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# Binary, ternary and quaternary mixture toxicity of benzo[a]pyrene, arsenic, cadmium and lead in HepG2 cells

Sasikumar Muthusamy [1, 2], Cheng Peng[1, 2], Jack C. Ng[1, 2,*]

[1]The University of Queensland, National Research Centre for Environmental Toxicology (Entox), Coopers Plains, Brisbane, QLD 4108, Australia.

[2]CRC CARE, The University of Newcastle, University Drive, Callaghan NSW 2308, Australia.

*To whom correspondence should be addressed:

Professor Jack Ng, PhD, DABT,

Professorial Research Fellow,

The University of Queensland,

Entox (National Research Centre for Environmental Toxicology),

Faculty of Health and Behavioural Sciences,

39 Kessels Road, Coopers Plains, Brisbane, Queensland, Australia 4108.

Tel: +61 414 747 147; Fax: +61 7 32739003.

Email: j.ng@uq.edu.au

## ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) and heavy metal/loid(s) are common environmental pollutants. Toxicological interaction data on benzo[a]pyrene (B[a]P) and heavy metal/loid(s) are lacking. In this study, we have determined the combined toxicity of B[a]P, arsenic (As), cadmium (Cd) and lead (Pb) in HepG2 cells. Binary, ternary and quaternary mixture toxicity of B[a]P and heavy metal/loid(s) was predicted by using the combination index (CI)- isobologram method. This method is useful to predict quantitative nature of interaction between chemicals at different effect (inhibitory concentration) levels from 0.1 to 99% using computerised quantitation. A total of 11 mixtures including six binary mixtures, four ternary and one quaternary mixtures of B[a]P and heavy metal/loid(s) were evaluated for their interactions. Cytotoxicity of individual and multi-component mixtures was evaluated by MTS assay. The selected concentrations for individual dose response study was 0-100 μM - B[a]P; 0-40 μM – Cd; 0-400 μM – As and Pb. The individual dose response results showed that all four chemicals were toxic to liver cells with Cd being the most potent toxicant. Mixtures of B[a]P and heavy metal/loid(s) were prepared based on their individual $D_m$ concentration using 1:1 ratio and exposed to HepG2 cells. By using the CI-isobologram method, the predicted interactions between these chemicals were synergism, additivity or antagonism at different effect levels. All the mixtures except ternary mixture of B[a]P + As + Pb displayed synergism at lower effect level ($IC_{10}$-$IC_{30}$), additivity, synergism or antagonism at 50-90% effect levels. Among these mixtures, mixtures of heavy metal/loid(s) (both binary and ternary combinations) and quaternary mixture of B[a]P + As + Cd+ Pb showed strong synergistic response at lower effect levels compared to other mixtures. The predicted interaction response by the CI method was compared with classical models of concentration addition and independent action. The CI method displayed improved prediction power compared to classical models. The predicted synergistic interaction between B[a]P and heavy

metal/loid(s) may have important implication on human health risk assessment of these mixed chemicals mixtures at contaminated sites.

## ABBREVIATIONS

As – Arsenic

ATCC- American type culture collection

ATSDR- Agency for toxic substances and disease registry

B[a]P- Benzo[a]pyrene

CA- Concentration addition

Cd- Cadmium

CI- Combination index

Cr- Chromium

$D_{m-}$ Concentration for 50% effect (e.g., 50% inhibition of cell viability or inhibitory concentration)

DMEM- Dulbecco's Modified Eagle Medium

DMSO- Dimethyl sulfoxide

EDTA- Ethylene diamine tetra acetic acid

H- hours

HepG2- Human hepatocellular carcinoma cell

IA- Independent action

IARC- International agency for research on cancer

IPQ- Index on prediction quality

MT- Metallothionein

MTS – tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

OD- Optical density

PAHs- Polycyclic aromatic hydrocarbons

Pb- Lead

PBS- Phosphate buffered saline

r- Linear correlation coefficient of the median-effect plot

µM- Micro molar

## INTRODUCTION

Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH) is formed during the process of incomplete combustion of organic material (eg. gasoline and wood) and found widely in tobacco smoke and grilled foods. Anthropogenic and industrial activities results in elevated concentration of B[a]P in environment.[1] B[a]P has been classified as class I carcinogen by IARC and it also causes teratogenicity, neurotoxicity and immunotoxicity. [2]

