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CYTOTOXIC EFFECT OF INK EXTRACTS OF CUTTLEFISH AND SQUID ON CHICK EMBRYO FIBROBLASTS

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ABSTRACT: The aim of the present study is to evaluate the cytotoxic effect of the ink extracts of different species of cuttlefish (*Sepia pharaonis* female, *Sepia pharaonis* male, *Sepiella inermis* female, *Sepia aculeata* female) and squid (*Loligo duvauceli* female). The ink extracts were prepared by delipidation with acetone and extracted by Tris HCl buffer. The cytotoxic effect of the drug was studied using Trypan blue cell viability assay and ethidium bromide / acridine orange staining. The ink extracts from different species of squid and cuttlefish inhibited cell proliferation and induced cell death in a dose dependent manner. The cytotoxic effects induced by cuttlefish ink extract on chick embryo fibroblast cells involve typical apoptotic changes like nuclear condensation, studied using ethidium bromide and acridine orange staining. The ink extracts from the female Cuttlefish, *Sepia pharaonis* (25 and 75 µg/ml) showed the highest antiproliferative activity. The results suggested the potent antiproliferative effect of the ink of cuttlefish, *Sepia pharaonis* on chicken embryo fibroblasts and thereby the therapeutic potential of the ink as an anticancer agent.

INTRODUCTION: Squid and cuttlefish come under class Cephalopoda (phylum- Mollusca) which form one of the major marine resources of India. They are exploited mainly for export. Visceral organs including the ink sac form the major waste material in squid and cuttlefish processing industry. Squid and cuttlefish eject ink from their ink sac to escape from their predators¹. The ejected ink functions as a warning signal or alarm substance that confuses predators and alerts conspecifics to the presence of danger².

Attempts are now-a-days being made to utilize these waste materials to produce bioactive substances such as antibacterial, anticancer and antiviral agents. Cephalopod ink has shown potential antiretroviral activity³. The peptidoglycan and acetone delipidated ink of squid (*Illex argentinus*) showed antitumour activity^{4,5}. The ink and tissue extracts of Cuttlefish and squid showed antibacterial effect^{6,7,8,9,10}.

Measurement of cell proliferation rates are often used to determine the response of cells to a particular stimulus or toxin. Because of the large size of the chick embryo, it is easier to dissect out individual organs to generate specific cell types such as hepatocytes, fibroblasts, cardiac muscle and lung epithelium¹¹. The present study was designed to investigate the cytotoxic effects of the cuttlefish and squid ink extracts using chick embryo fibroblasts.



MATERIALS AND METHODS:

Extraction of Cuttlefish and Squid ink: Three species of cuttlefish (*Sepia pharaonis* -female and male, *Sepiella inermis* -female, *Sepia aculeata* – female) and squid (*Loligo duvauceli* -female) were collected from the fishing harbour, Kochi, Kerala, India and brought to the laboratory in ice cold condition. The animals were carefully dissected to remove the ink sac. The ink from different species of cuttlefish and one species of squid were used for extraction. Known weight of the ink was delipidated with acetone at -20°C for 96 h.

The acetone was changed every 24 h after vacuum filtration. The defatted residue was vacuum dried to give a black powder. This powder was extracted with 40 volumes of 0.1 M Tris-HCl buffer (pH 6.8) at 4°C for 48 h, followed by centrifugation at 13,000 x g at 4°C for 30 min. The supernatant was dialysed against distilled water at 4°C for 48 h and then lyophilized to obtain an off-white powder. This powder was dissolved in normal saline (500 µg/ml) and was used as stock solution for cell culture studies.

Embryonated chicken eggs: Embryonated chicken eggs were purchased from the poultry farm of the College of Veterinary and Animal Sciences, Kerala Agricultural University, Kerala and brought to the laboratory. The eggs were incubated at 37°C in a humidified incubator till they were nine days old. The eggs were candled to select those with healthy embryos.

Preparation of chick embryo fibroblasts and cell culture: Embryos from nine days old embryonated eggs were collected by breaking the eggs under aseptic condition in a sterile inoculation hood. For this the eggs were initially cleaned with 70% ethanol and the broad end of each egg was opened with sterile scissors and the piece of shell with the white membrane underneath was carefully removed. The embryos were removed by sterile forceps and transferred to Petri dish containing sterile Hanks Balanced Salt Solution (HBSS).

The head and internal organs were discarded and the muscle tissue was washed with HBSS to remove the erythrocytes. This was chopped into small pieces and again washed with HBSS. The slices were then treated with 0.25% trypsin-EDTA solution and shaken to loosen the cells.

