Evaluation of Toxicity and Antioxidant Property of *Cassia fistula* Stem Bark Extracts in Zebrafish

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Abstract

Objective This study was aimed at evaluating the toxicity and the antioxidant property of *Cassia fistula* stem bark extracts in zebrafish.

Materials and Methods Crude aqueous and ethanol extracts of *C. fistula* stem bark were obtained following a standard solvent-based extraction method. The toxicity of these extracts on zebrafish embryonic development was determined and the LC_{50} values were calculated. Finally, the antioxidant property of *C. fistula* stem bark extracts was determined in arsenic-induced oxidative stress models of zebrafish.

Results The aqueous extract of *C. fistula* stem bark showed a slightly larger LC_{50} value (213.6 ppm) compared with the ethanol extract (LC_{50} = 63.5 ppm), suggesting a lower toxicity of the aqueous extract. A significant reduction of reactive oxygen species (ROS) signal was observed in arsenic-exposed embryos treated with the aqueous extract, but not the ethanol extract, indicating that the antioxidant activity is present only in the aqueous extract of *C. fistula* stem bark.

Conclusion Identification of antioxidants from natural sources is desirable because of increasing safety concerns associated with synthetic antioxidants. This study demonstrated that aqueous extract from *C. fistula* stem bark possesses antioxidant properties, which can be further characterized for mechanism of action and potential applications.

Keywords
- *Cassia fistula* stem bark
- embryonic toxicity
- ROS
- zebrafish
- natural antioxidant

Introduction

Cassia fistula is a fast-growing small tree found throughout the tropical countries of the world and is commonly called as Indian Laburnum or pudding pipe tree. In traditional Indian medicine system, almost all the parts of the plants are used for the treatment of various ailments.[1] Previous studies have analyzed several parts of the plant for their medicinal attributes including wound healing properties of leaves,[2,3] antitumor property of seed,[4] antimalarial, anti-inflammatory and antioxidant activities of bark and flower.[5-7] The most abundant active compound of the plant is flavonoids which are directly associated with its antioxidant activities.[8,9]

Oxidative stress, which is an imbalance between the production of free radicals and antioxidants in the body, is an important contributor to the pathophysiology of a variety of diseases, including cardiovascular dysfunctions, cancer, diabetes, atherosclerosis, inflammatory diseases, and neurodegenerative diseases.[10,11] In living cells, multiple mechanisms, especially enzymatic and nonenzymatic antioxidant systems, function to protect cells and their constituents against reactive oxygen species (ROS)-induced damage.[12] Therefore ROS activity is an important measure of oxidative stress and the search for effective antioxidants has been an ongoing effort. In the case of severe and continuous oxidative stress, our antioxidant defense system alone cannot overcome the stress and hence, exogenous antioxidants are required. Side effects of many synthetic antioxidants in use are well documented. Hence there is an increasing interest in screening for compounds with antioxidants properties from natural sources.

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Assessment of toxicity is very important for any natural or synthetic compound before its consideration for potential applications. Although cell line studies provide valuable information about the potential side effects of a compound, the results may not be relevant in a multicellular animal model where the pharmacokinetics of a compound are influenced by several other factors. Thus, for a compound to be considered safe, the toxicity studies should be done in a physiological context.[13]

Zebrafish (*Danio rerio*) is a well-established model system for toxicological studies. The model has been used to evaluate the in vivo effect of chemicals on redox homeostasis and oxidative stress.[14-16] The production of ROS, a hallmark of oxidative stress, can be measured by 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), a cell permeant, which gets converted into a fluorescent form due to the release of esterase from acetate groups during oxidation.[17,18]

In this study, we have obtained two types of extracts from *C. fistula* bark and investigated their toxicity in zebrafish. Further, we evaluated the antioxidant property of these extracts in arsenic-induced oxidative stress models of zebrafish. Here, using a live animal model system where the production of ROS was evaluated in real time, we show that *C. fistula* stem bark possesses high antioxidant property. Further characterization of the aqueous crude extract could lead to the identification of active ingredients and develop it for potential applications.

