Middle East Journal of Pure and Applied Sciences (MEJPAS)
Online ISSN: 3062-343X
Volume 2, Issue 2, 2026, Page No: 18-23
https://mideastjournals.com/index.php/mejpas

In-vitro cytotoxicity Assessment of gasoline on primary Gill and Liver Cell Cultures of Shield head fish (*Synodontis schall*)

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تقييم السمية الخلوية للجازولين في المختبر على مزارع الخلايا الأولية للخياشيم والكبد لأسماك القرقور (سينودونتيس شال)

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Received: June 23, 2026	Accepted: August 09, 2026	Published: August 16, 2026

Abstract
The aim of the study was to identify the toxicity of gasoline in the aquatic environment by studying its effects on the primary cell cultures of the gills and liver of shieldhead fish (*Synodontis schall*). Primary cell cultures were created from adult shieldhead fish. The cell cultures were carried out following a method modified from that of Gaurav *et al.* [1]. Thin slices of fish gills and liver were cultured in Petri dishes containing Omega-3 medium. The bioassays were carried out when the cultures reached 100% confluence, with multiple cell layers formed after 28 days.
A biochemical test was used to determine changes in the total protein content of gill and liver primary cells after 48 hours of exposure to gasoline. The effects were determined according to the Bovine Serum Albumin (BSA) standard curve and correlated with the dose values. These changes in protein concentrations were directly correlated with the dose. A cytotoxicity test was used to measure the cellular metabolic activity of gill and liver primary cells after 48 hours of exposure to gasoline. The deep purple color indicated cell viability in the control group. Cell viability decreased with increasing doses in both liver and gill primary cells, as determined by the reduction of MTT reagent to formazan.

Keywords: Cellular viability, gill and liver primary cell cultures, gasoline, shieldhead fish (*Synodontis schall*).

الملخص:
كان الهدف من هذه الدراسة هو تحديد السمية الناتجة عن البنزين في البيئة المائية من خلال دراسة تأثيره على مزارع الخلايا الأولية لخياشيم وكبد سمكة سينودونتس شال. تم إنشاء مزارع الخلايا الأولية من أسماك سينودونتس شال البالغة، وذلك باتباع طريقة معدلة عن المنهج الذي وصفه قوارف وآخرون [1].وقد زُرعت شرائح رقيقة من أنسجة الخياشيم والكبد في أطباق بتري تحتوي على وسط غذائي Omega-3. أُجريت الاختبارات الحيوية عندما وصلت المزارع الخلوية إلى نسبة 100% من التغطية الخلوية مع تكوين طبقات خلوية متعددة بعد 28 يومًا من الزراعة.
استُخدم اختبار كيميائي حيوي لتحديد التغيرات في إجمالي محتوى البروتين في الخلايا الأولية للكبد والخياشيم بعد 48 ساعة من التعرض للبنزين. وتم تقدير تركيز البروتين بالاعتماد على المنحنى القياسي لألبومين مصل الأبقار(BSA) ، وربط النتائج بقيم الجرعات المستخدمة. وأظهرت النتائج أن التغيرات في تركيز البروتين كانت مرتبطة ارتباطًا مباشرًا بالجرعة.
كما تم تقييم السمية الخلوية باستخدام اختبار MTT لقياس النشاط الأيضي لخلايا الكبد والخياشيم الأولية بعد 48 ساعة من التعرض للبنزين. وقد دلَّ ظهور اللون البنفسجي الداكن في مجموعة السيطرة على حيوية الخلايا. في المقابل، انخفضت حيوية الخلايا في كلٍّ من خلايا الكبد والخياشيم مع زيادة جرعات البنزين، وهو ما ظهر من خلال انخفاض قدرة الخلايا على اختزال كاشف MTT إلى مركب الفورمازان.

الكلمات المفتاحية: الحيوية الخلوية، خلايا الخياشيم والكبد الأولية المستزرعة، البنزين، أسماك القرقور (سينودونتيس شال).