The cell suspension was then filtered through cheese cloth to remove debris. The suspension was centrifuged at 5000 rpm for 3 min. The supernatant was discarded and the cells were dispensed in growth medium [DMEM enriched with 10% FBS, Penicillin (100 units/ml), Streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml)]. The cells were dispensed into T-25 cm² tissue culture flasks and allowed to become 80% confluent (incubated at 37°C in an atmosphere of 5% CO₂). The cells were then trypsinised and seeded into 96-well plates and 12-well plates for viability assay and dual staining (acridine orange / ethidium bromide staining) respectively.

Cell viability assay using Trypan Blue: Viability is a measure of the metabolic state of a cell population, which is indicative of the potential of the cells for growth. The fibroblast cells (5000 cells/well) in microtitre plates were treated with different concentrations of cuttlefish (*Sepia pharaonis* female and male, *Sepiella inermis* female, *Sepia aculeata* female) and squid (*Loligo duvauceli* female) ink extract (0, 25, 50 and 75 µg/ml). Each treatment was carried out in quadruplicate. The cells were incubated at 37°C in an atmosphere of 5% CO₂ for 24 h. The cells were then trypsinised using 100 µl of 0.25% Trypsin EDTA solution for 1 minute and neutralized using 100 µl of FBS. The viability was determined by Trypan blue dye exclusion method¹². The percentage of cells that were not stained with Trypan blue (viable cells) is the measure of the viability. The percentage viability of cells over the control was calculated using the formula

$$\text{Percentage Viability} = \left(\frac{\text{Number of viable cells in drug treated}}{\text{Mean number of viable cells in control}} \right) \times 100$$

Ethidium bromide/acridine staining: Loss of cell viability and nuclear condensation are a result of necrosis or apoptosis¹³. Cells (5 x 10⁵ cells/well) were seeded in 12-well plates and the cells were incubated with different concentrations (0, 25 and 75 µg/ml) of cuttlefish (*Sepia pharaonis* female) ink extract for 24 h. After washing once with phosphate-buffered saline (PBS), the cells were stained with 100 µl of a mixture of acridine orange/ethidium bromide (1:1) solutions. The cells were immediately washed once with PBS and viewed under microscope using a suitable filter¹⁴.

Statistical analysis: The data were subjected to analysis of variance and differences determined at 5% level of probability¹⁵.

RESULTS:

Effect of ink extracts of different species of Squid and Cuttlefish on Chick Embryo Fibroblasts: The graph, **Figure.1** showed that the cell viability of chick embryo fibroblasts treated with ink extract from *Sepia pharaonis* female was decreased to a significant extent in 24 h, whereas those treated with other four ink extracts (*Sepia pharaonis* male, *Sepiella inermis* female, *Sepia aculeata* female, *Loligo duvauceli* female) showed less significant effect. From the above results, it may be concluded that, among different species of squid and cuttlefish tested, ink extract from *Sepia pharaonis* female shows the most significant effect on cell viability. The significant reduction in the number of live cells in the treated samples compared to the control is a clear indication of the cytotoxic effect of cuttlefish ink extract. The extract inhibited cell proliferation and induced cell death in a dose dependent manner. Since the ink extract from the female Cuttlefish, *Sepia pharaonis* showed the highest cytotoxic activity, further studies were made using this extract.

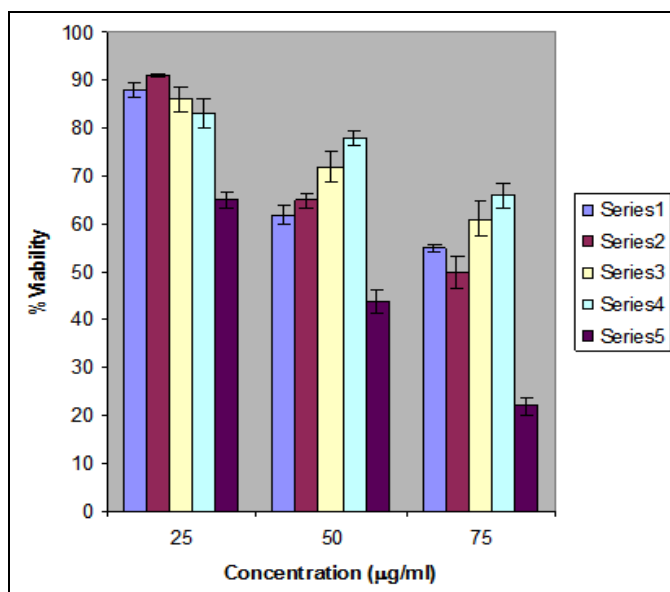


FIGURE: 1: EFFECT OF INK EXTRACTS OF DIFFERENT SPECIES OF SQUID AND CUTTLEFISH ON CHICK EMBRYO FIBROBLASTS

Series 1- *Sepia pharaonis* male, Series 2- *Sepiella inermis* female, Series 3- *Sepia aculeata* female, Series 4-*Loligo duvauceli* female, Series 5- *Sepia pharaonis* female. The results obtained in using triplicate samples were taken and percentage viability over control was calculated and expressed as Mean \pm SD. Each experiment was repeated two times with similar results.