Materials and Methods

Sample Collection and Extraction

C. *fistula* tree barks were collected from places in and around Mangalore. About 1 kg of the tree bark was taken and washed thoroughly using distilled water. The tree bark was then broken into smaller bits and dried overnight in a hot air oven at 40°C. The dried bits were powdered and then stored in an airtight container for further use. The powder was then subjected to solvent extraction using standard method.[19] Briefly, 10 g of bark powder was mixed in 100 mL of the solvent (ethanol and water) individually and was kept in a rotary shaker at 110 rpm and 37°C overnight. Post homogenization, the contents were filtered using a clean muslin cloth and air-dried in glass petri dishes overnight. The residue was again dissolved in 50 mL of the extraction solvent and kept in a rotary shaker maintained at 37°C for another night. The filtrates were combined, collected in petri dishes, and were subjected to drying in a hot air oven at 45°C. After drying, the residue was collected by scraping out from the petri dishes and it was stored at 4°C in airtight containers after finely powdering it.

Stock solution of 2,500 ppm was prepared using ethanol and distilled water, respectively and stored at 4°C. This stock solution was then used to prepare working solutions of various concentrations in E3 medium.

Zebrafish Husbandry and Rearing

Adult zebrafish were reared in an aquaculture system with the temperature maintained at around 28°C with a fixed 14/10 h day/night cycle. Embryo preparation was done by pairwise mating of the fishes in breeding chambers overnight. The fertilized embryos were collected the next morning and were transferred into petri dishes containing E3 medium and were maintained at 28°C in a cooling incubator.[20] Healthy embryos were treated with the desired compound at 10 hours post fertilization (hpf).

Determination of LC_{50}

A semi-static methodology, where media change with renewal of test compound was done twice a day, was followed. Kärber's method[21] was employed to calculate the LC_{50} values of the extracts. The concentration at which all the embryos were dead (LC_{100}) was considered as the upper limit and the concentration at which all the embryos survived (LC_0) was considered as the lower limit. The experiments were performed in microtiter plates using ten embryos (10 hpf) in each well and were monitored for 5 days. All the experiments were performed in triplicate using appropriate controls (distilled water and ethanol for aqueous and ethanol extract, respectively) and the resultant images were captured using a stereomicroscope (Leica S9D, Germany).

Evaluation of Developmental Toxicity of Aqueous and Ethanol Extracts

The evaluation of developmental toxicity for both aqueous and ethanol extracts was performed by observation and recording of lethal and sublethal end points at each day post fertilization for a total of 5 days. Developmental abnormalities like survivability rate, cardiotoxicity, neurotoxicity, and hepatotoxicity were assessed in embryos exposed to both types of extracts. The experiments were performed in triplicates with 10 embryos in each concentration. Data from three replicates for each end point for each concentration were recorded using a stereomicroscope (Leica S9D, Germany) and were compared with the control (unexposed) embryos. The *p*-value was calculated using a *t*-test, and a value of <0.05 was considered statistically significant.

Assessment of Antioxidant Property of *C. fistula* Bark Extracts

To determine the antioxidant property of the aqueous and ethanol extracts, an oxidative stress model was first generated by exposing zebrafish embryos to 5, 10, and 15 ppm of sodium arsenite (Merk, Germany). The arsenic-exposed embryos were treated with three different concentrations of aqueous and ethanol extracts of *C. fistula* stem bark and the concentrations below LC_{50}, at which there were no effects on embryonic development, were chosen. The exposure to arsenic was done at 6 hpf and the extracts were added at 10 hpf. At 24 and 48 hpf, the treated embryos were carefully dechorionated, washed twice in 1× PBS (phosphate buffered saline), and treated with 5 µM H_2DCFDA (Sigma, United States). Post permeant addition, the embryos were again washed with 1× PBS and then mounted onto a cavity slide using methylcellulose and checked for fluorescence using a Green Fluorescent Protein filter in a fluorescence microscope (Leica DMC4500, Germany). Zebrafish embryos exposed only

to arsenic were used as positive controls. All the experiments were performed in triplicates.