Arsenic (As), cadmium (Cd) and lead (Pb) are naturally occurring substances in the environment. Besides their natural occurrence, manufacturing and industrial activities such as mining, smelting operations, storage and disposal of waste from these process results in elevated concentrations of these contaminants in the environment.[3] Human exposure to heavy metal/loid(s) has been associated with a wide range of toxic effects.[4] Exposure to arsenic causes skin toxicity, liver injury, peripheral neuropathy, cardiovascular diseases and immunotoxicity.[5] Cd exposure results in testicular damage, pulmonary edema, osteomalacia, renal and hepatic dysfunction in humans.[6] Pb causes reduction in haemoglobin synthesis, neurological, neurobehavioral and developmental effects in children.[7] Furthermore, As and Cd are classified as class I carcinogen and Pb is classified as Class II carcinogen.

As, Pb, Cd and B[a]P have been ranked as 1, 2, 7 and 8$^{th}$ priority pollutants as per the Agency for Toxic Substances and Disease Registry (ATSDR) substance priority list.[8] Mixed contamination of PAHs and heavy metal/loid(s) are ubiquitous in environment and has received significant attention due to their known adverse health effects to humans and other organisms.[9-12] Soil is one of the major sink for these mixed contaminants and their co-occurrence at various contaminated sites around the world has been reported.[13-17]

Humans are very rarely exposed to individual contaminants of B[a]P or heavy metal/loid(s), exposure is more likely to mixture of chemicals from the contaminated sites. The available

toxicity data for B[a]P and heavy metal/loid(s) mixtures is very limited for their risk assessment. Human health assessment of B[a]P and heavy metal/loid(s) is carried out based on their individual toxicity. The reported interaction toxicity data for mixtures of As, Cd and Pb is limited to their binary combinations and the data are inconsistent for same endpoints from study to study and less relevant in terms of end points compared to their individual toxicity data.[18] In the literature, only two reports were available for metal mixtures toxicity beyond their binary combinations.[19-20] In case of B[a]P and metal mixtures, few studies were reported on As and Cd effect on B[a]P's genotoxicity and metabolism.[21-24] To the best of our knowledge there is no detailed study available with respect to assessment of multi-component mixture toxicity of B[a]P and heavy metal/loid(s).

One possible route for human exposure for these chemicals from soil is by oral ingestion. Liver, the organ for biotransformation is one of the main target organs for exposed chemicals. B[a]P and heavy metal/loid(s) accumulate in liver followed by their absorption and cause toxicity to liver cells.[25-33] In this study, we have used HepG2 (hepatocellular carcinoma) cell line to evaluate the interaction toxicity. This cell line is well established for toxicological research and possesses a liver-like enzyme pattern including the enzymes for biotransformation and the level of CYP enzymes expression although it is lower compared to primary hepatocytes.[34-35]

Concentration addition (CA) and independent action (IA) models are extensively used to predict the mixture toxicity of chemicals.[36-38] Combination Index (CI) method developed by Chou and Talalay[39] is commonly used for drug combination studies and in recent years this method has been used by various researchers to determine the interaction between environmental chemicals for ecotoxicity risk assessment. [40-42] One distinct advantage of this model is prior knowledge about chemicals mode of action is not required in order to determine their interaction.

The objective of this study is to determine the interaction cytotoxicity of B[a]P, As, Cd and Pb in HepG2 cells. Binary, ternary and quaternary mixture toxicities of B[a]P and heavy metal/loid(s) were predicted by using the combination index (CI)- isobologram method.

## MATERIALS & METHODS

### Chemicals

Cell culture medium DMEM (Dulbecco's Modified Eagle Medium), Trypsin-EDTA (0.25%), penicillin-streptomycin solution and fetal bovine serum (FBS) were purchased from Gibco® (Life Technologies, VIC, Australia). CellTiter 96 ® Aqueous One Solution Cell Proliferation Assay (G3581) was purchased from Promega Corporation, Madison, USA. Benzo[a]pyrene (B[a]P) (CAS ID-50-32-8), cadmium chloride (CAS ID-10108-64-2), lead acetate (CAS ID-6080-56-4) and sodium arsenite (CAS ID-7784-46-5) were purchased from Sigma-Aldrich, St. Louis, USA.