Introduction:

Fish cell cultures have gained prominence due to advancements in research and ethical pressure to reduce animal testing. Animal activist organizations, environmental organizations and the cosmetics industry promote alternative *in-vitro* models, offering benefits such as simple medication dosing, repeatability, quick test findings and economic viability Aarattuthodi *et al.*[2].

Aquatic ecosystems are scavengers for human-caused contamination, industrial waste, and leaks, whether they are solid or chemical contaminants. Such wastes for example: heavy metals, detergents, microfibers, petroleum waste, origin of plastic or non-plastic and contribute to problems with aquatic pollution Bashir *et al*.,[3].

Oil or petroleum (a word derived from the Latin Petra which means rock "oleum" which means oil) it is also called crude oil, and it has the common name (black gold) which is a thick, flammable, liquid, dark brown or Greenish brown found in the upper crust. Water contaminated by industrial and municipal sewage has also been responsible for developing skin lesions, teratogenic effects, skin ulceration, and fungal disease in fish communicable in humans. Feeding contaminated food leads to bioaccumulation in the food chain. Defective proteins affect the functioning of an organism by altering the physiology of cells Shahid *et al*.,[4].

Fish exposed to contaminants like crude oil experience identical histological alterations in their gills, liver, kidney and spleen. The stressor and the potency of the harmful substance affect these histological alterations differently; Fish tissue exhibits the histological changes that have been well-documented. Due to the toxic chemicals in crude oil and its water-soluble fraction, crude oil can be fatal at acute or chronic levels, even though these effects may not be immediately apparent. This can result in a high mortality rate for aquatic communities. They may result in fish moving erratically, losing their stability, increased biological activity, and eventually death. The internal organs and tissues of fishes are severely damaged when these crude oil toxicants enter their bodies during oil spills or leakages through the gills, digestive tract, and general body surface Ebonwu *et al*.[5].

Materials and methods:

1. Establishment of Gills and Liver Primary Cell Culture:

Materials:

Glass aquaria, Omega-3, Tissue culture Petridishes, Scissors, Phosphate buffer solution (PBS), EDTA, 20% streptomycin and penicillin, foil, candle, and refrigerator.

Method:

The cell culture was established according to the scientific method modified by Gaurav et al. Gills and liver tissues were cut into small fragments of 1.0 mm, washed twice with phosphate buffer solution (PBS) and transferred to a Petri dish containing 20% streptomycin and penicillin for 5 minutes. The cells were dissociated using 0.2 g of EDTA dissolved in 10 ml of phosphate buffer solution (PBS) for 2 minutes, and then the cells were centrifuged at 2000 rpm for 1 minute. Finally, the pellets were seeded in Petri dishes containing omega-3 medium and given CO2 by burning out a candle. The Petri dishes were covered with foil and kept in the refrigerator at 4 °C. The culture medium was changed every 6 days for 24 days and counted every 7 days for 28 days. After 24 days, the primary cell culture formed a full monolayer and reached 100% confluence.

2. Total Cell Protein Assay:

Materials:

Phosphate Buffer Solution, 0.1 mm NaOH, Coomassie Blue solution and Spectrophotometer.

Method:

The cell protein assay adopted from Shopsis and Eng [6] to measure the total cellular protein after incubation in descending concentrations of medium containing gasoline (7.4mg/ml, 3.7mg/ml, 1.85mg/ml, 0.925mg/ml and 0.4625mg/ml) and tubes containing only omega-3 medium were kept as controls. After 48 hours of incubation the medium was removed, the cells were washed with PBS, and 1 ml of NaOH was added to each tube. The plate was incubated for 1 hour at 4°C, then 1 ml of Coomassie Blue solution was added to each tube and incubated at room temperature for 20 min. The absorption of each tube was measured at 595nm. Serial dilutions of 1 to 100 mg/ml BSA dissolved in 0.1m NaOH were used for the protein standard. All experiments were performed three times and the average absorbance at each dose was calculated for each experiment. The value of protein was determined according to Bovine Serum Albumin (BSA) standard curve ($y = 0.0167x + 0.0561$).