Results

Effects of *C. fistula* Stem Bark Extracts on Zebrafish Development

The effects of aqueous and ethanol extracts on embryonic development were assessed using a set of previously identified lethal and sublethal morphological end points for a period of 5 days post exposure of the extracts. Based on the results obtained, the LC_{50} values for both type of extracts was calculated using the method developed by Kärber.[21] The LC_{50} value of aqueous extract was estimated to be 213.6 ppm, whereas, it was 63.5 ppm for ethanol extract. In case of aqueous extract, there was no mortality at concentration below 100 ppm (data not shown). Even at concentrations above 100 ppm, there was no significant difference in survivability till day 5 up to 210 ppm (►**Fig. 1A**). However, at 214 ppm onward, there was a corresponding decrease in survivability of embryos on day 5 with increasing concentrations of the extract. At 280 ppm, the survivability was 0% at day 5. The developmental defects associated with the bark extracts included incomplete development of the digestive system, pericardial edema, and incomplete yolk absorption (►**Fig. 1B**).

In contrast, the effects of ethanolic extract were very severe at concentrations above 100 ppm resulting in extensive mortality of the embryos. Even at 100 ppm, the survivability showed a steady decrease with each day and by day 5, 100% of treated embryos were dead. The concentrations between 10 and 20 ppm were safe for the embryos. However, at concentrations of 60 ppm onward, the survivability dropped to 40% and less (►**Fig. 2A**). Although, the developmental defects associated with ethanol extracts were similar to those seen with aqueous extract (►**Fig. 2B**), the toxicity

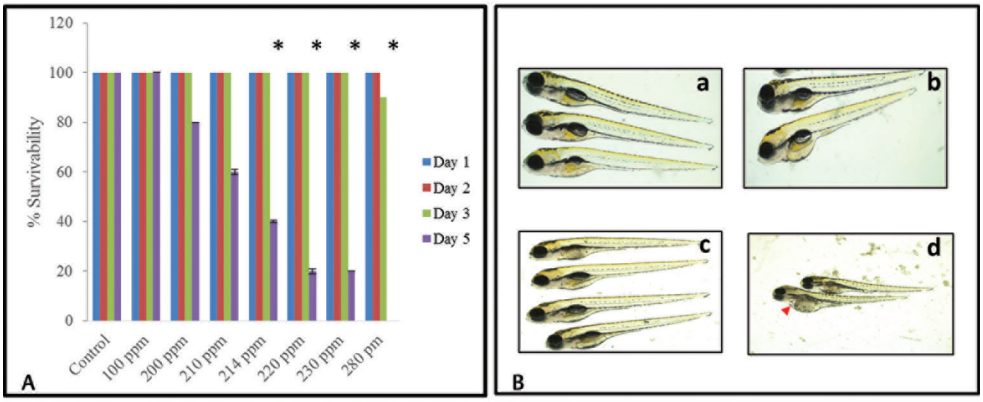


Fig. 1 Survival rate and developmental toxicity of zebrafish embryos treated with *Cassia fistula* stem bark aqueous extract. (**A**) Survival rates of zebrafish embryos treated with Cassia fistula stem bark extract at different days post fertilization. The embryos were exposed at 10-hours post fertilization (hpf) and the survival rates were recorded at 24, 48, 72 and 120 hpf. * indicates *p*-value <0.05. (**B**) Representative images of zebrafish embryos at 120 hpf exposed to different concentrations of aqueous extract: (**a**) control, (**b**) 100 ppm, (**c**) 210 ppm, and (**d**) 214 ppm. Morphological anomalies such as pericardial edema (arrowhead), incomplete yolk absorption (roundish yolk), and defective swim bladder were evident in treated embryos.

