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Comparison of *in vitro* and *in vivo* acute toxicity assays in *Etroplus suratensis* (Bloch, 1790) and its three cell lines in relation to tannery effluent

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ABSTRACT

Cell lines of *Etroplus suratensis* established in our laboratory were evaluated for their potential use as screening tools for the ecotoxicological assessment of tannery effluent. The cytotoxic effect of tannery effluent in three cell lines derived from eye, kidney and gill tissue of *E. suratensis* was assessed using multiple endpoints such as Neutral Red (NR) assay, Coomassie Blue (CB) protein assay and Alamar Blue (AB) assay. Acute toxicity tests on fish were conducted by exposing *E. suratensis* for 96 h to tannery effluent under static conditions. The toxic effect of tannery effluent on the survival of fish was found to be concentration and time dependent. The tannery effluent at the concentration of 15% caused 100% mortality at 96 h whereas the lower concentration (0.5%) caused 13.33% mortality. The cytotoxicity of tannery effluent was found to be similar in the three cell lines tested, independent of the toxic endpoints employed. EC_{50} values, the effective concentration of tannery effluent resulting in 50% inhibition of cytotoxicity parameters after 48 h exposure to tannery effluent were calculated for eye, kidney and gill cell lines using NR uptake, AB and cell protein assays. Statistical analysis revealed good correlation with $r^2 = 0.95–0.99$ for all combinations between endpoints employed. Linear correlations between each *in vitro* EC_{50} and the *in vivo* LC_{50} data, were highly significant $p < 0.001$ with $r^2 = 0.977$, 0.968 and 0.906 for AB_{50}, NR_{50}, and CB_{50}, respectively.

1. Introduction

Following the report of the first fish cell line in literature in 1962 (Wolf and Quimby, 1962), at least 157 fish cell lines have been established (Fryer and Lannan, 1994) in different laboratories worldwide for various applications including *in vitro* cytotoxicity tests for aquatic hazard assessment to replace *in vivo* bioassays with fish. *In vitro* tests using fish cell lines for ecotoxicological assessment are preferred for various reasons such as cost-effectiveness, speed early reproduction and easy adaption to automated high-throughput screening technologies (Fent, 2001; Castano et al., 2003; Castano and Gomez-Lechon, 2005; Tan et al., 2008). In addition, the *in vitro* tests would also satisfy a societal desire to avoid the use of whole animals in toxicological tests. Efforts have been made over the past three decades to find out whether *in vitro* cytotoxicity assays can be used for aquatic hazard assessment as an alternative to *in vivo* tests (Isomaa et al., 1994; Segner, 1998; Castano et al., 2003; Gulden and Seibert, 2005; Knauer et al., 2007; Na et al., 2009). Rachlin and Perlmutter (1968) were the first to make an attempt to use a fish cell line to study aquatic toxicants and examine the possibility of using the muscle cells of fathead minnow to assess the toxicity of Zn^{2+}. Various *in vitro* systems using different cell lines derived from marine and freshwater fish have been used to test a variety of chemicals and industrial wastes worldwide (Babich and Borenfreund, 1987; Matlova et al., 1995; Repetto et al., 2001; Segner, 2004; Gulden et al., 2005; Knauer et al., 2007; Tan et al., 2008; Na et al., 2009). The study conducted by Ekwall (1995) forms the principal basis for relating *in vitro* assay to *in vivo* acute toxicity tests. Following this work, research has been carried out to explore the potential use of fish cell lines based on cytotoxicity tests as an essential alternative to whole animal experiments (Castano and Tarazona, 1995; Fent, 2001; Repetto et al., 2001; Segner, 2004; Tan et al., 2008; Na et al., 2009). In future, fish cell lines will be utilized as a biological model for evaluating the cytotoxicity of pollutant chemicals in environmental samples and will also become a standard practice in toxicological study.

The *in vitro* tests using fish cell lines provide data on cytotoxicity, both basal and selective, genotoxicity and effects on cell-specific functions and parameters (Castano et al., 2003; Bols et al., 2005; Knauer et al., 2007). The basal toxicity in fish cell line has been determined by membrane integrity using Neutral Red test (Borenfreund and Puerner, 1985), energy metabolism by tetrazolium salt reduction (MTT) assay (Mossman, 1983), cell proliferation by bromodeoxyuridine (BrdU) assay (Gratzner, 1982) and protein content by crystal violet assay (Saad et al., 1993).

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Tanning industry contributes significantly towards exports, employment generation and occupies an important role in the Indian economy while on the other hand, tannery wastes are ranked as the highest pollutants among all the industrial wastes (Soyaslan et al., 2007). Damage to the environment by the hazardous tannery effluent is becoming an acute problem in India.

E. suratensis is an important brackish water fish distributed along the coastal regions of Tamil Nadu, Kerala, Pondicherry and Orissa. It is an excellent food fish with a good market demand in Kerala. *In vivo* toxicity studies using this species have been carried out for evaluating the toxicity of various chemicals (Das and John, 1999; Mercy et al., 2000; Nair et al., 2000; Anikuttan, 2004; Sobhana et al., 2006). Recently, Sarath Babu et al. (2011) have developed cell lines from tissues of eye, gill and kidney of *E. suratensis*. The main purpose of this study was to evaluate the potential of these cell lines for use as Tier I screening tools in the ecotoxicological assessment of tannery effluent. The cytotoxic effect of tannery effluent was assessed using multiple endpoints, thus permitting detection of differential responses of the fish cells to contaminants. Neutral Red (NR) assay was employed to assess the lysosomal function of the cells, followed by total protein analysis using the Coomassie Blue (CB) cytotoxicity assay. In addition, Alamar Blue (AB) was used as an indicator of mitochondrial function. The results of *in vitro* studies were correlated with the results of *in vivo* lethality test using fish and recommend the use of these cell lines for toxicity assessment of industrial effluents instead of living fish.

