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In Vitro Evaluation of Cytotoxicity Induced by Indoxacarb and its Amelioration with *Cassia fistula* Bark Extract

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Abstract

The primary objective of the study is to evaluate the cytotoxic effect and biochemical alteration on exposure to the Indoxacarb (IDC) in the Madin-Darby Bovine Kidney (MDBK) cell line and its amelioration by Ethanolic Bark Extract of *Cassia fistula* (EBECF). The MTT assay was used to study the cell viability and determine the IC_{50} value. The cells were further treated with IC_{25}, $IC_{12.5}$ and $IC_{6.25}$ values of IDC alone and in combination with EBECF for 24, 48 and 72 hours. IDC-induced cytotoxicity was evident by cellular morphological changes such as rounding of cells, cellular swelling, blebs formation, nuclear degradation, intracytoplasmic changes and loss of intracytoplasmic contents. The biochemical alteration was observed as a significant increase ($p<0.05$) in the levels of Lipid Peroxidation (LPO) and cytotoxicity marker enzymes namely Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALKP), Acid Phosphatase (ACP) and Creatine Kinase (CK). These changes were dose-dependent in IDC-treated groups. EBECF intervention in IDC-treated cells ameliorated the toxicity against lower concentrations. Thus, the present study suggested that *C. fistula* bark has the potential to protect the cells from IDC-mediated toxicity to a limited extent.

Keywords: Biochemical Parameters, *Cassia fistula,* Cytotoxicity, Indoxacarb, MBDK

1. Introduction

Pesticides are used worldwide to manage agricultural pests and have aided production by lowering losses caused by weeds, diseases and insect pests. The United States Environmental Protection Agency(EPA)[1] defines a pesticide as, "Any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest." Unfortunately, less than 1% of the pesticides used reach their target species while working non-specifically. Thus, the life of non-target organisms is adversely affected in the ecosystem. Long-term exposure to such substances, even at low concentrations, can harm the body system, leading to irreversible harmful effects such as carcinogenicity and teratogenicity[2].

Insecticides are a major group of pesticides used in agriculture[3], which control the insect-pest manifestation to optimise the food-grain production and fulfil the demands of an ever-increasing animal as well as human population. This leads to a rise in the demand for the

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production of effective, selective and safe insecticides. In 2000, Du Pont Ltd., US, developed and marketed IDC, the first commercialised pesticide under the oxadiazine class of insecticides. It is widely used because of its environmental compatibility and outstanding field insecticidal activity against lepidopteron pests of vegetables, tree fruits, cotton, corn, peanut, soybean, alfalfa, etc. The EPA has classified IDC as a "low-risk" pesticide alternative to an organophosphate[4].

IDC is highly selective and competent against various agricultural pests. It is a neurotoxic agent and acts by blocking the voltage-dependent sodium channel, thereby inhibiting action potential. In mammals, the target site of action is the n-Acetylcholine receptors (nAChRs), which modulates its actions. It has been discovered to be more efficient against lepidopteran pests, cockroaches, fire ants, termites, fleas and houseflies[5].

Plants have always been used for medical purposes and still constitute an inexhaustible source for treating many diseases in humans and animals. According to various literatures, most plants have high antioxidant and Reactive Oxygen Species (ROS) scavenging power due to the presence of many bioactive compounds[6], thereby, minimising the adverse effects of oxidative stress caused by xenobiotics. *C. fistula* Linn. is a medicinal plant under the *Leguminosae* family. It is a flowering plant, endemic to the Indian subcontinent and the neighbouring areas of Southeast Asia. The plant possesses multiple pharmacological benefits, with its bark demonstrating the most prominent antioxidant properties, followed by its leaves, flowers and pulp[7]. This plant is commonly known as '*Amaltas*', 'Indian Laburnum' or the 'Golden Shower Tree'. It has long been utilised in Ayurvedic medicine to treat a variety of diseases and hence named as "disease killer". It has many pharmacological properties such as hepatoprotective, anti-inflammatory, antitussive, antifungal, antibacterial, wound-healing, hypoglycaemic, hypocholesterolemic and antidiabetic[8].

Keeping in view the increasingly inadvertent use of IDC in agricultural practices, rapid exposure to non-targeted animal species through a variety of mediums may lead to potential health hazards. Thus, IDC was selected to evaluate its cytotoxic potential through an *in vitro* screening system using the MDBK cell line and its possible protection by *C. fistula* bark extract.