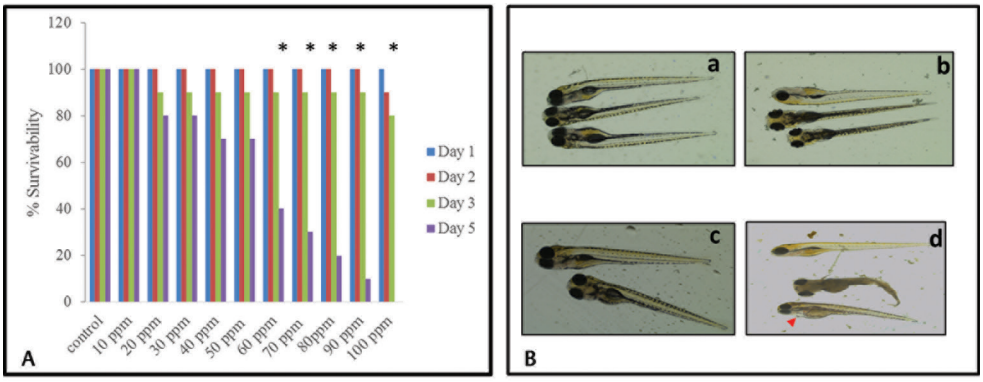


Fig. 2 Survival rate and developmental toxicity of zebrafish embryos treated with *Cassia fistula* stem bark ethanol extract. (**A**) Survival rates of zebrafish embryos treated with *Cassia fistula* stem bark extract at different days post fertilization. The embryos were exposed at 10 hours post fertilization (hpf) and the survival rates were recorded at 24, 48, 72, and 120 hpf. * indicates *p*-value <0.05. (**B**) Representative images of zebrafish embryos at 120 hpf exposed to different concentrations of ethanol extract: (**a**) control, (**b**) 20 ppm, (**c**) 50 ppm, (**d**) 80 ppm. Morphological anomalies such as pericardial edema (*arrowhead*), incomplete yolk absorption (*roundish yolk*), and defective swim bladder were evident in treated embryos.

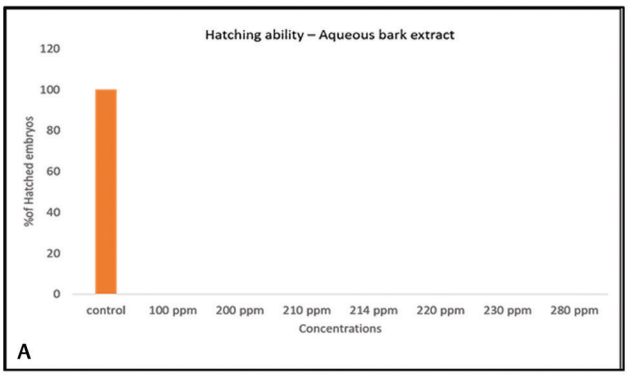
of the ethanol extract was higher than that of the aqueous extract, which was also evident from the larger LC_{50} value (213.6 ppm) observed for aqueous extract as compared with the ethanol extract (63.5 ppm).

Effects of *C. fistula* Stem Bark Extracts on Hatching of the Embryos

Zebrafish embryos come out of the chorion (hatching) on their own without any external force by 48 to 72 hpf. The ability of the embryos to hatch out on their own is considered an important milestone in the development and interference with hatchability is an indication of defects in later stages of development. The hatchability (percentage of embryos that have completed hatching by the third day) was monitored for both aqueous and ethanol extract treated embryos. As shown in ►**Fig. 3A** and **B**, the effect of the aqueous extract on hatching ability was very severe even at 100 ppm where none of the embryos could hatch out on their own as compared with the untreated controls. In contrast, for ethanol extract there was no significant difference in hatching ability up to 30 ppm. However, at 40 ppm onward, the hatching ability was severely disrupted and the percentage of the embryos that hatched was 0%.

