

MTT assay

Cytotoxicity of the arsenate, was evaluated using the MTT assay. 80–90% confluent cells were trypsinized and seeded at a density of 1×10^4 cells per well and treated with sodium arsenate, *Ixora coccinea* extract alone and arsenate in combination with *Ixora coccinea* extract to evaluate the cytotoxicity. 0.1 ml of DMSO was added at the end to dissolve formazan crystals, and absorbance was recorded at 550 nm (Mosmann et al. 1983).

Determination of reactive oxygen species

To measure the intracellular ROS production, cells (seeded at a density of 3×10^5) were treated with arsenate, *Ixora coccinea* extract and combination of extract as well as arsenate with fresh medium and the cultures were incubated for 1 h (Bai and Cederbaum 2003). After the incubation, the medium was removed and supplemented with 5 μM DCFDA for 30 min at 37 °C in 5% CO₂ incubator. Cells were trypsinized and suspended in PBS, analyzed using flow cytometer (Becton Dickinson, USA).

Estimation of mitochondrial membrane potential ($\Delta\Psi_m$)

To measure the mitochondrial membrane potential cells were seeded at a density of 3×10^5 in 6 cm² Petri dishes. Cells were incubated for 24 h with arsenate, *Ixora coccinea*

flower extract and in a combination of arsenate with extract, followed by the addition of Rhodamine 123 (5 μg/ml) and incubated at 37 °C for 30 min in 5% CO₂. Cells were trypsinized, collected, washed twice with PBS and centrifuged at 1000 rpm for 10 min (Scaduto and Grotyohann 1999). Cells were finally suspended in 1 ml of cold PBS and samples were read on FACS Calibur (Becton Dickinson, USA).

Single cell gel electrophoresis (SCGE)

Single strand DNA breaks were quantified by comet assay in an alkaline medium. 7×10^5 cells were seeded in 6 cm² petri plates. Cells were exposed to arsenate, *Ixora coccinea* and in combination kept for 24 h, later cells were trypsinized and collected in PBS. Agarose covered slides were layered with suspended cells again recoated with agarose. The slides were dipped in the lysing solution maintained at 4 °C overnight, lysed cells were subjected to electrophoresis for 28 min (300 mA, 20V). The slides were washed with neutralizing buffer. Ethidium bromide stained slides were immediately observed under the fluorescence microscope and photographed with coomat, analyzed with software (Singh et al. 1988).

AO/EtBr dual staining

3×10^5 cells were seeded per well in a 6 well plate overnight and kept in an incubator at 37 °C, in 5% CO₂, then treated with arsenate, *Ixora coccinea* and in combination. Later 300 μl of AO/EtBr (30/20 μg/ml) was added to each well then the plate was kept at 37 °C for 30 min. Finally washed with PBS and visualized under the fluorescence microscope for nuclear fragmentation and condensation (Renvoize et al. 1998).

Catalase activity estimation

3×10^5 cells were seeded per well in a 6 well plate overnight kept in an incubator at 37 °C, in 5% CO₂, then treated with arsenate, *Ixora coccinea* and in combination kept for 24 h, later cells were trypsinized and collected in PBS. The catalase assay was performed as described by (Aebi 1974). The phosphate buffer (prepared by mixing 50 mM KH₂PO₄ and 50 mM Na₂HPO₄ in a 1:1.5 v/v ratio) having pH 7.0 was mixed with 30 mM H₂O₂ to get an absorbance value of around 0.5 using a spectrophotometer. This reading was taken as the blank. If the value was higher than 0.5 then the amount of buffer in the reaction mixture was increased and if it showed a lower value than the H₂O₂ content was increased. The volume of the buffer and H₂O₂ mixture was kept constant at 0.9 ml throughout the experiment. 50 μl of sample was added to the cuvette containing the reaction

mixture and the decrease in absorbance per unit time was measured for each of the samples. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance (A_{240}) per unit time is a measure of catalytic activity.