2. Materials and Methods

2.1 Chemicals and Reagents

IDC (99.9% purity) was procured from Sigma-Aldrich, USA. Medium-Dulbecco's Modified Eagle Medium (DMEM), Foetal Bovine Serum (FBS), Dulbecco's Phosphate Buffered Saline/Phosphate Buffered Saline and Antibiotic-Antimycotic solutions were procured from HiMedia Laboratories Pvt. Ltd. (India). Trypsin-EDTA, trypan blue and MTT were procured from Sigma, USA. Dimethylsulphoxide (DMSO), ethanol (96%) and glacial acetic acid (100%) were bought from Merck (Darmstadt, Germany). The sterile culture flasks and culture plates were procured from Nest Biotech Co. Ltd., China. Different buffers and the experiment's reagents were autoclaved Milli-Q water and filtered through Millipore membrane filters-0.22µm (Molsheim, France).

2.2 Cell Culture

A commercial cell line MDBK procured and maintained by the College of Animal Biotechnology, GADVASU, Ludhiana, was used in the study. A flask containing the MDBK cell line was brought to the Department of Veterinary Pharmacology and Toxicology, GADVASU, Ludhiana and sub-cultured for the experiment. The culture flask was incubated in an incubator at 5% CO_2, set at 37°C with >50% humidity. After the cells reached >80% confluency, the spent media was removed and washed with Phosphate-Buffered Saline (PBS). The adherent cells were dispersed with 0.25% trypsin-EDTA and the activity was inhibited by 10% FBS. Cells were harvested and pelleted by centrifugation at 1000 RPM for 10 minutes. The resultant pellet was resuspended in PBS for washing and centrifuged three times as done earlier. Cell viability was assessed using the 0.1% trypan blue dye exclusion method. The live cells were adjusted to a concentration of $1x10^5$ cells/mL of growth media (Dulbecco's Modified Eagle's Medium+10% FBS+0.1% antibiotic-antimycotic solution) and used in subsequent experiments.

2.3 Plant Materials

The bark of the plant *C. fistula* was collected from the campus of Punjab Agricultural University, Ludhiana, Punjab, India. The collected plant material was cleaned by simple water rinsing and dried at 40°C temperature in an oven dryer. The bark was then ground into a

fine powder, weighed (100 grams) and soaked in 99% ethanol (400ml) in a stoppered flask for more than 72 hours at room temperature with intermittent shaking. The mixture was first filtered by using a clean and sterile four-fold muslin cloth and then twice with Whatman filter paper no. 1. The filtrate obtained was evaporated in an oven dryer at 40˚C to obtain the dry extract.

2.4 *In vitro* Experimental Protocols

2.4.1 Inhibitory Concentration (IC_{50}) Determination

The IC_{50} was determined by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay[9] to assess the number of metabolically active cells and determine the cytotoxicity and growth rate indirectly. MDBK live single-cell suspension was seeded in a 96-well cell culture plate at a concentration of $2x10^4$ cells/mL (5,000 cells/well) and incubated for 12 hours for *cellular* attachment. Cells were exposed to different concentrations of IDC (100-1000µg/mL) for the next 48 hours. At the end of the exposure period, spent media was removed and replaced with 20µl of 5mg/ml MTT dissolved in PBS and 180µl of maintenance media per well. One set of wells served as a control, containing only MTT and medium with no cells. The cells were incubated for 4 hours at 37°C in the dark in an incubator. Thereafter, needle-shaped formazan crystals were found intracellularly. These were visualised under a microscope, which was dissolved by replacing the medium with 150µl of MTT solvent (DMSO). The microplate was covered with tin foil and the flask was agitated on an orbital shaker for 30 minutes. The absorbance was read at 570nm. Each assay was repeated at least three times with each group of triplicates.

Table 1. Experimental design with groups and exposure time

Group	Treatment	Exposure Period (Hours)		
I	Control	24h	48h	72h
II	Vehicle Control	24h	48h	72h
III	EBECF	24h	48h	72h
IV	IDC-$IC_{6.25}$	24h	48h	72h
V	IDC- $IC_{12.5}$	24h	48h	72h
VI	IDC- IC_{25}	24h	48h	72h
VII	IDC-$IC_{6.}$25 + EBECF	24h	48h	72h
VIII	IDC-$IC_{12.5}$ + EBECF	24h	48h	72h
IX	IDC-IC_{25} + EBECF	24h	48h	72h
X	Positive Control (Cyclophosphamide)	24h	48h	72h