Evaluation of Antioxidant Property of *C. fistula* Stem Bark Extracts

The antioxidant property of the bark extracts was evaluated using arsenic-induced oxidative stress models of zebrafish. Arsenic exposure induces the generation of intracellular ROS, which leads to oxidative stress. Amelioration of arsenic-induced ROS would indicate potential antioxidant property of the test compound. The ability of the bark extracts to bring about an antioxidant effect was estimated by checking the reduction in the ROS signal induced by arsenic. The generation of ROS was detected by addition of H_2DCFDA, a cell permeable dye, which undergoes a conversion from a nonfluorescent to a fluorescent form in presence of oxidative stress. As shown in ►**Fig. 4**, a significant reduction in ROS signal was observed in arsenic-exposed embryos treated with the aqueous extract of *C. fistula* stem bark (100–210 ppm). In contrast, the treatment with the ethanol extract (30–50 ppm) did not result in significant reduction in ROS signal, suggesting the lack of antioxidant property in the ethanol extract of *C. fistula* stem bark (►**Fig. 5**).



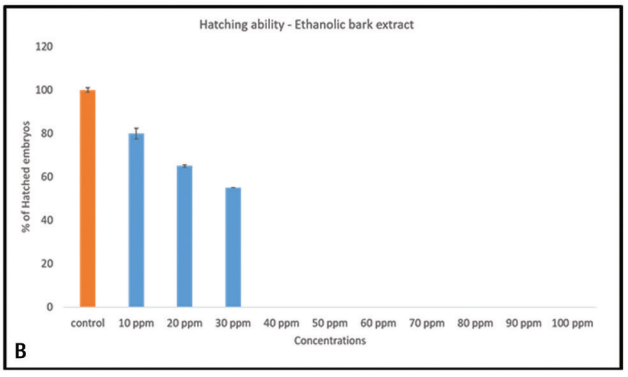


Fig. 3 Hatching rate of zebrafish embryos treated with *Cassia fistula* stem bark aqueous and ethanol extract. (**A**) Graphical representation of percentage of hatched zebrafish embryos treated with various concentrations (100–280 ppm) *Cassia fistula* stem bark aqueous extract. The embryos were exposed at 10 hours post fertilization (hpf) and the hatching ability was recorded at 72 hpf. (**B**) Graphical representation of percentage of hatched zebrafish embryos treated with various concentrations (10–100 ppm) of *Cassia fistula* stem bark ethanol extract. The embryos were exposed at 10 hours post-fertilization (hpf) and the hatching ability was recorded at 72 hpf.

Discussion

Over the years, there has been an increase in safety concerns associated with synthetic compounds among the consumers. This has shifted the focus toward a search for natural alternatives. The generation of ROS and the resulting oxidative stress are linked to the clinical manifestation in several diseases.[22,23] Thus, natural antioxidants which are rich in polyphenols and carotenoids, are being promoted for applications as additives or supplements. Although these phytochemicals are presumed to be safe,[24] scientific studies on the toxicity and side effects of these alternatives are limited.

Originally described as a model organism for studying embryogenesis almost 3 decades ago, the zebrafish system has now become a powerful in vivo platform with applications in diverse areas of biological research. The advantage of zebrafish over other animal models lies in its several inherent qualities such as high fecundity, short generation time, embryonic transparency, *ex vivo* development, and low set up cost. These attributes together with the availability of complete genome information, the ease of large-scale genetic screens, and existence of several gene manipulation techniques make zebrafish ideally suited to biomedical research. This system is also a suitable vertebrate animal model to study oxidative stress dynamics.[25]

C. *fistula* bark is known to possess significant antioxidant potential compared with other vegetative parts of the plant.[9,26] In this study, we have evaluated the toxicity and teratogenic effects of *C. fistula* bark extracts in zebrafish embryos. The results revealed that the aqueous extract of *C. fistula* bark is less toxic compared with the ethanol extract and no mortality or development defects were observed for concentrations below 100 ppm. In contrast, the ethanol extract was more toxic and developmental anomalies were evident from 60 ppm onward. The LC_{50} values for both the extracts were

