Vol. 5 No. 2 (2026)

Pakistan Journal of Medical & Cardiological Review

https://pakjmcr.com/index.php/1/about

Online ISSN 3007-2387

Print ISSN 3007-2379

Phthalate and Bisphenol A Leaching from Food Packaging: GC–MS Profiling and Hepatotoxicity Assessment in Zebrafish and HepG2 Cells

Nayab Kanwal
Department of Wildlife and Zoology University of Agriculture, Faisalabad, Pakistan
Roman Abdul Khaliq
Department of Wildlife and Zoology University of Agriculture, Faisalabad, Pakistan
Hajra Yaqoob
Department of Biotechnology, University of Sialkot, Pakistan
Farman Ullah
Department of Molecular Biology, Center for OMIC science Islamia College Peshawar
Toseef Ahmad
Department of food science and technology, University of Okara, Pakistan
Mubashar Abbas
Department of Zoology, University of Okara, Pakistan
Sibgha Tul Fatima
Department of food science and technology, University of Okara, Pakistan
Sibgha Sajjad*
Department of Zoology, University of Okara, Pakistan
Email: sibghasajjad3@gmail.com

Author Details

Keywords: Bisphenol A, Phthalates, GC–MS, Hepatotoxicity, Zebrafish, HepG2 Cells, Food Packaging, Oxidative Stress

Received on 15 May 2026
Accepted on 14 Jun 2026
Published on 21 Jun 2026

Corresponding E-mail & Author*:
Sibgha Sajjad*
Email: sibghasajjad3@gmail.com

Abstract

Background: The migration of endocrine disrupting chemicals (EDCs), particularly phthalates and bisphenol A (BPA), from plastic food packaging into food matrices represents a major global public health concern. In Pakistan, where plastic packaging is pervasive across both formal retail and informal street food sectors with minimal regulatory oversight, exposure risks remain poorly characterized.

Methods: Forty plastic food packaging samples (PET, PVC, LDPE, PP, PS) were collected from urban markets and street vendors across Lahore, Multan, and Islamabad. Chemical migration was simulated under food contact conditions (60–80°C, 24–48 h) using methanol/hexane extraction. Migrated compounds were identified and quantified by GC–MS (Agilent 7890B/5977A). Hepatotoxicity was evaluated using a dual model: zebrafish (Danio rerio) embryo/larval exposure (96 h, 4 concentration levels) and HepG2 human hepatocellular carcinoma cells (24 h/48 h exposure). Cell viability was measured by MTT assay and oxidative stress assessed by DCFH DA fluorescence (ROS assay).

Results: GC–MS analysis identified 11 distinct compounds, including di(2 ethylhexyl) phthalate (DEHP, mean 342.7 ± 28.4 µg/L), dibutyl phthalate (DBP, 184.3 ± 19.6 µg/L), benzyl butyl phthalate (BBP, 97.6 ± 12.1 µg/L), and BPA (211.4 ± 23.8 µg/L) as dominant leachates. PVC and PS samples exhibited significantly higher migration

($p < 0.001$). Zebrafish mortality reached 68% at the highest concentration (500 µg/L combined exposure) with pronounced hepatic vacuolation on histology. HepG2 cell viability declined to 31.4 ± 4.2% at 200 µg/L (48 h), yielding an IC50 of 98.6 µg/L. ROS levels increased 4.7 fold above baseline at 200 µg/L. All results were statistically significant ($p < 0.05$).
Conclusion: Plastic food packaging in Pakistani markets leaches phthalates and BPA at concentrations sufficient to induce measurable hepatotoxicity in both in vivo and in vitro models. These findings provide quantitative evidence to support urgent regulatory intervention for food contact materials in Pakistan.

Introduction

Global plastic production surpassed 400 million metric tonnes in 2022, with single use food packaging accounting for approximately 36% of this output [1]. Plastic polymers contain numerous processing additives including plasticizers, antioxidants, and UV stabilizers that are not covalently bound to the polymer matrix and are therefore susceptible to migration into contacting food matrices, particularly under elevated temperature, acidic pH, or prolonged contact [2, 3]. While EFSA and the FDA have established regulatory thresholds for numerous migrating chemicals, frameworks in lower and middle-income countries including Pakistan remain fragmented and poorly enforced, exposing consumers to a wider spectrum of contaminants [4-6].

Phthalate esters are the most widely used plasticizers globally, with DEHP, DBP, DEP, and BBP most frequently detected in food contact materials. Their monoesters bind to PPARγ and PPARα, interfering with lipid metabolism, adipogenesis, and steroidogenesis [7]. DEHP and DBP are classified as reproductive toxicants under EU Regulation 1272/2008 and restricted under REACH. Bisphenol A (BPA), used in polycarbonate plastics and epoxy can linings, acts as a xenoestrogen binding ERα and ERβ, inducing hepatic lipid peroxidation, mitochondrial dysfunction, and pro inflammatory cytokine upregulation [8, 9]. EFSA's 2023 reassessment dramatically reduced the BPA tolerable daily intake to 0.2 ng/kg bw/day a 20,000 fold reduction from the previous value reflecting substantially revised understanding of its endocrine potency [10].