Estimation of SOD activity

3×10^5 cells were seeded per well in a 6 well plate overnight kept in an incubator at $37^\circ C$, in 5% CO_2 , then treated with arsenate, *Ixora coccinea* and in a combination of arsenate with extract kept for 24 h, later cells were trypsinized and collected in PBS. 1850 μ l of sodium carbonate buffer was mixed with 50 μ l of the sample. Further 100 μ l of epinephrine was added to the reaction mixture and the increase in the absorbance which is proportional to the rate of autoxidation of epinephrine to adrenochrome was read immediately at 480 nm using a spectrophotometer.

Results and discussion

The role of natural products and folk medicine in the amelioration of the toxic effects of arsenic is scanty (Bhattacharya 2017). *Ixora coccinea* extract was prepared and analyzed for phytochemical constituents and its potential to ameliorate arsenic-induced cytotoxicity in CHO cells. The phytochemical evaluation of the aqueous extract showed antioxidant activities providing the mechanistic answer to the observed amelioration of arsenic-induced toxicity in cells.

Phenols are known as reducing agents, free radical scavengers and metal chelators. They are an important class of antioxidants because of their chemical structure. There are reports of flavonoids being scavengers of free radicals and has antioxidant properties. Phenol compounds and flavonoids were detected in *Ixora coccinea* flower aqueous extract.

Reactive nitrogen species have a role to play in metal induced toxicity. In spite of the defense system existing in cells, an imbalance in redox status leads to oxidative stress and DNA damage. *Ixora coccinea* aqueous extract possesses the nitric oxide scavenging capacity indicating its quenching ability of free radical. ABTS scavenging indicates that the presence of phenol-related compounds is responsible for ABTS free radical scavenging activity. The total antioxidant activity of aqueous flower extract was measured using the phosphomolybdenum complex.

CHO cells were exposed to different concentrations of sodium arsenate for 24 h to obtain the inhibitory concentration (IC_{50}) i.e., 48 μ M. Various concentrations of *Ixora coccinea* flower aqueous extract did not have any adverse effect on cell viability when treated for 24 h. Increase in cell viability was observed when the cells were pretreated with

Ixora coccinea extract for about 2 h before the exposure to sodium arsenate (IC_{50}). Maximum survival was observed in 125 μ g extract and was significant ($p < 0.01$) when compared to other concentrations of the extract (Fig. 1).

ROS concentration was measured using DCFHDA (2', 7'-dichlorofluorescein diacetate). High concentration of ROS induces lipid peroxidation and oxidative stress. However, the pretreatment of cells with *Ixora coccinea* extract significantly reduced the concentration of ROS in CHO cells in the presence of sodium arsenate (Fig. 2). Transmembrane potential dissipation increases with the increase in mitochondrial damage. CHO cells treated with sodium arsenate showed alteration in membrane potential. Pretreatment of CHO cells with *Ixora coccinea* extract restored the membrane potential and change was significant ($p < 0.01$) compared to sodium arsenate treated cells (Fig. 3).

Comet assay (alkaline) was used to measure the DNA damage induced by sodium arsenate. Sodium arsenate induced single strand breaks in CHO cells when exposed to its inhibitory concentration. Pretreating of CHO cells with *Ixora coccinea* flower extract before treatment of sodium arsenate showed significant ($p < 0.01$) decrease in DNA damage (Fig. 4). Apoptotic execution is followed by chromatin condensation and morphological changes in cells. Apoptotic index was calculated counting random 100 cells (Fig. 5). Sodium arsenate induced apoptosis was reduced significantly ($p < 0.01$) after the CHO cells were pretreated with *Ixora coccinea* extract.