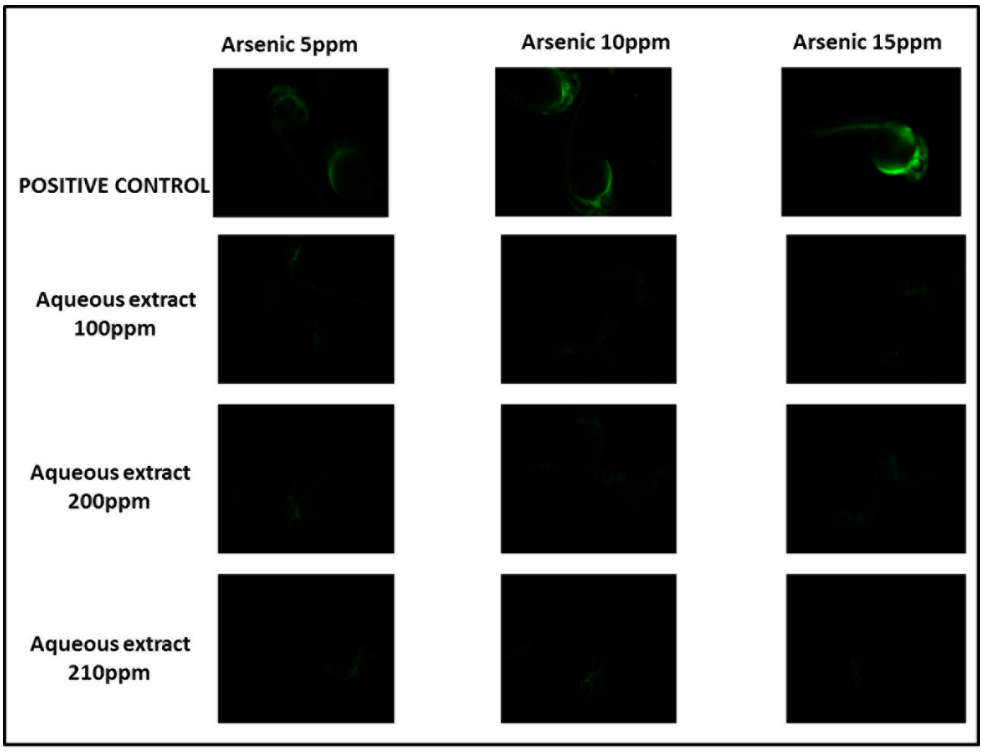


Fig. 4 Detection of ROS in zebrafish embryos treated with Cassia fistula bark aqueous extract. Representative images of H_2DCFDA staining in arsenic-exposed zebrafish embryos treated with different concentrations of aqueous extract and observed at 24 hpf. H_2DCFDA, 2', 7'-dichlorodihydrofluorescein diacetate; hpf, hours post fertilization; ROS, reactive oxygen species.