Note: IDC-Indoxacarb; EBECF: Ethanolic Bark Extract of *C. fistula)*

2.4.2 Experimental Design

The MDBK cells were treated with IC_{25}, $IC_{12.5}$ and $IC_{6.25}$ concentrations of IDC (74.12, 37.06, 18.53µg/ml, respectively) at different intervals of time (24, 48 and 72 hours). MDBK cells were seeded at a concentration of 2 x 10^4 cells/mL (20,000 cells/well) in 24-well cell-culture plates with culture growth media. The cells were then incubated in a 37˚C, 5% CO_2 in a humidified incubator for 12 hours. The untreated cells with growth media only were used as the control and vehicle control (DMSO 0.1% in growth media). The cells treated with a cytotoxic drug- Cyclophosphamide were used as a positive control. The EBECF was tested as an ameliorative agent. The cells were treated with a concentration of 125µg/ml alone as *C. fistula* control (The dose was determined in a pilot experiment as the best suitable dose), three doses of IDC alone and in combinations (Table 1). After completion of 24, 48 and 72 hours of exposure period, cellular morphology was studied by visualising under an inverted microscope and graded accordingly. Spent culture growth media was removed from adherent cells and stored at -20°C until further analysis of cytotoxicity enzymes. Further, cells were harvested and used for LPO estimation.

2.4.3 Cellular Morphology

After completion of the exposure period, cells were observed under an inverted phase contrast microscope (Nikon ECLIPSE-Ti, Japan) to evaluate the cellular morphological changes. These changes were graded as per the standard procedure[10] and tabulated in Table 2.

2.4.4 Biochemical Parameters

After the treatment period, the media collected was used for the analysis of cytotoxicity marker enzymes. The different biochemical parameters, LDH, ALKP, ACP and CK were estimated using standard commercial kits (ERBA diagnostics Mannheim GmbH Mallaustre, Germany).

Table 2. Scoring of morphological changes in MDBK cells after 48-hour exposure to IDC and EBECF alone as well as in combination

Morphological Features	Treatment Groups									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Cellular Swelling	1	1	1	3	3	4	2	2	4	5
Cellular Blebbing	1	1	1	2	4	5	1	2	4	5
Cell Rounding	1	1	2	2	3	4	1	2	3	5
Cell Shrinkage	1	1	1	3	4	4	2	3	4	5
Chromatin Condensation	1	1	1	2	3	5	1	2	4	5
Nuclear Degradation	1	1	1	1	3	4	1	2	4	5
Plasma Membrane Disintegration	1	2	1	3	3	5	2	3	4	5
Release of Cytoplasmic Content	1	1	1	2	3	4	1	2	4	5

Where; 1: Normal morphology; 2: Low level changes; 3: Moderate level changes; 4: High level changes; 5: Very high-level changes

2.4.5 Estimation of Lipid Peroxidation

LPO was measured in the MDBK cells harvested after the completion of treatment time and with exposure to different treatments using a commercial kit as per the manufacturer's instruction by the MDA-586 method (Cat# 250070; Real Gene). A standard curve was prepared by using the internal standard provided in the kit and an unknown sample concentration was estimated by using equation $y = 0.0129x + 0.0432$ with an R^2 value of 0.9948. LPO values were expressed in nmol of TBARS/mg of protein.

2.4.6 Statistical Analysis

Finally, the data obtained was subjected to statistical analysis. The data analysis was done using two-way ANOVA, wherever applicable, using SPSS version 26.0 software. Data were expressed as Mean ± SE. The significance was assessed at $p<0.05$.

3. Results

3.1 Effect on Inhibitory Concentration (IC_{50}) of IDC

The cell viability assay/MTT assay was performed and the log dose-response curve was plotted (Figure 1) to determine the IC_{50} value of the drug. MDBK cells showed significant cytotoxicity after 48 hours of exposure to IDC, with an IC_{50} value of 148.25µg/ml. Thereafter, the IC_{25}, $IC_{12.5}$ and $IC_{6.25}$ values were calculated as 74.13µg/ml, 37.06µg/ml and 18.53µg/ml, respectively.

3.2 Effect on Cellular Morphology of MDBK Cells

The cellular morphology of MDBK cells upon exposure to IDC and EBECF, alone and in combination was examined under an inverted microscope (Figure 2). The control group showed no morphological malformations after 24, 48 and 72 hours and there was normal proliferation of the cells. However, cytotoxic effects were evident in the form of several morphological changes in IDC-treated cells alone and combinations of EBECF. Cellular swelling, bleb development, cell rounding, cell shrinkage, chromatin condensation, nuclear degradation, plasma membrane disintegration and loss of cytoplasmic contents were among the cellular abnormalities noted. These changes were graded on a scale of 0 to 5 depending on the severity of the changes (Table 2).