Cuttle fish ink extract induces Nuclear Condensation: To see whether the cytotoxic effects induced by cuttlefish ink extract on chick embryo fibroblast cells involve typical apoptotic changes, cells were first examined for changes in nuclear condensation. Control cells showed bright green chromatin with organized structure, indicative of viable cells.

Upon treatment with different concentrations (25 and 75 µg/ml) of cuttlefish *Sepia pharaonis* female ink extract, nuclear condensation was visible in chick embryo fibroblasts at 24h whereas the untreated control cells were healthy with very less staining. The results were shown in **Figure 2**.

Chick embryo fibroblasts treated with 25 µg/ml of cuttlefish ink extract, a few cells lose their membrane integrity and takes up ethidium bromide, which stains orange (mixed with green colour of acridine orange). Chick embryo fibroblasts treated with 75 µg/ml of cuttlefish ink extract the cell loses their membrane integrity and takes up ethidium bromide, which stains orange. Cells undergoing apoptosis exhibit nuclear condensation.

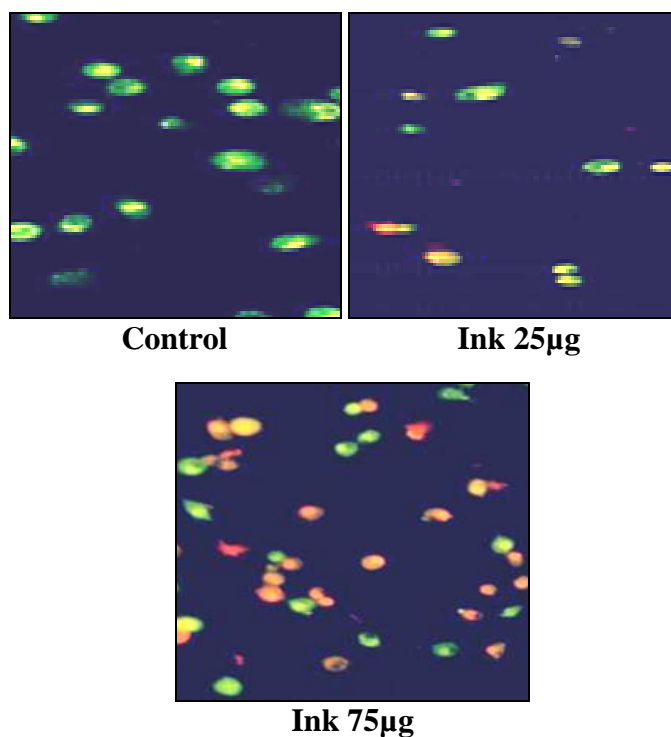


FIGURE 2: CUTTLEFISH (*SEPIA PHARAONIS*) INK EXTRACT INDUCES NUCLEAR CONDENSATION IN CHICK EMBRYO FIBROBLASTS (ACRIDINE ORANGE / ETHIDIUM BROMIDE STAINING)

DISCUSSION: The cytotoxicity of the melanin free ink of cephalopod, *Sepia officinalis* was reported¹⁶. Cuttlefish ink finds wide applications in homeopathic medicine (medicinal name 'Sepia'). In homeopathy it was used to treat hormonal imbalances especially in woman¹⁷. In the present study tris extracted ink of cuttlefish *Sepia pharaonis*, female showed the highest cytotoxic activity. The antiproliferative activity of the ink extract revealed the cytotoxic effect of the ink extract of cuttlefish *Sepia pharaonis*. Squid ink has been reported for its antitumour activity¹⁸. The squid ink extract have protective effect towards hemopoietic injuries from chemotherapeutics¹⁹.

There is evidence that methanolic extract of squid had anticancer activity.²⁰ Our investigation also reveals that the ink can also induce nuclear condensation which is the characteristic feature of apoptosis²¹. Both chick embryo fibroblasts and cancerous cells are undifferentiated immature cells and they share certain common characters. So the cytotoxic effect of the ink extract renders its therapeutic potential as an anticancer agent.

CONCLUSION: The result of the present study reveals the cytotoxic effect of the ink extract of cuttlefish (*Sepia pharaonis*) on chick embryo fibroblasts. The finding renders its therapeutic potential as an anticancer agent. Since the ink is a cheap and readily available waste material of processing industry, it will be meaningful to look at ink as a potential raw material for drug production through further studies.

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