3. Mitochondrial Suicidical Dehydrogenate Assay (MTT):

Materials:

Tetrazolium Dye MTT, Dimethyl Sulfoxide and Spectrophotometer

Method:

The method was done according to Borenfreund *et al.* [7] that based on the reduction of the soluble yellow tetrazolium salt (MTT) to a blue insoluble MTT formazan. The reduction done by the suicidical dehydrogenate that was produced from the mitochondria that had been injured by descending concentrations of containing

gasoline (7.4mg/ml, 3.7mg/ml, 1.85mg/ml, 0.925mg/ml and 0.4625mg/ml) and tubes containing only omega-3 medium were only kept as controls. After 48-hour exposure period the tested medium was removed, and 20 ml of 5.0 mg/ml MTT was added to phosphate buffer solution (PBS) for incubation for 4 hours at 4°C. The cells were rapidly washed twice with PBS, and then 1ml of dimethyl sulfoxide was added to each tube to dissolve the purple formazan crystals that had been produced. The absorbance of each tube was measured at 490 nm. Three times the average absorbance at each dose was calculated for each experiment and expressed as a percentage of the absorbance of cells treated with different doses of gasoline compared to the control. The concentrations of Gasoline that killed 50% and 90% of the cell population (LC50, LC90) were determined.

3. Statistical Analyses:

Data were subjected to Analysis of variance (ANOVA) using SPSS to compare the results between the control and cells exposed to different concentrations of gasoline. Linear correlation between variables was also carried out using the Pearson correlation coefficient.

Results:

1. Establishment of Gills and Liver Primary Cell Culture:

Primary cell cultures have been established from tissues and have shown an increase in the number of live cells every 7 days. The viability of gills and liver primary cells showed that omega-3 was a suitable medium for growing and maintaining the viability of gill and liver primary cell cultures (Figures 1, 2 and 3).