The cellular morphological changes observed were determined by the dose and time of exposure. The changes became more pronounced as the time of IDC exposure increased in comparison to the control group. The IDC-treated cells showed cell damage with respect to dose and time. The rate of cell proliferation decreased with time in IDC-treated groups, while it was found to improve in *C. fistula*-intervened groups. However, the cellular morphology was normal or improved in EBECF alone or IDC combined treated groups in comparison with IDC alone treated cells. Among the EBECF-treated groups, the lowest dose of IDC with *C. fistula* extract showed the least cellular morphological alterations. The highest degree of cellular changes was noticed in the cyclophosphamide-treated group.

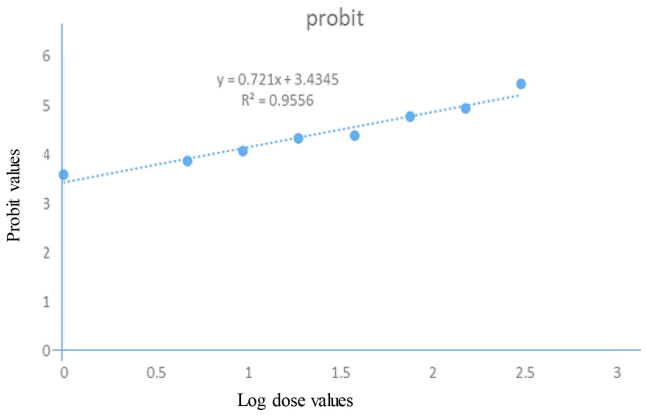


Figure 1. Probit analysis method or graphical method for estimation of IC-50 of IDC.

3.3 Effect on the Lipid Peroxidation

The present investigation revealed a significant increase in LPO in all the treatment groups at different time points in comparison to the control groups. The IDC-treated groups showed a dose-dependent increase in the extent of LPO (Table 3). The highest IDC concentration (Group VI) showed the maximum increase ($p<0.05$) in LPO at 72-hour exposure period in comparison to the control group (Group I). The ameliorative effect of EBECF was notable ($p<0.05$) in all the co-treated groups as compared to the IDC alone treated groups.

3.4 Effect on Biochemical Parameters

A dose-dependent increase ($p<0.05$) in the LDH activity was observed in IDC-treated groups.

The highest IDC concentration (Group VI) showed 54.10±0.55 LDH activity at a 48-hour exposure period. Upon exposure of the cells to a combination of IDC and EBECF, the level of LDH declined (Table 4). Not many significant ($p<0.05$) changes in the LDH activity were noticed with respect to the time-dependent effect. A significantly highest ($p<0.05$) LDH activity was noticed in the cyclophosphamide-treated group at all time intervals when compared with all other treatment groups.

The Alkaline Phosphatase (ALP) activity in IDC-treated groups increased with the inclining concentration significantly ($p<0.05$) in all the time intervals (24, 48 and 72 hours) when compared with the control group. EBECF co-treated groups did not show significant ($p<0.05$) change in ALP activity when compared between groups, while they showed significantly lower ($p<0.05$) ALP values when compared with IDC alone treated groups (Table 5). The cyclophosphamide-treated group showed significantly ($p<0.05$) very high APL activity at all time intervals when compared with the other groups. The ACP activity of IDC-treated groups increases in a dose-dependent manner (Table 6). The highest IDC dose (Group VI) showed a significant ($p<0.05$) ACP activity and showed an incline in enzymatic activity with respect to time. After co-treatment with EBECF, a significant ($p<0.05$) reduction in ACP activity was observed in the IDC- $IC_{12.5}$ treatment group, which showed amelioration by declining the ACP activity in the *C. Fistula* treated groups. Co-administration of EBECF was able to partially ameliorate this effect against higher concentrations.

An increase in the value of CK was observed in all groups exposed to IDC, in a dose-dependent manner. A significant ($p<0.05$) increase in CK values were observed in the highest dose of IDC-treated cells (Group VI). When co-treated with EBECF, a significant reduction ($p<0.05$) in CK activity was noticed in all the groups (Groups VII, VIII and IX), when compared with IDC alone treated groups. EBECF co-exposure reduced the IDC-induced increase in CK activity but was not fully able to ameliorate the effects in all the groups (Table 7).