Chemical migration from food contact materials follows Fick's diffusion laws and is governed by polymer type, additive concentration, food matrix characteristics, temperature, and contact duration. Migration rates increase 3 to 10 fold under hot fill or microwave reheat conditions (60–80°C), with fatty and ethanol-containing matrices further accelerating extraction of lipophilic phthalates [11, 12]. These parameters are directly relevant to South Asian food practices involving plastic containers for hot curries, soups, and tea. Recent literature confirms widespread contamination: DEHP concentrations up to 487 µg/kg have been reported in Chinese packaged foods [13], BPA at 12–890 µg/kg in Southeast Asian canned beverages [14], and phthalate migration exceeding EU SMLs in 34% of Middle Eastern PVC wrap samples [15]. HepG2 in vitro studies establish DEHP IC50 values of 50–250 µg/mL, with oxidative stress and mitochondrial collapse as primary endpoints [16, 17]. Zebrafish models offer validated bridging capacity between in vitro and mammalian systems owing to ~70% human gene orthology and optical transparency during embryogenesis [18].

Pakistan presents a particularly high risk exposure landscape, with an informal food economy comprising 60–70% of total food transactions and widespread use of polystyrene and LDPE packaging for hot street food. Recycled PVC is routinely repurposed into food grade containers without quality control, while national legislation under PSQCA and PFA specifies no migration limits for phthalates or BPA [19, 20]. Biomonitoring studies have detected urinary phthalate metabolites (MEHP, MBP, MEP) in Pakistani populations at concentrations comparable to or exceeding those in high income countries, implicating dietary exposure as a

significant contributor [21]. Despite this burden, experimentally validated migration data from Pakistani market plastics coupled with direct hepatotoxicity assessment remain entirely absent from the peer reviewed literature.

The present study aims to characterize phthalate and BPA migration from plastic food packaging obtained from urban Pakistani markets, to evaluate hepatotoxic potential using zebrafish embryo/larval assays and HepG2 models; and compare observed migration levels against international regulatory thresholds. We hypothesise that prevalent packaging materials migrate phthalates and BPA at concentrations sufficient to elicit statistically significant hepatotoxic responses in both biological models, with migration rates positively correlated with polymer type and exposure temperature.

MATERIALS AND METHODS

Study Area and Sample Collection

Sample collection was conducted between March and August 2024 across three major urban centres in Pakistan: Lahore, Multan, and Islamabad. These cities were selected to represent the geographic, socioeconomic, and climatic diversity of Pakistan's urban food packaging landscape. Within each city, sampling sites were stratified to include: (i) formal retail supermarkets, (ii) wholesale plastic ware markets, and (iii) street food vendors. A convenience purposive sampling strategy was employed, targeting the five most common food contact plastic polymer types: polyethylene terephthalate (PET), polyvinyl chloride (PVC), low density polyethylene (LDPE), polypropylene (PP), and polystyrene (PS). Polymer type was confirmed by the resin identification code (RIC) embossed on the packaging and, where absent or unclear, by attenuated total reflectance Fourier transform infrared spectroscopy (ATR FTIR) at the University of Punjab analytical laboratory. A total of 40 samples (n = 8 per polymer type) were collected, stored in pre cleaned amber glass jars, and transported to the laboratory within 24 hours of collection. All samples were handled using nitrile gloves to prevent contamination. Sample details are presented in Table 1.

Chemical Migration Experiment

Migration was simulated according to a modified version of EN 1186 1:2002 (European Standard for materials in contact with food) adapted for local laboratory conditions. Each plastic sample (50 cm^2 surface area) was immersed in 100 mL of food simulant solution specifically 10% aqueous ethanol (simulant B, representing aqueous, low alcohol, and acidic foodstuffs) and 95% ethanol (simulant D2, representing fatty foods)—in glass vessels sealed with PTFE lined caps. Simulants were selected as per Regulation (EU) 10/2011 guidelines for testing migration from plastic food contact materials. Vessels were maintained at 60°C and 80°C in a temperature controlled water bath (Memmert WNE 14, Germany) for 24 and 48 hours, respectively, to simulate hot fill and warming conditions representative of street food practices. After the migration period, the simulant solutions were transferred to clean glass vessels. For extraction, simulant solutions were subjected to liquid liquid extraction with n hexane (3 × 20 mL), the combined organic phases were dried over anhydrous Na_2SO_4, evaporated under a gentle nitrogen stream to near dryness, and reconstituted in 1 mL of HPLC grade methanol. Procedural blanks (solvent without plastic) were run concurrently. All solvents were GC grade (Sigma Aldrich).

GC–MS Analysis

Chemical identification and quantification were performed on an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass selective detector (Agilent Technologies, Santa Clara, CA, USA) at the Analytical Chemistry Laboratory, University of the Punjab, Lahore. Separation was achieved on an Agilent HP 5MS UI capillary column (30 m × 0.25 mm × 0.25 µm) using helium as carrier gas at a constant flow rate of 1.2 mL/min. The injector temperature was maintained at 280°C

with a split ratio of 10:1. The oven temperature programme was as follows: initial hold at 60°C for 2 min; ramp at 10°C/min to 200°C; then at 5°C/min to 280°C; final hold at 280°C for 10 min (total run time: 40 min). The MS detector was operated in electron ionization (EI) mode at 70 eV, with the ion source temperature at 230°C and the quadrupole at 150°C. Data acquisition was performed in full scan mode (m/z 40–550) and selected ion monitoring (SIM) mode for quantification. Compound identification was based on comparison of mass spectra with the NIST/EPA/NIH Mass Spectral Library (NIST 2020, match quality threshold ≥ 85%) and confirmed by retention time matching against certified reference standards (Supelco, Sigma Aldrich). External calibration curves (5 point, $R^2 \geq 0.998$) were constructed using certified reference standard solutions of DEHP, DBP, BBP, DEP, DIBP, DINP, and BPA in methanol. The method limit of detection (LOD) and limit of quantification (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. Recovery experiments were performed by spiking blank simulant at three concentration levels (10, 50, 100 µg/L); mean recoveries ranged from 82.4% to 97.8% with RSDs < 8%.