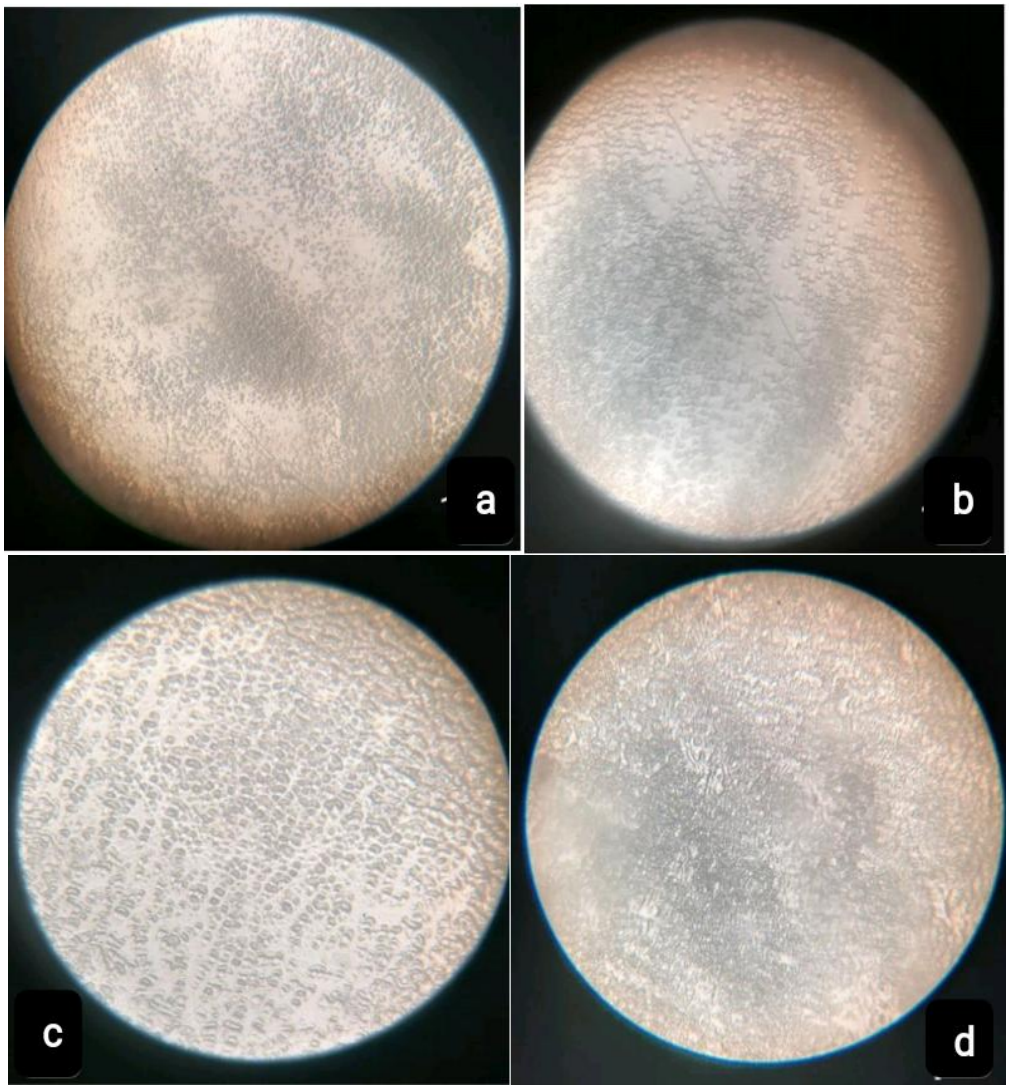

Figure (1) Gill primary cell culture grown in omega-3 medium in (a) 6 days (b) 12 days (c) 18 day (d) 24 days (Eosinx100).