Table 3. Effect of Indoxacarb exposure alone and in combination with *Cassia fistula* on LPO in MDBK cells at 24, 48 and 72 hours

Group	24 hours	48 hours	72 hours
I	0.83^{Aa}± 0.05	0.85^{Aa}±0.03	0.80^{Aa}±0.03
II	0.83^{Aa}±0.04	0.84^{Aa}±0.06	0.82^{Aa}±0.05
III	0.88^{Aa}±0.06	0.86^{Aa}±0.05	0.96^{Aa}±0.01
IV	2.51^{Ad}±0.09	3.17^{Bd}±0.08	3.21^{Bc}±0.07
V	4.18^{Af}±0.1	4.49^{Af}±0.10	4.57^{Ad}±0.16
VI	4.70^{Ag}±0.06	5.16^{Bg}±0.13	5.88^{Cf}±0.10
VII	2.23^{Ac}±0.08	2.11^{Ab}±0.07	2.55^{Cb}±0.12
VIII	3.82^{Ce}±0.08	2.73^{Ac}±0.08	3.33^{Bc}±0.12
IX	2.27^{Acd}±0.07	4.18^{Be}±0.07	5.07^{Ce}±0.12
X	6.72^{Ah}±0.15	7.17^{ABh}±0.1	7.62^{Bg}±0.25

Values given represent the mean±S.E.; LPO: Lipid Peroxidation
Values given are expressed as nmol of TBARS/mg of protein.
Values with different superscripts in capitals (between groups) and lowercase (within groups) differ significantly ($p<0.05$)