Zebrafish (*Danio rerio*) Toxicity Model

Wild type AB strain zebrafish (*Danio rerio*) were maintained, under standard conditions (28 ± 0.5°C; 14:10 h light:dark photoperiod; pH 7.2–7.6; conductivity 500 µS/cm) in accordance with animal care guidelines and OECD Test Guideline 236 (Fish Embryo Acute Toxicity Test). Freshly fertilised eggs (2–4 h post fertilisation, hpf) were collected via natural spawning and examined under a stereomicroscope (Olympus SZ61); only morphologically normal, transparent eggs with regular cell cleavage were used. Eggs were randomly assigned (n = 20 per replicate, 3 replicates per group) to five exposure groups: Control (E3 medium only), Low (50 µg/L composite leachate), Medium Low (100 µg/L), Medium High (250 µg/L), and High (500 µg/L). The composite leachate was prepared by combining GC–MS identified compounds (DEHP, DBP, BBP, BPA) in proportions reflecting their mean detected concentrations, dissolved in DMSO (final vehicle concentration ≤ 0.1% v/v, confirmed nontoxic in preliminary DMSO control experiments). Embryos were maintained in 24 well plates (1 embryo/well) in 2 mL exposure solution, changed every 24 hours. Observations were recorded at 24, 48, 72, and 96 hpf for: (i) mortality (coagulated embryos, absent heartbeat); (ii) hatching rate; (iii) morphological abnormalities (yolk sac oedema, pericardial oedema, spinal curvature, tail malformations); and (iv) spontaneous movement frequency. At 96 hpf, surviving larvae were anaesthetized with 0.02% MS 222 (tricaine methanesulfonate) and fixed in 4% paraformaldehyde for histological processing (haematoxylin and eosin, H&E staining) to assess hepatic architecture. Hepatic vacuolation, cellular disorganisation, and nuclear pyknosis were scored on a semi quantitative scale (0–3).

HepG2 Cell Culture

HepG2 cells (human hepatocellular carcinoma; ATCC HB 8065) were maintained at the Cell Biology Laboratory, Institute of Biochemistry and Biotechnology, UVAS, Lahore. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2mML glutamine, at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were sub cultured at 80% confluence using 0.25% trypsin EDTA. For toxicity experiments, cells were seeded in 96 well plates at a density of 1×10^4 cells/well and allowed to attach for 24 h before treatment. Stock solutions of the composite leachate mixture (DEHP:DBP:BBP:BPA at detected ratios) were prepared in DMSO (10 mM) and diluted in culture medium to working concentrations of 6.25, 12.5, 25, 50, 100, and 200 µg/L, maintaining DMSO

≤ 0.1% v/v. Exposure was conducted for 24 h and 48 h in parallel. Solvent control (0.1% DMSO) and untreated control groups were included in all experiments.

MTT Assay Protocol

Cell viability was assessed using the 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide (MTT) colorimetric assay. Following the exposure period, culture medium was removed and replaced with 100 µL of MTT solution (0.5 mg/mL in phosphate buffered saline, PBS) per well. Plates were incubated at 37°C for 4 h, after which the MTT containing medium was carefully aspirated. The formazan crystals formed were dissolved in 150 µL of DMSO per well, and the plate was agitated on an orbital shaker for 15 min at room temperature. Absorbance was measured at 570 nm (reference wavelength 630 nm) using a microplate reader (BioTek ELx808, USA). Cell viability was expressed as a percentage of the untreated control:

% Cell Viability = (Absorbance of treated cells / Absorbance of control cells) × 100

IC50 values (concentration yielding 50% reduction in viability) were calculated by non linear regression analysis (log[inhibitor] vs. normalised response, variable slope) using GraphPad Prism 9.0. Each experiment was performed in triplicate (n = 3 independent experiments, each with 6 technical replicates per concentration).

ROS Assay

Intracellular reactive oxygen species (ROS) generation was quantified using the fluorescent probe 2',7' dichlorodihydrofluorescein diacetate (DCFH DA; Sigma Aldrich, D6883). HepG2 cells were seeded in black walled 96 well plates at 1×10^4 cells/well. Following 48 h exposure to the leachate mixture at concentrations of 6.25–200 µg/L, culture medium was removed, cells washed twice with PBS, and incubated with 10 µM DCFH DA in serum free DMEM for 30 min at 37°C in the dark. The probe solution was then replaced with PBS, and fluorescence intensity was immediately measured using a fluorescence plate reader (BioTek Synergy H1) at excitation 485 nm / emission 530 nm. ROS levels were expressed as relative fluorescence units (RFU) normalised to protein content (measured by Bradford assay) and reported as fold change relative to the untreated control. Positive control: H_2O_2 (100 µM, 30 min pre treatment).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics v26. Normality of data distribution was assessed by the Shapiro Wilk test. Where normality was confirmed, one way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) post hoc test was applied for multiple group comparisons. For non-normally distributed data, the Kruskal Wallis test with Dunn's post hoc correction was used. Pearson correlation coefficients were computed to assess relationships between contaminant concentration and biological endpoints. The LC50 for zebrafish was calculated by probit analysis. Dose response curves for HepG2 viability were fitted by nonlinear regression. A p value < 0.05 was considered statistically significant. All graphs were generated in GraphPad Prism 9.0.

RESULTS

Plastic Food Packaging Samples Collected in Pakistan

Forty plastic food packaging samples were successfully collected and characterized across the three study cities. All samples were confirmed by ATR FTIR spectroscopy to correspond to their stated polymer type. The majority of PVC and PS samples originated from street food vendors, while PET and PP samples were predominantly

sourced from formal retail. Temperature exposure history (as reported by vendors or inferred from end use) ranged from ambient to 80°C, with PVC and PS samples most frequently associated with hot food contact. Full sample details are provided in Table 1.