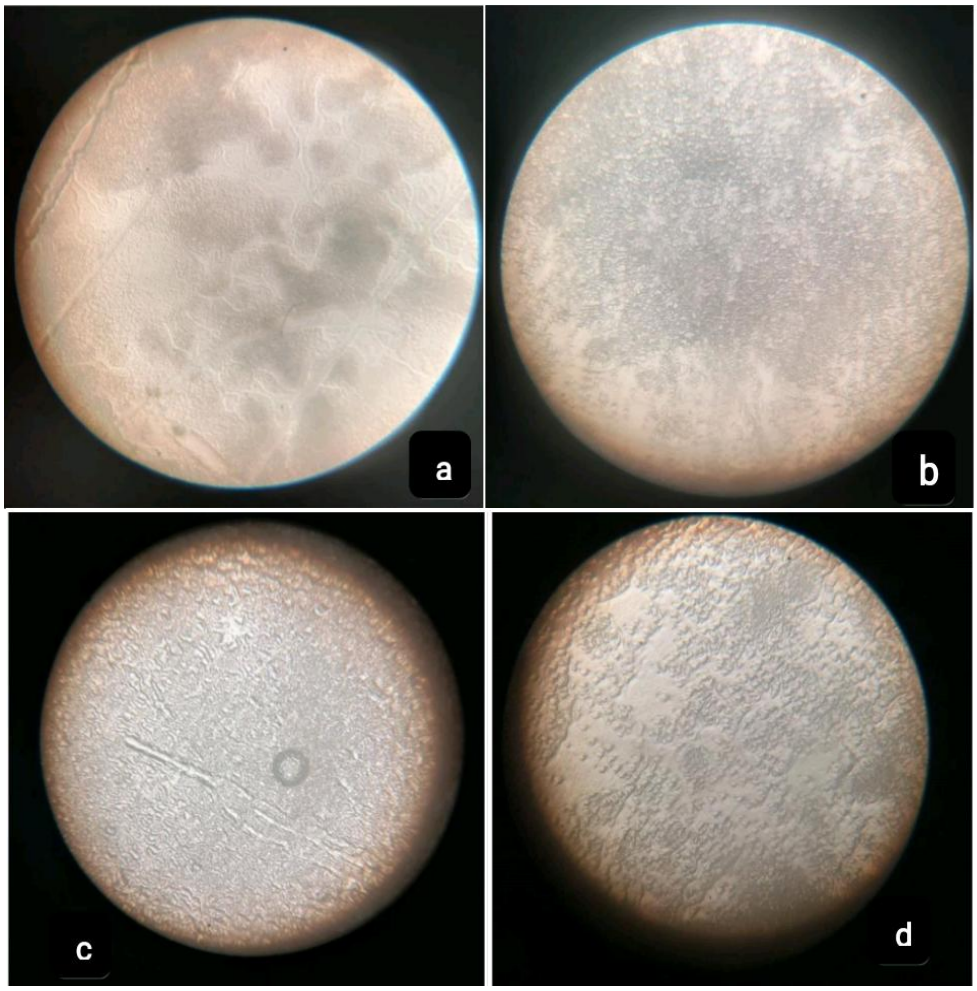

Figure (2) Liver primary cell culture grown in omega-3 medium in (a) 6 days (b) 12 days (c) 18 days (d) 24 days (Eosinx100).

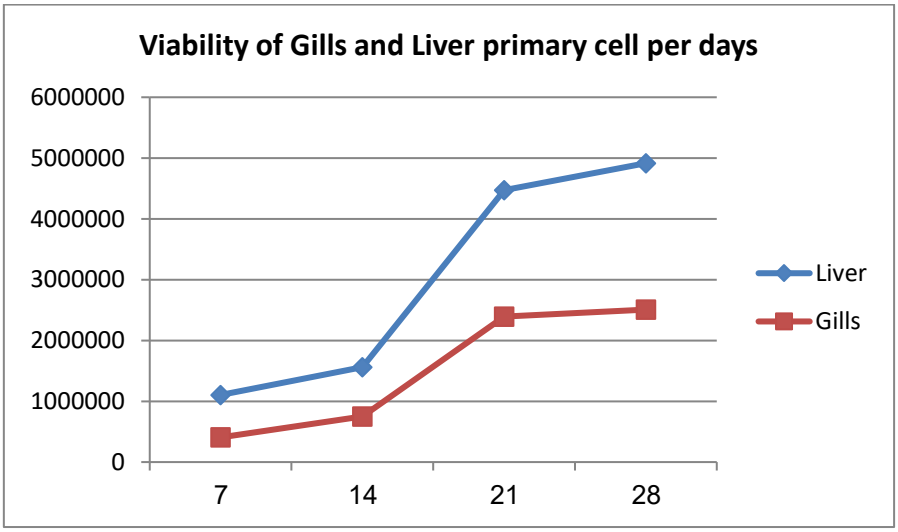

Figure (3) Viability of gills and liver primary cells in omega-3 medium per day: 7 days, 14 days, 21 days, and 28 days.

2. *In-vitro* Total Protein in Bioassay:

The total protein of gills and liver primary cells was affected by doses after 48 hours of exposure. The value of protein was determined according to the Bovine Serum Albumin (BSA) standard curve as shown in Table 1.

Table (1) Effect of Gasoline on liver and gill cells culture on protein concentration after 48 hours treatment:

Dose in mg/mL	Type of cell culture	Absorption of cells	Value of cell protein	Concentration of cell protein	Significance
0.00	Liver	0.455	24.93	**100**	**.018**
7.4		0.083	1.68	6.73	
3.7		0.129	4.56	18.28	
1.85		0.144	5.5	22.06	
0.92		0.150	5.87	23.54	
0.46		0.156	6.25	25.07	
0.00	Gills	0.257	12.56	100	
7.4		0.022	- 2.12	- 16.87	
3.7		0.050	- 0.375	- 2.98	
1.85		0.095	2.43	19.34	
0.92		0.101	2.81	22.37	
0.46		0.239	11.43	91	

3. Mitochondrial Suicidal Dehydrogenase Assay (MTT):

This colorimetric assay used the reduction of tetrazolium salt as a means to measure cellular metabolic activity for cell viability. Viable cells contained the NADpH-dependent oxidoreductase enzyme which reduced the MTT reagent to formazan and the deep purple colour was formed. The effect is also expressed by the degree of cell viability, as shown in table 2 which shows a reduction of viable cells and thus a reduction of formazan.