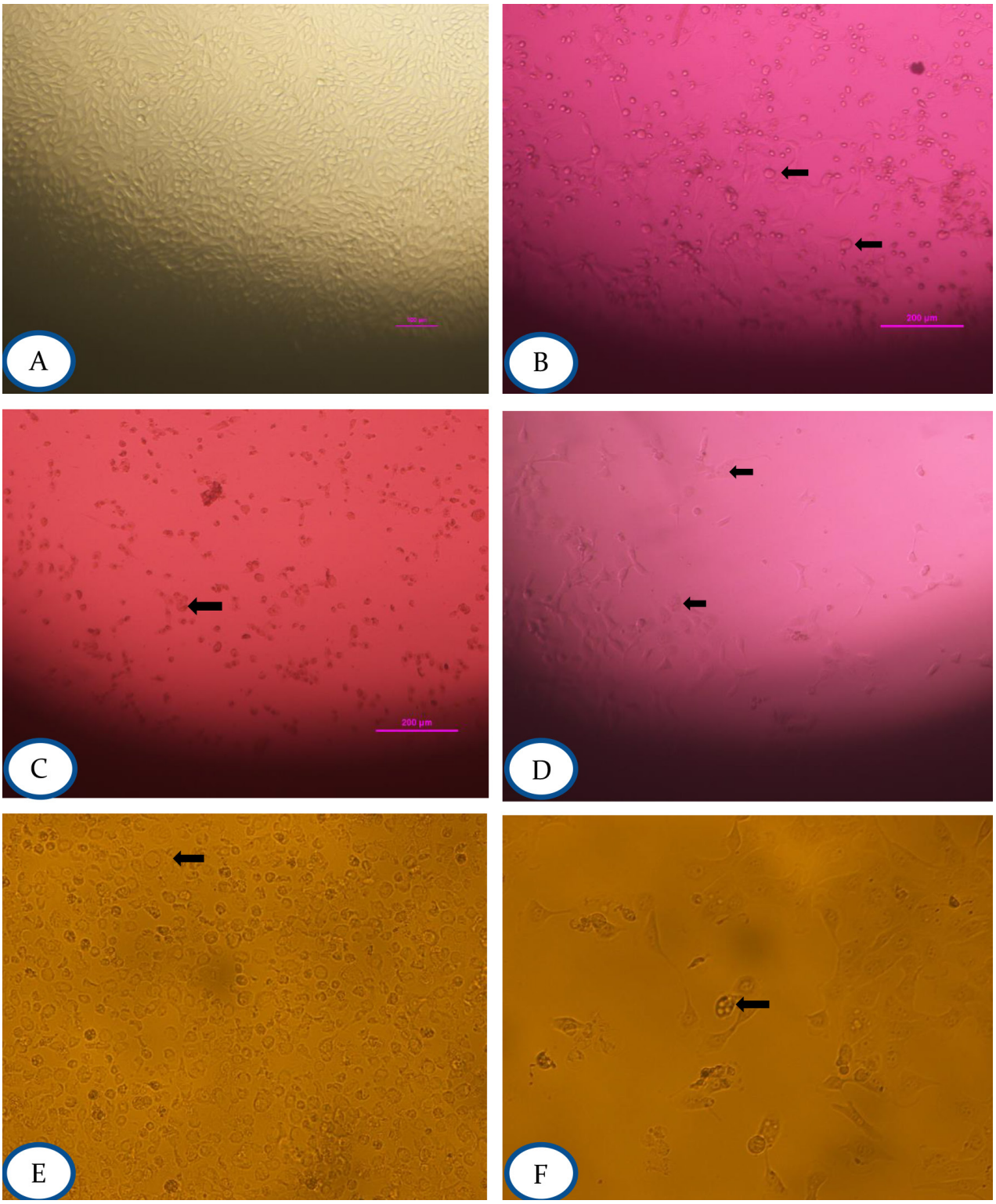


Figure 2. Representative photomicrographs of changes in MDBK cells after exposure to Indoxacarb under an inverted microscope (Original magnification: 10X). **(A)**Normal MDBK cells showing epithelial morphology, **(B)** Rounding of cells, (**C)** DNA degradation, **(D)** Changes in intracytoplasmic contents, **(E)** Loss of cytoplasmic content, **(F)** Vacuolisation.

Table 4. Effect of Indoxacarb exposure alone and in combination with *Cassia fistula* on LDH in MDBK cells at 24, 48 and 72 hours

Group	24 hours	48 hours	72 hours
I	15.44^{Aa}±0.16	15.29^{Aa}±0.16	15.40^{Aa}±0.19
II	16.52^{Ba}±0.27	15.56^{Aa}±0.17	16.11^{ABa}±0.28
III	15.96^{Ba}±0.17	14.72^{Aa}±0.23	15.25^{ABa}±0.31
IV	25.00^{Ac}±0.53	24.11^{Ab}±0.5	24.30^{Ab}±0.32
V	35.93^{Ad}±0.48	38.09^{Bd}±0.45	38.62^{Bd}±0.78
VI	52.95^{Af}±0.54	54.10^{Ae}±0.55	54.09^{Ae}±0.54
VII	21.84^{Ab}±0.34	23.07^{Ab}±0.47	23.21^{Ab}±0.6
VIII	34.99^{Ad}±0.86	34.87^{Ac}±0.41	35.10^{Ac}±0.43
IX	49.98^{Ae}±0.52	52.73^{Be}±0.57	53.30^{Be}±1.10
X	72.03^{Bg}±0.97	62.53^{Af}±0.8	71.9^{Bf}±1.04

Values given represent the mean±S.E.; LDH: Lactate Dehydrogenase
Values given are expressed in IU/L
Values with different superscripts in capitals (between groups) and lowercase (within groups) differ significantly ($p<0.05$).

Table 5. Effect of Indoxacarb exposure alone and in combination with *Cassia fistula* on ALP in MDBK cells at 24, 48 and 72 hours

Group	24 hours	48 hours	72 hours
I	4.66^{Aa}±0.06	4.76^{Aa}±0.07	4.87^{Aa}±0.07
II	4.54^{Aa}±0.08	4.70^{ABa}±0.1	4.88^{Ba}±0.10
III	4.49^{Aa}±0.13	4.43^{Aa}±0.11	4.65^{Aa}±0.05
IV	5.90^{Ab}±0.3	6.60^{Ac}±0.10	7.38^{Bc}±0.23
V	8.52^{Ad}±0.18	8.67^{Ae}±0.18	8.49^{Ad}±0.11
VI	10.44^{Af}±0.21	10.43^{Ag}±0.13	10.59^{Af}±0.21
VII	5.50^{Ab}±0.21	6.10^{ABb}±0.13	6.33^{Bb}±0.21
VIII	7.71^{Ac}±0.16	7.78^{Ad}±0.09	7.71^{Ac}±0.14
IX	9.66^{Ae}±0.21	9.73^{Af}±0.2	10.03^{Ae}±0.13
X	12.16^{Ag}±0.51	12.42^{Ah}±0.13	12.54^{Ag}±0.23

Values given represent the mean±S.E.; ALP: Alkaline Phosphatase
Values given are expressed in IU/L
Values with different superscripts in capitals (between groups) and lowercase (within groups) differ significantly ($p<0.05$)

Table 6. Effect of Indoxacarb exposure alone and in combination with *Cassia fistula* on ACP in MDBK cells at 24, 48 and 72 hours

Group	24 hours	48 hours	72 hours
I	0.37^{ABa}±0.02	0.39^{Ba}±0.01	0.34^{Aa}±0.01
II	0.37^{Aa}±0.01	0.44^{Ba}±0.01	0.36^{Aa}±0.01
III	0.38^{Aa}±0.02	0.40^{Aa}±0.01	0.43^{Aa}±0.01
IV	0.75^{Ab}±0.01	0.76^{Ab}±0.02	0.78^{Ab}±0.01
V	1.23^{Ac}±0.04	1.42^{Bc}±0.02	1.28^{Ac}±0.03
VI	2.38^{Ae}±0.06	2.50^{ABd}±0.05	2.67^{Be}±0.06
VII	0.71^{Ab}±0.02	0.74^{Ab}±0.01	0.73^{Ab}±0.04
VIII	1.09^{Bc}±0.01	1.37^{Cc}±0.02	0.83^{Ab}±0.03
IX	1.86^{Ad}±0.02	1.91^{Ad}±0.04	1.85^{Ad}±0.05
X	3.49^{Af}±0.14	3.85^{Ae}±0.04	3.85^{Af}±0.17