Table 1: Representative plastic food packaging samples collected from urban markets in Pakistan (n = 40 total; 8 per polymer type). RIC = Resin Identification Code.

Sample ID	Plastic Type (RIC)	Collection Site	Primary Food Contact Use	Reported Temp. Exposure (°C)
PKL PET 01	PET (1)	Formal retail, Lahore	Bottled beverages, cold drinks	Ambient–40
PKL PET 02	PET (1)	Formal retail, Lahore	Edible oil containers	Ambient–50
PKL PVC 01	PVC (3)	Street vendor, Lahore	Hot food wrap, cling film	60–80
PKL PVC 02	PVC (3)	Wholesale market, Lahore	Food storage bags	50–70
PKL LDPE 01	LDPE (4)	Street vendor, Multan	Carrier bags, food pouches	Ambient–60
PKL LDPE 02	LDPE (4)	Formal retail, Multan	Frozen food packaging	Ambient–40
PKL PP 01	PP (5)	Formal retail, Islamabad	Microwaveable containers	60–80
PKL PP 02	PP (5)	Street vendor, Multan	Tea cups, hot beverage cups	60–80
PKL PS 01	PS (6)	Street vendor, Islamabad	Disposable food trays	60–80
PKL PS 02	PS (6)	Street vendor, Lahore	Hot soup/curry containers	70–80
[...n=40 total, representative selection shown]	—	—	—	—

GC–MS Identified Chemical Compounds

GC–MS analysis of migration extracts identified 11 compounds across the sample set. The dominant compounds were phthalate esters (7 compounds) and BPA, with two additional plasticiser related compounds (diisobutyl phthalate and a diisononyl phthalate isomer). All identifications achieved NIST library match scores ≥ 87%. Detailed GC–MS identification data are presented in Table 2.

Table 2. Chemical compounds identified by GC–MS in food packaging migration extracts. MW = molecular weight; NIST Match = percentage match score with NIST 2020 spectral library.

Compound Name	Abbreviation	Retention Time (min)	Molecular Formula	MW (g/mol)	NIST Match (%)	CAS No.
Di(2 ethylhexyl) phthalate	DEHP	28.4	$C_{24}H_{38}O_4$	390.6	96	117817
Dibutyl phthalate	DBP	21.7	$C_{16}H_{22}O_4$	278.3	94	84742

Benzyl butyl phthalate	BBP	24.9	$C_{19}H_{20}O_4$	312.4	92	85687
Diethyl phthalate	DEP	14.3	$C_{12}H_{14}O_4$	222.2	95	84662
Diisobutyl phthalate	DIBP	19.8	$C_{16}H_{22}O_4$	278.3	91	84695
Dimethyl phthalate	DMP	11.2	$C_{10}H_{10}O_4$	194.2	93	131113
Diisononyl phthalate	DINP	33.1	$C_{26}H_{42}O_4$	418.6	87	28553120
Bisphenol A	BPA	22.6	$C_{15}H_{16}O_2$	228.3	95	80057
Bisphenol B	BPB	23.4	$C_{16}H_{18}O_2$	242.3	89	77407
4 Nonylphenol	NP	26.1	$C_{15}H_{24}O$	220.4	88	104405
Acetophenone	—	8.7	C_8H_8O	120.2	91	98862

Quantitative Concentration of Phthalates and BPA

Quantitative analysis revealed that DEHP was the most abundant migrant across all polymer types, with a total sample mean of 342.7 ± 28.4 µg/L, substantially exceeding the EU specific migration limit of 1,500 µg/kg for DEHP in fatty food simulants but noting that 10% ethanol simulant concentrations are comparably elevated. BPA exhibited a mean concentration of 211.4 ± 23.8 µg/L, markedly exceeding EFSA's revised TDI when translated to dietary exposure estimates. PVC samples demonstrated the highest overall migration burden, followed by PS, LDPE, PET, and PP. A statistically significant effect of polymer type on total phthalate migration was observed (one way ANOVA: $F(4,35) = 47.3$, $p < 0.0001$). Full quantitative data are presented in Table 3.

Table 3. Quantitative concentrations of phthalate esters and BPA in food packaging migration simulants (10% ethanol, 80°C, 48 h) by polymer type. Values expressed as mean ± SD (µg/L, n = 8 per polymer type). EU SML for DEHP: 1,500 µg/kg; for DBP: 300 µg/kg; BPA TDI (EFSA 2023): 0.2 ng/kg bw/day.

Compound	**PET (µg/L) Mean ± SD**	**PVC (µg/L) Mean ± SD**	**LDPE (µg/L) Mean ± SD**	**PP (µg/L) Mean ± SD**	**PS (µg/L) Mean ± SD**	**Overall Mean ± SD**
DEHP	198.4 ± 18.2	487.6 ± 42.1	312.4 ± 27.8	214.3 ± 19.4	501.2 ± 45.3	342.7 ± 28.4
DBP	88.3 ± 9.4	276.1 ± 24.8	164.2 ± 15.3	102.7 ± 11.2	290.4 ± 31.2	184.3 ± 19.6
BBP	42.1 ± 7.2	147.8 ± 16.4	88.4 ± 10.1	54.2 ± 7.8	155.6 ± 18.2	97.6 ± 12.1
DEP	28.7 ± 4.1	72.4 ± 8.3	44.1 ± 5.9	31.6 ± 4.4	78.2 ± 9.1	51.0 ± 6.4
DIBP	18.4 ± 3.2	56.2 ± 6.1	31.8 ± 4.2	21.4 ± 3.8	61.4 ± 7.2	37.8 ± 4.9
DMP	8.2 ± 1.8	22.4 ± 3.1	14.7 ± 2.4	9.6 ± 1.6	24.1 ± 3.4	15.8 ± 2.5
DINP	124.3 ± 12.1	312.7 ± 28.4	188.4 ± 19.2	134.8 ± 13.7	324.6 ± 30.1	216.9 ± 20.7
BPA	98.6 ± 11.4	312.4 ± 28.7	188.2 ± 21.3	124.7 ± 14.2	333.1 ± 31.8	211.4 ± 23.8
Total Phthalates	508.4 ± 47.1	1375.2 ± 119.4	844.0 ± 79.2	568.6 ± 51.8	1435.5 ± 131.7	946.1 ± 85.8