Table (2) Effect of four different concentrations of gasoline on Shield head (*Synodontis schall*) gill and liver primary cells on cell viability after 48 hours of treatment.

Dose in mg/mL	Type of cell culture	Absorption of cells	(Absorption of cells blank (DIMSO:0.002)	Viable cells%	Significance
0.00	Liver	0.147	0.0145	100	**.001**
7.4		0.019	0.017	11.7	
3.7		0.017	0.015	10.3	
1.85		0.023	0.021	15.1	
0.92		0.034	0.032	22	
0.46		0.067	0.065	44.8	
0.00	Gills	0.231	0.229	**100**	
7.4		0.019	0.021	7.4	
3.7		**0.**017	0.015	6.5	
1.85		**0.**078	0.076	33.1	
0.92		0.109	0.107	46.7	
0.46		0.157	0.155	67.6	

Discussion:

Primary cell cultures have been derived from gills and liver tissues of Shield head fish (Synodontis schall) and kept in Petri dishes containing omega-3 medium. After 28 days, the cell culture formed multiple layers. The results showed the omega-3 is a suitable medium for maintaining the viability of liver and gill primary cell cultures for more than 30 days. The results agreed with Fallahi [8], who studied some samples from goldfish (Carassiusauratus) internal organs, including the kidney, liver, muscles, ovary and external organs including fins, snout connective tissue and gills, collected and prepared according to standard procedures. The methods used include: enzymatic disaggregation by short- and long-term trypsinisation and without enzyme, by planting minced tissues and the ordinary method. The EMEM enriched by FBS was used for growing cells in 25oC incubation. The cells had suitable growth and reached complete confluency, and several subcultures were obtained from them. The best result was obtained from long time trypsinization of ovaries and muscles.

In a biochemical test of gills and liver primary cells, there was a decrease in total protein levels after 48 hours of exposure to gasoline. The results agreed with Salazar -Coria et al [9], who reported that Crude oil (CO) is a super mixture of chemical compounds whose toxic effects are reported in fish species according to international guidelines. In their study, a proteomic analysis of oxidised proteins (ox) was performed on the brain and liver of Nile tilapia exposed to WAF obtained from relevant environmental loads (0.01, 0.1 and 1.0 g/L) of Maya CO.

Results have shown that oxidation of specific proteins was a newly discovered organ-dependent process able to disrupt key functions in Nile tilapia.
Oxidative stress by gasoline was observed in mitochondrial activity dysfunction and DNA damage due to strand breaks. Viable cells contained the NAD pH-dependent oxidoreductase enzyme, which reduced the MTT reagent to formazan, and the deep purple colour was formed. The consequences of gasoline cytotoxicity on the liver and gill primary cells of Shield head fish were also expressed by the degree of cell viability, which showed a reduction of viable cells and thus a reduction of formazan. The results agreed with Aldoghachi et al [10] their results showed that changes in the activity of the enzyme can be considered as a pointer of the pollution impact in fish.

Conclusion:

Oil pollutants are considered one of the most widespread and influential sources of water pollution. Oil pollution occurs when petroleum products leak into bodies of water and spread to the surface of ocean water and deeper water layers. There are many causes of water pollution by oil, including accidents involving offshore oil wells.
In the present study, significant damage to the gill and liver cells of *Synodontis schall* was demonstrated, even at low doses of gasoline. In conclusion, the results indicated that gasoline induced cytotoxicity and toxicity through biochemical disturbances and cellular injuries in different types of cells of shieldhead fish (*Synodontis schall*) at different doses over time.

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