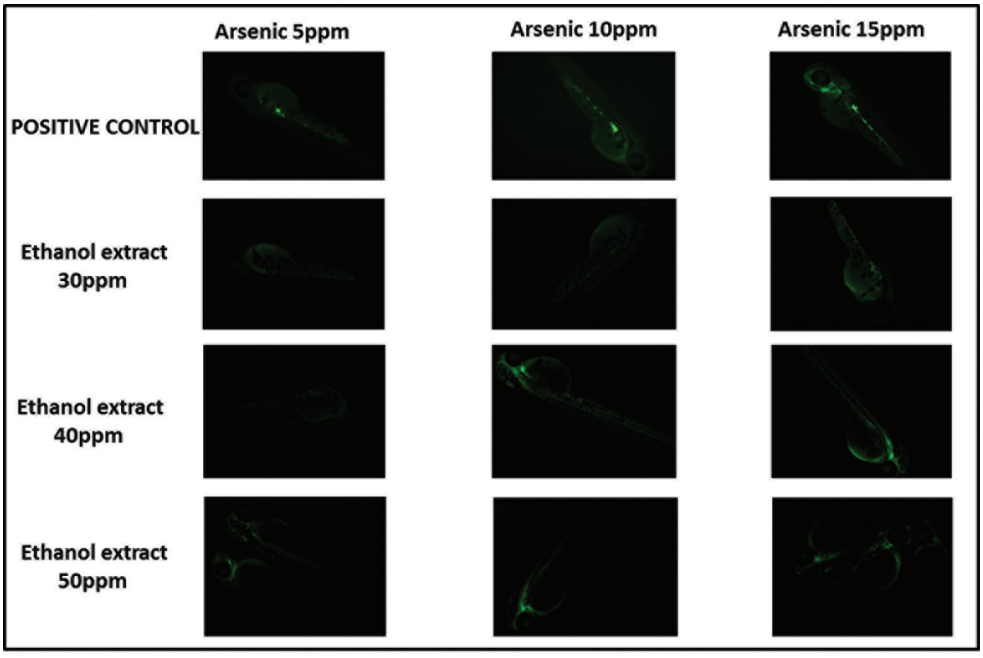


Fig. 5 Detection of ROS in zebrafish embryos treated with Cassia fistula bark ethanol extract. Representative images of H_2DCFDA staining in arsenic-exposed zebrafish embryos treated with different concentrations of ethanol extract and observed at 48 hpf. H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; hpf, hours post fertilization; ROS, reactive oxygen species.

also quite different with aqueous extract showing a larger value compared with the ethanol extract. Previous studies have shown that the LD_{50} of *C. fistula* fruit extract tested on mice and rat was estimated to be greater than 5,000 mg/kg and are considered quite safe.[6,27,28]

Intriguingly, the effects of the extracts on the hatching ability of the embryos were quite severe. The zebrafish embryos are protected by chorion till day 2 or 3 post fertilization. The chorion is a thin envelope covering embryos that is permeable. The hatching (coming out of the chorion

naturally) process is usually complete by day 3 post fertilization. In case of aqueous extracts, the effects were seen in all the concentrations tested (100 ppm onward) whereas in case of ethanol extract, it was severe from 40 ppm onward. Although highly speculative, it can be concluded that the addition of these extracts renders the chorion thicker, which results in the inability of the embryos to hatch by themselves. Future studies can be focused on understanding the exact underlying mechanism.

To determine the antioxidant property of the bark extracts, a live animal system was used where the generation of ROS and its reduction in presence of the test compound could be traced at real time. Generation of ROS such as superoxide and hydrogen peroxide is common during arsenic exposure. Here, the strategy was to first induce ROS in zebrafish through arsenic and then expose these embryos to crude extracts. Reduction in ROS signal in embryos treated with arsenic and test compounds compared with only arsenic treated embryos would suggest a potential antioxidant property of the test compound. The results indicated that the aqueous extract of *C. fistula* (200–210 ppm) possessed potent antioxidant activity as evident from the dramatic reduction in the ROS signal in double-exposed zebrafish embryos. Our data corroborate with previous studies where aqueous extracts of *C. fistula* flowers, barks, and seeds were shown to have significant antioxidant, anti-inflammatory, hepatoprotective, and anti-infertility properties.[7,29,30] However, there was no significant change in the ROS signal intensity when treated with ethanol extract, suggesting an absence of antioxidant property. Our results corroborate with a previous study where dose-dependent protective effects of *C. fistula* extracts from different parts of the plant were reported.[25] However, the antioxidant activities of *C. fistula* bark extracts also depend on the different age classes of the tree.[31] Although the data presented here are preliminary, they nevertheless, demonstrate that the crude aqueous extract of *C. fistula* possesses antioxidant property. In contrast, the ethanol extract, which was more toxic, did not show any antioxidant property. The study also highlights the utility of the zebrafish system as an effective animal model for high throughput screening of natural compounds for their antioxidant properties. In-depth characterization of the aqueous extract of *C. fistula* barks and identification of the active ingredients could pave the way for development of novel antioxidants with potential applications in food and pharmaceutical industries.

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Conflict of Interest

None declared.

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