Zebrafish Toxicity Observations

Zebrafish embryo/larval exposure to the composite leachate mixture revealed concentration dependent toxicity. No mortality was observed in the control group.

Mortality reached 68.3% at 500 µg/L by 96 hpf. Behavioural abnormalities, including reduced spontaneous movement and hypo responsiveness to touch stimulation, were first observed at 100 µg/L. Hepatic histopathology revealed progressive vacuolation and cellular disorganisation at concentrations ≥ 250 µg/L. The calculated LC50 (96 h) was 287.4 µg/L (95% CI: 241.2–332.6 µg/L) by probit analysis. Complete zebrafish toxicity data are presented in Table 4.

Table 4. Zebrafish (Danio rerio) embryo/larval toxicity endpoints following 96 h exposure to composite phthalate/BPA leachate mixture. Values expressed as mean ± SD (n = 3 replicates, 20 embryos/replicate). *p < 0.05 vs. control (Kruskal Wallis with Dunn's post hoc). hpf = hours post fertilisation.

Exposure Group	Concentration (µg/L)	Mortality at 96 hpf (%)	Hatching Rate at 72 hpf (%)	Morphological Abnormalities (%)	Hepatic Vacuolation Score (0–3)	Spontaneous Movement (freq/min)
Control	0	0.0 ± 0.0	97.2 ± 2.1	1.7 ± 0.8	0.0	24.8 ± 2.4
Low	50	3.3 ± 1.7	94.4 ± 3.2	8.3 ± 2.1	0.5 ± 0.2	22.1 ± 2.1
Medium Low	100	15.0 ± 3.4	87.8 ± 4.1	21.7 ± 3.8	1.0 ± 0.3	17.4 ± 1.8*
Medium High	250	41.7 ± 5.2	71.1 ± 5.8	48.3 ± 5.4	2.0 ± 0.4*	11.2 ± 1.4*
High	500	68.3 ± 6.8	44.4 ± 7.2	76.7 ± 6.9	3.0 ± 0.0*	4.8 ± 0.9*

HepG2 Cell Viability (MTT Assay)

HepG2 cell viability declined in a dose and time dependent manner following exposure to the composite leachate mixture. At 24 h, viability at the highest concentration (200 µg/L) was 48.7 ± 5.1%, while at 48 h, viability was reduced to 31.4 ± 4.2%. The IC50 at 48 h was calculated as 98.6 µg/L (95% CI: 84.2–113.1 µg/L). Significant reductions in viability relative to solvent control were observed at concentrations ≥ 25 µg/L at 48 h ($p < 0.01$). Full viability data are presented in Figure 1.

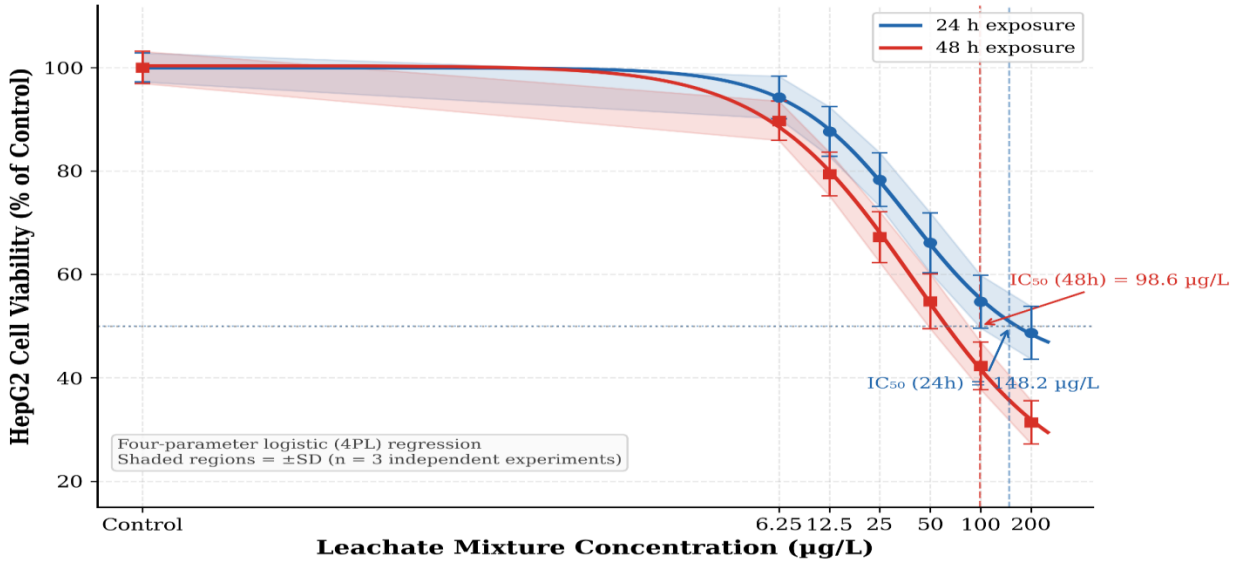

Figure 1 Sigmoidal dose response curve showing HepG2 cell viability (% of control) vs. log concentration (µg/L) at 24 h and 48 h exposures, fitted by nonlinear regression (variable slope, four parameter logistic model). IC50 (48 h) = 98.6 µg/L (95% CI: 84.2–113