Values given represent the mean±S.E.; ACP: Acid Phosphatase
Values given are expressed in IU/L
Values with different superscripts in capitals (between groups) and lowercase (within groups) differ significantly ($p<0.05$).

Table 7. Effect of Indoxacarb exposure alone and in combination with *Cassia fistula* on 58CK in MDBK cells at 24, 48 and 72 hours

Group	24 hours	48 hours	72 hours
I	0.88^{Aa}±0.02	0.92^{Aa}±0.03	0.94^{Aa}±0.02
II	0.89^{Aa}±0.02	0.91^{Aa}±0.02	0.92^{Aa}±0.02
III	0.85^{Aa}±0.01	0.87^{Aa}±0.01	0.91^{Aa}±0.03
IV	1.61^{Ac}±0.04	1.62^{Ab}±0.04	1.60^{Ab}±0.04
V	2.43^{Ae}±0.08	2.46^{Ac}±0.05	2.50^{Ac}±0.12
VI	3.53^{Ag}±0.06	3.71^{ABe}±0.1	3.83^{Be}±0.05
VII	1.25^{Ab}±0.05	1.59^{Bb}±0.05	1.48^{Bb}±0.03
VIII	1.87^{Ad}±0.1	2.04^{Bc}±0.01	2.45^{Bc}±0.13
IX	3.19^{Af}±0.4	3.23^{Ad}±0.08	3.24^{Ad}±0.05
X	5.19^{Bh}±0.11	5.27^{Bf}±0.11	4.52^{Af}±0.14

Values given represent the mean±S.E.; CK: Creatine Kinase
Values given are expressed in IU/L
Values with different superscripts in capitals (between groups) and lowercase (within groups) differ significantly ($p<0.05$)

4. Discussion

Normal MDBK cell line has an epithelial morphology and is an adherent cell line derived from adult steer[11]. MDBK cell line was selected in the current investigation as the kidney is an important organ for purification. Most of the drugs are eliminated or excreted *via* urine. The chances of kidney cells coming in contact with toxicants are more. The cells are exposed to toxicants and their reaction can be studied *ex-vivo* using cell culture to predict toxic effects or renal toxicity. The exposure time of MDBK cells to IDC was 24, 48 and 72 hours which represent the sub-chronic or chronic exposure as in *in-vivo* condition. With exposure to IDC, normal cellular morphology was altered and the maximum changes were observed at the highest dose of IDC treated group. The MDBK cell line

no longer adhered to the flask which resulted in rounding of cells and bleb formation. IDC treatment might cause an increase in the ROS and LPO responsible for the initiation of cell damage or cellular changes. Cytoplasmic vacuolisation of mammalian cells occurs due to exposure to a toxicant and could result in cell death[12]. IDC exposure also led to cellular swelling due to altered cell membrane's permeability indicated by increased LPO resulting in uncontrolled movement of ions and water across the membrane[13]. Due to the plasma membrane disintegration, the cytoplasmic contents ooze out of the cell and hence, there was loss of cytoplasmic contents. The morphological alterations of the IDC-treated cells such as cell shrinkage, chromatin condensation, plasma membrane and nuclear degradation are part of the programmed cell death[14].

The oxidation of Polyunsaturated Fatty Acids (PUFAs) by ROS is known as Lipid Peroxidation (LPO). It involves auto-oxidation by molecular oxygen (O_2) and hence, is self-sustainingly capable of producing extensive tissue injury. Oxidative stress results in lipid peroxidation by producing hydroperoxides, which are the main source of free radicals. ROS forms adducts with DNA and proteins, resulting in the disruption of their normal function and hence, oxidative damage, thereby disturbing the normal cellular function[15]. The cell membrane is made up of PUFA which are the main target for ROS, eventually causing cell membrane damage. Besides, it leads to a reduction of membrane fluidity as well as decreased membrane barrier functions[16]. The observations of the current investigation also support similar findings by Mudaraddi *et al.*[17] who reported that exposure to IDC in Swiss albino mice resulted in an elevation of LPO and lowered antioxidant enzyme activities. Further, the hepatoprotective effect of *C. fistula* against paracetamol-induced hepatotoxicity in rats has been reported[18]. Similarly, a limited extended decrease in the LPO was noticed in the present study with the intervention of *C. Fistula* in IDC-treated cells.