Superoxide dismutase is thought to be present in all aerobic cells wherein it has a crucial function of protecting living organisms against unrestrained reactivity of the superoxide

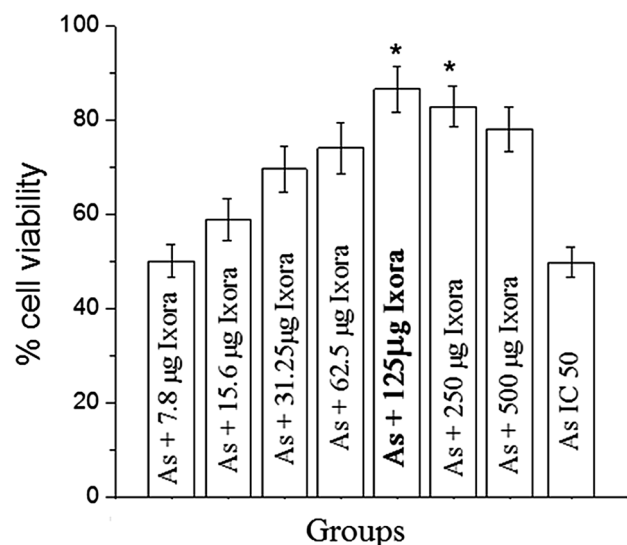


Fig. 1 Alterations in cell viability of CHO cells treated with different concentrations of *Ixora coccinea* flower aqueous extract and Arsenate (iAs) using MTT assay

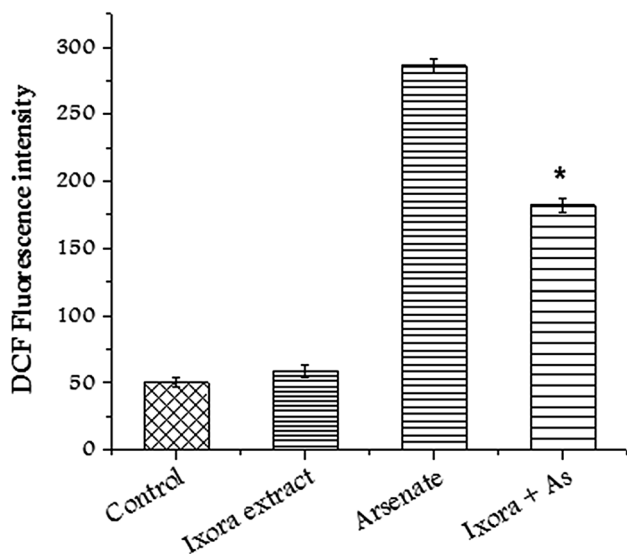


Fig. 2 Alterations in intracellular ROS after treating CHO cells with *Ixora coccinea* extract (125 µg/mL), Arsenate (IC₅₀) and in combination. Significant (*p* < 0.01) alteration observed in treated group with respect to arsenate

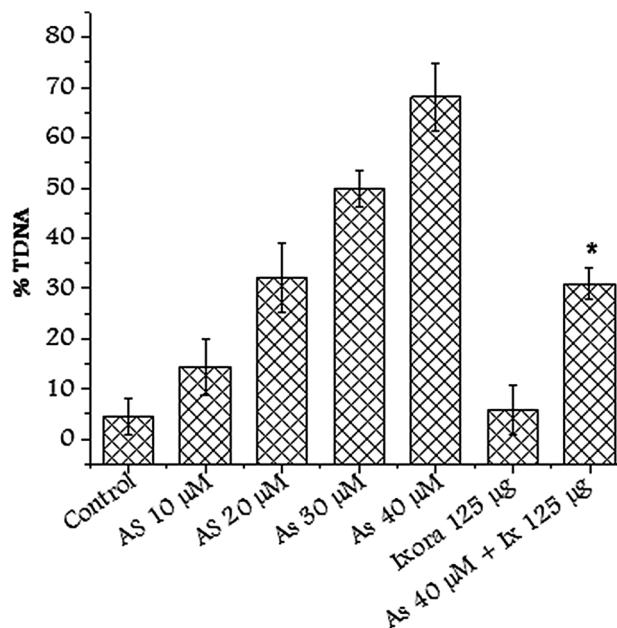


Fig. 4 Altered DNA damage after treating CHO cells with *Ixora coccinea* extract (125 µg/mL), arsenate (IC₅₀) and in combination. Significant (*p* < 0.01) alteration observed in treated group with respect to arsenate