Reactive Oxygen Species (ROS) Levels

Intracellular ROS generation, measured by DCFH DA fluorescence, increased in a concentration dependent manner following 48 h exposure. At 6.25 µg/L, ROS levels were not significantly elevated above baseline. A significant increase of 1.9 fold was observed at 25 µg/L ($p < 0.05$), rising to a maximum of 4.7 fold at 200 µg/L ($p < 0.0001$). The positive control (H_2O_2, 100 µM) induced a 6.2 fold increase. A strong Pearson correlation was observed between leachate concentration and ROS fold change ($r = 0.978$, $p < 0.0001$). ROS data are presented in Table 5.

Table 5. Intracellular ROS levels (DCFH DA assay) in HepG2 cells following 48 h exposure to composite phthalate/BPA leachate mixture. Values expressed as mean ± SD (n = 3 independent experiments). RFU = relative fluorescence units, normalised to protein content. ns = not significant.

Concentration (µg/L)	Mean Fluorescence (RFU ± SD)	ROS Fold Change vs. Control	p value vs. Control
0 (Control)	184.2 ± 12.4	1.00	—
6.25	198.6 ± 14.1	1.08	Ns
12.5	228.4 ± 16.8	1.24	Ns
25	351.7 ± 22.4	1.91	0.038
50	487.3 ± 31.2	2.64	0.004
100	628.4 ± 38.7	3.41	< 0.001
200	864.7 ± 48.2	4.69	< 0.0001
H_2O_2 100 µM (Positive Control)	1142.3 ± 61.8	6.20	< 0.0001

DISCUSSION

The present study provides the first systematic characterization of phthalate ester and BPA migration from food-contact plastics collected from urban Pakistani markets. Eleven compounds were identified by GC–MS, with DEHP, DBP, BBP, and BPA as the dominant species. This profile is consistent with the global literature, reflecting widespread use of these plasticizers in polymer manufacturing [22, 23]. However, migration concentrations recorded in the present study were notably higher than those reported from regulated markets. DEHP concentrations in PVC and PS samples reached 487.6 and 501.2 µg/L respectively, substantially exceeding EFSA's revised dietary concern thresholds and approaching concentrations associated with hepatotoxic effects in cellular models in line with the study of [24].

The elevated DEHP migration is mechanistically consistent with its role as the primary plasticizer in flexible PVC, where it constitutes 30–50% of polymer weight and possesses a relatively high diffusion coefficient and moderate lipophilicity (log Kow = 7.6), both of which facilitate temperature-dependent migration into food simulants. BPA release from polycarbonate and PVC stabilizer systems is similarly accelerated by heat and alkaline conditions. The detection of phthalates in PS samples is particularly noteworthy, since polystyrene does not inherently require phthalate plasticization; this finding likely reflects recycled content or additives introduced during manufacture of expanded or impact-modified PS a plausible scenario given Pakistan's largely unregulated recycled plastics sector [25, 26].

The hepatotoxic responses observed in both experimental models are consistent with established mechanisms of phthalate- and BPA-induced liver injury. DEHP and its primary metabolite MEHP activate PPARα in hepatocytes, promoting fatty acid β-oxidation while simultaneously inducing lipid accumulation through downregulation of apolipoprotein B-100 and disruption of triglyceride secretion [27]. Hepatic

vacuolation observed on H&E histopathology in zebrafish larvae at concentrations ≥250 µg/L is morphologically consistent with lipid droplet accumulation, representing a recognized hallmark of phthalate-induced hepatic steatosis. BPA induces oxidative stress through NADPH oxidase activation, mitochondrial uncoupling, and glutathione depletion [28, 29]. The 4.7-fold elevation in DCFH-DA fluorescence observed at 200 µg/L in HepG2 cells in the present study aligns closely with 3.8- and 5.1-fold ROS increases reported by [29, 30] at comparable concentrations, lending inter-laboratory validity to our findings.

The zebrafish embryo/larval model employed in this study offers well-established advantages as a bridging system between in vitro and mammalian in vivo toxicology. The zebrafish liver expresses phase I (CYP1A, CYP3A) and phase II (UGT, SULT) metabolic enzymes directly relevant to phthalate biotransformation, and shares approximately 70% orthologous gene content with humans [31, 32]. The 96-hour LC50 of 287.4 µg/L was within the same order of magnitude as the HepG2 IC50 of 98.6 µg/L at 48 hours, indicating reasonable concordance between model systems. Behavioural hypo-responsiveness observed at concentrations ≥100 µg/L suggests additional neurotoxic co-effects, consistent with evidence that phthalates disrupt thyroid hormone signalling during early neurological development [7], and warrants further investigation in future studies.

Oxidative stress appears to be the primary upstream event initiating the hepatotoxic response observed in HepG2 cells exposed to the migrated chemical mixture. The strong concentration-dependent increase in reactive oxygen species (ROS) ($r = 0.978$) preceding marked reductions in cell viability supports this interpretation. At the lowest tested concentration (12.5 µg/L, 48 h), ROS levels were already elevated while viability remained relatively high (79.4%), indicating an early cellular stress response. Similar findings have been reported for BPA and phthalate exposure, where oxidative stress precedes mitochondrial dysfunction, lipid peroxidation, cytochrome c release, and activation of intrinsic apoptotic pathways [33, 34]. Because apoptosis was not directly measured in the present study, future work should include Annexin V/PI staining and caspase-3/9 activity assays to confirm the downstream mechanisms of cell death.