The common indicator of cytotoxicity is the leakage of cytoplasmic enzymes into the surrounding medium. Elevated enzymatic activities can be measured in a secretory medium and the degree of cytotoxicity can be determined[19]. LDH is one of the key cytoplasmic enzymes ubiquitous in the body which is predominant in the liver, muscles, kidney, etc. It is an important indicator of abnormal metabolism of DNA, gluconeogenesis and non-specific markers of tissue injury as well as turnover[20]. When the cell loses its viability, the plasma membrane becomes leaky and LDH is released from the damaged cells, hence, it is used to assess cytotoxicity. Hepatocyte LDH activity increases with exposure to pesticides such as organochlorine, organophosphate and synthetic pyrethroids[21]. Our findings suggested that IDC causes significant cellular injury in MDBK cells, evidenced by an increase in the LDH activity and EBECF can produce amelioration in this enzymatic activity only at a lower concentration of IDC. Our results are corroborated well by Maiza *et al.*,[22] who reported an incline in LDH activity in cockroaches (*Blattella germanica)* after exposure to IDC.

ALP belong to a group of isoenzymes present on the outer layer of the cell membrane. Their function is to catalyse the extracellular hydrolysis of organic phosphate esters at an alkaline pH and are known as true isoenzymes because they catalyse the same reaction in different body tissues. ALP is found in the tissues such as the liver, bones, kidney, intestine, placenta and leukocytes. It is derived from abnormal conditions of the liver, bones and intestine and is a non-specific indicator. An increase in its value denotes a variety of diseases and cell damage[23]. The outcomes of our observations indicate that IDC causes dose and duration-dependent increase in ALP levels in MDBK cells and EBECF was able to minimise cell damage indicated by reduced ALP levels. Our findings were supported by Bhakta *et al.*[24], who reported a significant protective effect of *C. fistula* in rats by lowering the serum ALP level.

ACP is an enzyme which catalyses the hydrolysis of organic phosphates at an acidic pH. It is present in the lysosome of cells of the organs like liver, kidney, bone, spleen, blood and intestine. ACP test measures the amount of ACP released in the blood and a significant increase indicates cellular malfunctions and pathologic conditions of the body. Based on our findings, it was evident that IDC produces dose and duration-dependent toxic damage to the MDBK cells. The extent of damage was quite remarkable and comparable with the potent carcinogen cyclophosphamide. Co-administration of *C. fistula* was able to partially ameliorate this effect at a higher dose of IDC and compete against a lower dose of IDC. These findings are in corroboration with Gamil *et al.*[25] who reported a decline in the activity of ACP in 2nd instar and 4th instar larvae of *Spodoptera littoralis* (Boisd.) after treating them with IDC.

CK plays a critical role in the conversion of creatine and Adenosine Triphosphate (ATP) to phosphocreatine and Adenosine Diphosphate (ADP). This reaction

produces phosphocreatine, which provides ATP to the brain and other vital organs. Whenever there is tissue or cell injury, the CK leaks into the bloodstream which can be directly measured[26]. The estimation of CK activity provides substantial evidence of tissue damage when exposed to contaminants[27]. In the present study, the elevated CK activity suggests that IDC induces concentration and time-dependent toxicity in MDBK cells. EBECF co-administration reduces the IDC-induced incline in CK activity. Similarly, an increase in hepatic functional enzymes AST and ALT upon exposure to IDC has also been reported[28]. However, *C. fistula* contains phytochemicals such as anthraquinones, chromones, flavonoids, coumarins, phytosterols, long-chain hydrocarbons, alkaloids, Phenols. etc. which may contribute to the cellular protection against pesticide-mediated toxic effects[29].

IDC is an outstanding insecticide for agricultural applications used in selective control of pest infestation. Indiscriminate application may pose an adverse effect on the non-targeted animal species. The present investigation revealed that IDC produced changes in cellular morphology and lipid peroxidation, which leads to cell damage. Further, IDC caused alteration in cellular biochemical reaction and leaked the cytotoxicity marker indicators into the medium as indicated by their elevated level. When the ethanolic bark extract of *C. fistula* was added into the medium in the presence of IDC, it prevented the cells from toxic damage and cellular changes induced by IDC. The present study suggested that IDC has a marked cytotoxic effect on kidney cells, while ethanolic extract of *C. fistula* bark has the potential to ameliorate the cytotoxic effect caused by IDC.

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