2. Materials and methods

2.1. Collection of industrial effluent

The tannery effluents were collected from an identified tannery unit located at Ranipet, Vellore District, Tamil Nadu, India. Samples were collected and transported to the laboratory in sterile dark bottles within 3–6 h of collection in order to carryout *in vitro* and *in vivo* studies. The samples were stored at 4–6 °C until used. Tannery effluents were filtered through a 0.45 μm and 0.22 μm syringe filter for *in vivo* acute toxicity and *in vitro* cytotoxicity tests, respectively.

2.2. Physico-chemical characterization of effluent

The color, odor, temperature, pH, Dissolved oxygen (DO), Biochemical oxygen demand (BOD) and Chemical oxygen demand (COD) of the tannery effluent were determined using standard protocols. All chemical analyses were performed according to standard procedures described by APHA (1998). Osmolarity was measured using osmometer (Osmomat 030, Gonotec, Germany).

2.3. Fish

E. suratensis with body weight of 2–3 g were obtained from a fish farm of Central Institute of Brackishwater Aquaculture (CIBA) located near Chennai, India. There was no industry in that area. They were kept in a 500 L fiberglass tank and acclimatized in natural seawater adjusted to 5 ppt (23–28 °C) under an ambient photoperiod in the laboratory for 7 d prior to experiments. The fish were fed with commercial pellet feed twice a day and starved for 24 h before and during the experiments.

2.4. Fish cell lines

A total of three cell lines established from different organs (eye, gill and kidney) of *E. suratensis* were tested for their sensitivities to tannery effluent. These fish cells were propagated at 28 °C in Leibovitz's L-15 medium (pH 7.0–7.4) with 2 mM L-glutamine, 10% fetal bovine serum (FBS), penicillin 100 IU mL^{-1} and streptomycin 100 μg mL^{-1}. The Osmolarity ranged from 300 to 360 mO sm kg^{-1}. These cells were sub-cultured every 2–3 d using standard procedure. Cells at exponential growth phase were harvested and used for *in vitro* cytotoxicity tests.

2.4.1. In vivo fish acute toxicity test

Fish acute toxicity tests were conducted by exposing *E. suratensis* ($N = 10$ per aquarium) for 96 h to tannery effluent under static conditions (OECD 203, 1992). Six concentrations of tannery effluent (0.5%, 1.0%, 2.5%, 5.0%, 10.0% and 15.0%, v/v) diluted with seawater (5 ppt) and control with sea water alone were tested to determine the LC_{50} (concentration at which 50% of the fish population dies). The aquaria had a working volume of 30 L based on the body weight of fish (1 g L^{-1}). Dead fishes were counted and removed immediately every day. All the experiments were conducted in triplicates. Mortalities were recorded following the guideline for fish acute toxicity OECD 203 (1992).

2.4.2. In vitro cytotoxicity assay using fish-derived cell lines

Cells (eye, gill and kidney) of *E. suratensis* at exponential growth phase were harvested and diluted to a concentration of 10^5 cells mL^{-1} in Leibovitz's L-15 medium with 10% FBS. After agitation, the cells were added to each well of 96-well tissue culture plates at the concentration of 2×10^4 $well^{-1}$ and incubated overnight at 28 °C. After incubation, the medium was removed and the cells were re-fed with medium containing 0% (control), 0.5%, 1.0%, 2.5%, 5.0%, 10.0% and 15.0% (v/v) of tannery effluent for 48 h EC_{50} analysis. Then three endpoints for toxicity, i.e. Neutral Red (NR) uptake assay, Alamar Blue assay and protein concentration assay were determined after 48 h exposure.

2.4.2.1. Neutral Red uptake assay. NR uptake assay was carried out based on the procedure described by Borenfreund and Puerner (1985). This assay measures the inhibition of cell growth, which is based on the absorbance of the vital dye NR by living, but not by dead, cells. After 24 or 48 h exposure, the test medium in each well was replaced by 200 μL L-15 medium containing 50 μg mL^{-1} of NR and incubated in situ for 3 h at 28 °C. Then the cells were rinsed with warmed phosphate-buffered saline (PBS) to remove the NR dye and then destained with 200 μL solution containing glacial acetic acid, ethanol and water at the ratio of 1:50:49, respectively. After rapid agitation for 10 min at room temperature, the absorbance of the solution in each well was measured at 550 nm with a microplate reader (Multiscan EX Thermo Electron Corporation) and the EC_{50} value (concentration of test agent which causes a 50% inhibition in NR uptake) was determined.

2.4.2.2. Alamar Blue reduction assay. Alamar Blue assay was carried out to assess the metabolic activity using the water soluble reagent, Alamar Blue essentially as described by Dayeh et al. (2003) and by Trek Diagnostic Systems, Inc., Westlake, Ohio (reagent source). Alamar Blue was diluted with L-15 medium to 10% (v/v), filter sterilized and stored in the dark at 4 °C. After removing the test medium, 150 μL of this solution was added into each well and, 2 h later, the wells were read with a fluorometric multiwell plate reader (Multiscan EX Thermo Electron Corporation) at respective excitation and emission wavelengths of 530 (±30) and 595 (±35) nm.

2.4.2.3. Coomassie Blue dye protein assay. The cell protein assay using Coomassie Blue dye as described by Shopsis and Eng (1985) was carried out to investigate the cell growth by the alteration of the total cellular protein. After 48 h exposure, the test medium was removed, and the cells were washed with PBS and