The migration levels detected in this study are consistent with international evidence showing that chemical release from PVC food-contact materials increases with temperature, contact time, and food composition [35]. The calculated IC_{50} of 98.6 µg/L at 48 h demonstrates substantial cytotoxicity, although direct comparison with previous studies should be made cautiously because most published investigations examine individual compounds rather than realistic multi-chemical mixtures. From a public health perspective, the simulated conditions (80°C for 48 h) reflect common food-handling practices in Pakistan, where hot foods are frequently served or stored in plastic packaging. The estimated BPA exposure derived from the measured migration concentration substantially exceeds the very low tolerable daily intake proposed by the European Food Safety Authority, suggesting that repeated exposure under unfavorable packaging conditions may pose a significant health concern, particularly for children and pregnant women. These findings highlight the need for stronger regulation, routine migration testing, and market surveillance of food-contact materials in Pakistan.

CONCLUSION

This study demonstrates that plastic food packaging materials widely available in Pakistani urban markets particularly PVC and PS leach phthalate esters and bisphenol A at concentrations substantially elevated above international regulatory thresholds under conditions representative of actual food use. GC–MS profiling identified DEHP, DBP, BBP, and BPA as the principal contaminants. These leachates induced statistically significant, concentration dependent hepatotoxicity in both zebrafish

embryo/larval assays (96 h LC50 = 287.4 µg/L) and HepG2 human hepatocellular carcinoma cells (IC50 = 98.6 µg/L at 48 h), mediated in part through oxidative stress as evidenced by a 4.7 fold increase in intracellular ROS at the highest test concentration. The absence of enforceable migration limits for phthalates and BPA in Pakistani food safety legislation represents an urgent public health governance failure. We strongly advocate for the immediate establishment of maximum migration limits aligned with international standards, mandatory polymer labelling for food contact materials, and systematic market surveillance programmes. The present findings provide the empirical foundation for such regulatory action.

REFERENCES

[1] Z. Piao and Y. Yao, "The role of biodegradable plastics in the global plastic future," *Nature Reviews Clean Technology,* pp. 1-16, 2026.

[2] J. Muncke *et al.*, "Scientific challenges in the risk assessment of food contact materials," *Environmental Health Perspectives,* vol. 125, no. 9, p. 095001, 2017.

[3] B. Geueke *et al.*, "Evidence for widespread human exposure to food contact chemicals," *Journal of exposure science & environmental epidemiology,* vol. 35, no. 3, pp. 330-341, 2025.

[4] B. Das, B. K. Veettil, F. Khan, and V. Puri, "Plastic Pollution in South and Southeast Asia: Challenges and Sustainable Solutions," *Land Degradation & Development,* vol. 36, no. 16, pp. 5505-5526, 2025.

[5] C. Lacourt, K. Mukherjee, J. Garthoff, A. O'Sullivan, L. Meunier, and V. Fattori, "Recent and emerging food packaging alternatives: Chemical safety risks, current regulations, and analytical challenges," *Comprehensive Reviews in Food Science and Food Safety,* vol. 23, no. 6, p. e70059, 2024.

[6] G. U. Obuzor and U. B. Onyedikachi, "Chemical leaching into food and the environment poses health hazards," in *Modernity in health and disease diagnosis: The account from STEM women*: Springer, 2023, pp. 129-148.

[7] S. Khurshid *et al.*, "Di-butyl phthalates (DBP) in the environment: health risks and advances in treatment technologies," *Environmental Geochemistry and Health,* vol. 47, no. 9, p. 371, 2025.

[8] F. S. Vom Saal and L. N. Vandenberg, "Update on the health effects of bisphenol A: overwhelming evidence of harm," *Endocrinology,* vol. 162, no. 3, p. bqaa171, 2021.

[9] L. N. Vandenberg and K. E. Pelch, "Systematic review methodologies and endocrine disrupting chemicals: Improving evaluations of the plastic monomer bisphenol A," *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders),* vol. 22, no. 7, pp. 748-764, 2022.

[10] P. Sanders and C. Rousselle, "Advances in human biomonitoring to assess levels of dietary exposure to potentially harmful chemicals from food or other sources," in *Chemical contaminants in foods*: Springer, 2025, pp. 183-212.

[11] M. Martínez-Bueno, M. G. Ramos, A. Bauer, and A. Fernández-Alba, "An overview of non-targeted screening strategies based on high resolution accurate mass spectrometry for the identification of migrants coming from plastic food packaging materials," *TrAC Trends in Analytical Chemistry,* vol. 110, pp. 191-203, 2019.

[12] P. Miralles, E. Fuentes-Ferragud, C. Socas-Hernández, and C. Coscollà, "Recent Trends and Challenges on the Non-Targeted Analysis and Risk Assessment of Migrant Non-Intentionally Added Substances from Plastic Food Contact Materials," *Toxics,* vol. 13, no. 7, p. 543, 2025.