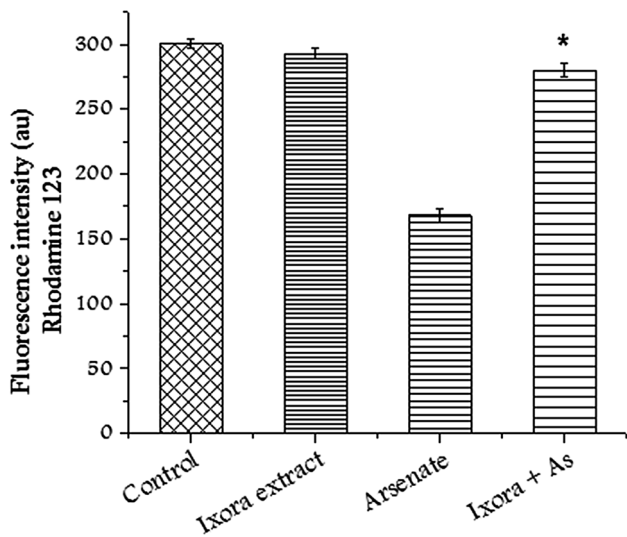


Fig. 3 Alteration in mitochondrial membrane potential after treating CHO cells with *Ixora coccinea* extract (125 µg/mL), arsenate and in combination. Significant (*p* < 0.01) alteration observed in treated group with respect to arsenate

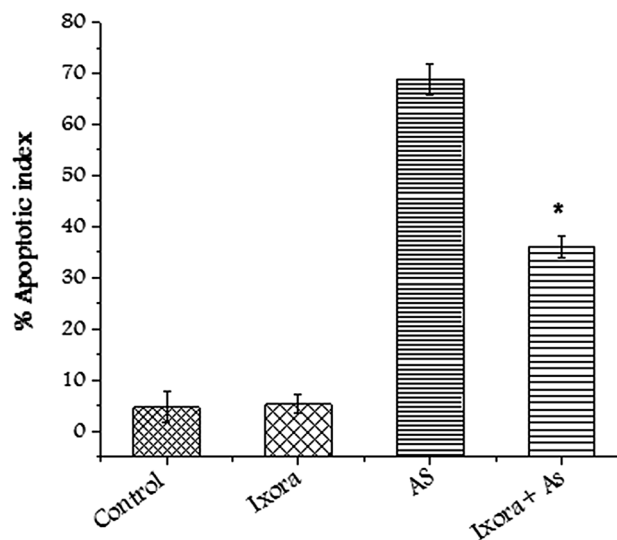


Fig. 5 Graph showing altered apoptotic index after treating CHO cells with *Ixora coccinea* extract (125 µg/mL), arsenate (IC₅₀) and in combination. Significant (*p* < 0.01) alteration observed in treated group with respect to arsenate

radical (O₂⁻) which is generated as a result of the univalent reduction of oxygen either chemically or enzymatically. SOD enzyme level was restored in the group pretreated with *Ixora coccinea* extract (Fig. 6). This could be attributed to free radical scavenging activity of the extract (Table 1).

Catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian aerobic cells containing a cytochrome system. Catalase enzyme level was restored in

the group pretreated with *Ixora coccinea* extract (Fig. 7). The antioxidant behavior of the extract helped in restoring the enzyme activity.

Dietary supplementation or intervention of naturally occurring nutrients is useful in preventing arsenic-induced toxicological effects and oxidative damage to the cells. A