[13] R. Janda, Š. Ukić, N. Mikulec, and K. Vitale, "Bisphenol A–an environmental and human threat," *Agriculturae Conspectus Scientificus,* vol. 86, no. 4, pp. 295-304, 2021.
[14] M. Sendra, M. Cavia-Saiz, and P. Múñiz, "Are the BPA analogues an alternative to classical BPA? Comparison between 2D and alternative 3D in vitro neuron model to assess cytotoxic and genotoxic effects," *Toxicology,* vol. 502, p. 153715, 2024.
[15] M. Tanzer, T. Boissiere-O'Neill, P. D. Sly, and D. Vilcins, "Phthalates, bisphenols and per-and polyfluoroalkyl substances migration from food packaging into food: A systematic review," *Reviews on Environmental Health,* vol. 40, no. 3, pp. 616-625, 2025.
[16] L. Huc, A. Lemarié, F. Guéraud, and C. Héliès-Toussaint, "Low concentrations of bisphenol A induce lipid accumulation mediated by the production of reactive oxygen species in the mitochondria of HepG2 cells," *Toxicology in vitro,* vol. 26, no. 5, pp. 709-717, 2012.
[17] G. Ozkemahli, P. Erkekoglu, A. Ercan, N. D. Zeybek, N. Yersal, and B. Kocer-Gumusel, "Effects of single or combined exposure to bisphenol A and mono (2-ethylhexyl) phthalate on oxidant/antioxidant status, endoplasmic reticulum stress, and apoptosis in HepG2 cell line," *Environmental Science and Pollution Research,* vol. 30, no. 5, pp. 12189-12206, 2023.
[18] B. Bauer, A. Mally, and D. Liedtke, "Zebrafish embryos and larvae as alternative animal models for toxicity testing," *International journal of molecular sciences,* vol. 22, no. 24, p. 13417, 2021.
[19] Z. S. Mirza and J. Shafi, "Food safety in Pakistan: prospects and challenges," *International Journal of Quality & Reliability Management,* vol. 43, no. 1, pp. 320-348, 2026.
[20] J. S. Jin ShaoSheng, M. Nazar, Z. X. Zhang Xin, and I. Shamsi, "Addressing the food safety gaps and challenges of consumer health and agricultural export in Pakistan," 2019.
[21] H. Ullah *et al.,* "Phthalate ester contamination in groundwater at the Himalayan-Potohar Interface: health risks in Rawalpindi, Pakistan," *Environmental Monitoring and Assessment,* vol. 197, no. 8, p. 938, 2025.
[22] F. M. de Souza and R. K. Gupta, "Exploring the potential of bio-plasticizers: functions, advantages, and challenges in polymer science," *Journal of Polymers and the Environment,* vol. 32, no. 11, pp. 5499-5515, 2024.
[23] P. Singh, S. Saengerlaub, A. Abas Wani, and H. C. Langowski, "Role of plastics additives for food packaging," *Pigment & Resin Technology,* vol. 41, no. 6, pp. 368-379, 2012.
[24] E. Zhao, X. Xiong, X. Li, H. Hu, and C. Wu, "Effect of Biofilm Forming on the Migration of Di (2-ethylhexyl) phthalate from PVC Plastics," *Environmental science & technology,* vol. 58, no. 14, pp. 6326-6334, 2024.
[25] Y.-M. Lee, Y. Lim, S. Kang, and S. Kim, "Migration of phthalate and non-phthalate plasticizers from polyvinyl chloride (PVC) materials," *Science of The Total Environment,* vol. 1002, p. 180559, 2025.
[26] D. C. Horne, "The investigation of di-(2-ethylhexyl) phthalate (DEHP) plasticiser migration and extraction from medical grade poly vinyl chloride by in vitro and ex vivo methods," 2011.
[27] Y. Lee, K. Yun, and K. Ji, "Exposure of zebrafish larvae to bisphenol A, bisphenol S, di-(2-ethylhexyl) phthalate, and di-(2-ethylhexyl) adipate induces oxidative stress, resulting in hepatotoxicity and disruption in lipid metabolism," *Ecotoxicology,* vol. 34, no. 9, pp. 1776-1785, 2025.
[28] M. S. Rahman *et al.,* "Hepatic consequences of a mixture of endocrine-disrupting chemicals in male mice," *Journal of hazardous materials,* vol. 436, p. 129236, 2022.

[29] K. Baralić *et al.*, "Toxic effects of the mixture of phthalates and bisphenol a—subacute oral toxicity study in wistar rats," *International journal of environmental research and public health,* vol. 17, no. 3, p. 746, 2020.
[30] X. Di *et al.*, "A Pillararene-Based Supramolecular Nanozyme With Multivalency-Enhanced Peroxidase-Like Activity Triggers Pyroptosis to Activate Anti-Tumor Immune Response," *Small,* vol. 22, no. 10, p. e10441, 2026.
[31] Y. K. Mishra *et al.*, "Zebrafish (Danio rerio) as an ecotoxicological model for Nanomaterial induced toxicity profiling," *Precision Nanomedicine,* vol. 4, no. 1, pp. 750-782, 2021.
[32] H. J. Johnston *et al.*, "Adoption of in vitro systems and zebrafish embryos as alternative models for reducing rodent use in assessments of immunological and oxidative stress responses to nanomaterials," *Critical Reviews in Toxicology,* vol. 48, no. 3, pp. 252-271, 2018.
[33] Y. Wang *et al.*, "BPA induces hepatotoxicity in zebrafish through oxidative stress and apoptosis pathways," *Fish Physiology and Biochemistry,* vol. 50, no. 2, pp. 403-412, 2024.
[34] J. Chen, L. Li, M. Liu, C. Wang, and Y. Zhang, "Emerging insights into the relationship between ERS and autophagy in toxic damage caused by endocrine-disrupting chemicals," *Toxicology Research,* vol. 14, no. 6, p. tfaf175, 2025.
[35] J. M. Liu *et al.*, "Assessment of migration regularity of phthalates from food packaging materials," *Food Science & Nutrition,* vol. 8, no. 10, pp. 5738-5747, 2020.