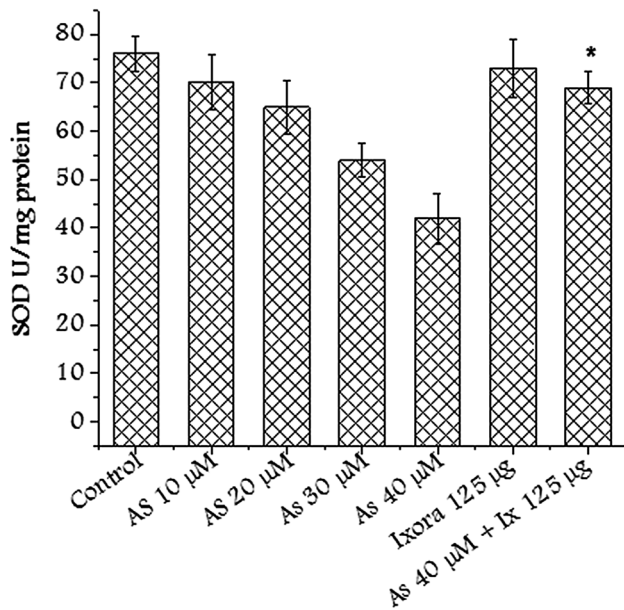


Fig. 6 SOD activity after treating CHO cells with *Ixora coccinea* extract (125 µg/mL), arsenate (various concentrations) and in combination. Significant ($p < 0.01$) alteration observed in treated group with respect to arsenate

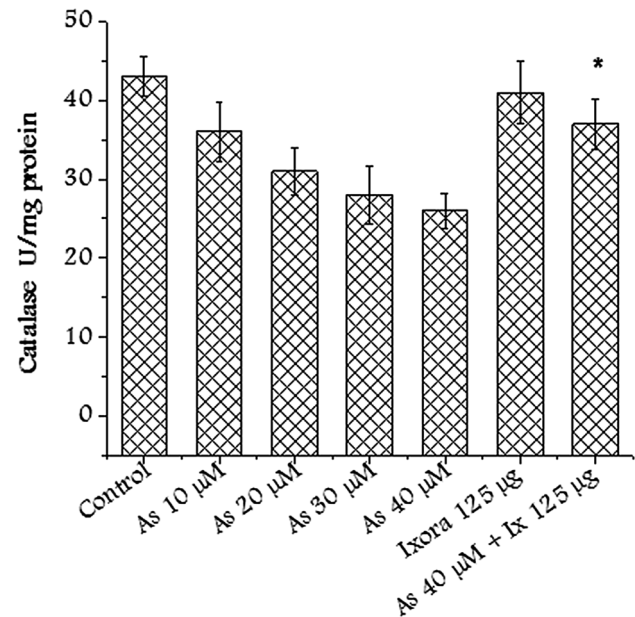


Fig. 7 Catalase activity after treating CHO cells with *Ixora coccinea* extract (125 µg/mL), arsenate (various concentrations) and in combination. Significant ($p < 0.01$) alteration observed in treated group with respect to arsenate

Table 1 Free radical scavenging and antioxidant capacity of the *Ixora coccinea* aqueous extract

Total phenols (mg GAE/g of flower extract)	42.21 ± 0.24
Total flavonoids (mg QE/g of flower extract)	2.4 ± 0.004
DPPH radical scavenging activity	98.42 ± 3.2
ABTS radical scavenging activity	81.10 ± 1.8
Nitric oxide scavenging assay	118.26 ± 8.2
Total antioxidant capacity	480 ± 1.34

number of edible plants has been listed to reverse the effect of arsenic-induced toxicity (Baliga and Kurian 2012). *Ixora coccinea* flower extract possesses ameliorative properties because of the presence of phenolics, flavonoids and rutin like known antioxidants. *Ixora coccinea* is used in traditional medicine and there are limited studies concerning metal toxicity or metalloids toxicity.

Conclusion

The present study is focused on amelioration of arsenic-induced cytotoxicity and genotoxicity by *Ixora coccinea* a known functional food. The protective effect of *Ixora coccinea* could be ascribed to its antioxidant properties of flavonoid and phenolic components of the extract. Aqueous extract of *Ixora coccinea* flower attenuated arsenic-induced oxidative stress, cytotoxicity and genotoxicity in CHO cells. Pretreatment of the extract also restored the activity of

antioxidant enzymes like SOD and catalase to near normal levels reducing the oxidative stress. The experimental results indicate the protective role of *Ixora coccinea* against arsenic (iAs) induced toxicological effects. The current study demonstrated the cytoprotective potential of the extract, which may be attributed to quenching of the ROS generated in the CHO cells due to oxidative stress induced by sodium arsenate. *Ixora coccinea* flowers can be used as functional food in the affected regions to reduce the harmful effects of the arsenic due to the consumption of arsenic contaminated drinking water.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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