### Cell culture

HepG2 cells were obtained from American type culture collection (ATCC), USA (ATCC No. HB-8065). This cell line is derived from a liver hepatocellular carcinoma of a 15 year old Caucasian male. After thawing, cells were maintained as sub confluent monolayer in a 75 $cm^2$ culture flask using the modified DMEM medium with 10% (v/v) FBS + penicillin-streptomycin (50 Units/ml). Cells were used for experiment after two week of thawing from cryopreserved stock. On the day of harvest, the cell monolayer was rinsed with 1 x PBS and pre-warmed 0.25% trypsin-EDTA solution was added. Cells ($1\times10^4$ cells/well) were seeded into 96 well plates and allowed to attach for 24 h before chemical treatment. During the experiment period, cells were maintained in a $CO_2$ incubator at 37 °C under 5% $CO_2$.

Stock solution of B[a]P was prepared in DMSO, whereas solutions of heavy metal/loid(s) (As, Cd and Pb) were prepared in MilliQ water respectively. Working solutions were prepared in DMEM medium and added to the plates reaching a final concentration of 1% v/v of vehicle control (DMSO or MilliQ water).

**Chemical Treatment**

Individual dose response was evaluated for B[a]P and heavy metal/loid(s) by exposing them to HepG2 cells for 24 h. Selected concentrations were 0 to100 μM- B[a]P, 0 to 40 μM -Cd and 0 to 400 μM for both As and Pb. Treatment was carried out in triplicate for vehicle (DMSO or MQ water) and chemicals. The details of selected individual concentrations were provided in Table S1 of supplementary information.

**Cytotoxicity assay**

Cell viability was determined using CellTiter 96® aqueous one solution, which is a colorimetric method for determining the number of viable cells. This solution contains a tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and an electron coupling reagent PES (phenazine ethosulfate). The principle of this assay is based on metabolic reduction of tetrazolium salts. Viable cells with active metabolism convert MTS salt into purple colored formazan product. The exact mechanism of metabolic reduction is not well understood, enzymes from endoplasmic reticulum, mitochondria and from cell surface is attributed to the observed metabolic reduction activity.

After a chemical treatment period of 24 h, the treatment medium was carefully aspirated using multi-channel micropipette and 20 μL of MTS reagent + 100 μL of DMEM was added

to each well and incubated for another 2-3 h. The plates were placed in a microplate reader and the absorbance was measured at 490 nm.

Cell viability was calculated as shown in equation (1) after blanking.

$$\text{Viability (\%)} = \frac{100 * \text{mean optical density (OD) in single chemical or mixtures treated samples}}{\text{mean OD in vehicle control treated samples}} \text{ ---- (1)}$$

**Mixture experiments**

Mixture studies were conducted for a total of 11 mixtures including six binary mixtures (As + Cd, As + Pb, Cd + Pb, B[a]P +As, B[a]P + Cd and B[a]P + Pb), four ternary mixtures (As + Cd + Pb, B[a]P + As + Cd, B[a]P + As + Pb and B[a]P + Cd+ Pb) and one quaternary mixture (B[a]P + As + Cd + Pb). Each mixture was prepared based on their individual Dm concentration and mixed at 1:1 ratio. The mixtures were further diluted serially at two fold for 8 times. Three independent experiments were conducted for chemical mixtures and treatment was carried out in triplicate for each concentration and vehicle control (DMSO/or MQ water). The details of individual chemical concentrations in mixtures were provided in Table S1 of supplementary information.

**Individual and mixtures toxicity determination**

Individual toxicity of B[a]P, As, Cd and Pb and their binary, ternary and quaternary interaction was determined by using the median effect and combination index (CI) isobologram respectively.[39] This method is based on median-effect principle (mass-action law) which defines the relationship between concentration of individual/mixtures and cytotoxic effect, independent of number of chemicals and also mechanism of action. In this method, response for individual and their combination is determined by using the median–effect equation (2).

$$\frac{f_a}{f_u} = (D/D_m)^m \quad \text{---------- (2)}$$

D is the concentration of chemicals,

$D_m$ is the concentration for 50% effect (e.g., 50% inhibition of cell viability or $IC_{50}$),

$f_a$ is the fraction affected by dose D (e.g., 0.50 if cell viability is inhibited by 50%),

$f_u$ is the unaffected fraction (therefore, $f_a = 1 - f_u$), and

m is the coefficient of the sigmoidicity of the dose-effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively.

$D_m$ and m values for each chemical were calculated by using median-effect plot by taking account of both potency ($D_m$) and shape (m) parameters. These parameters ($D_m$ and m) were used to calculate concentrations of individual chemical and their combination required to produce various effect levels (e.g. 10, 30, 50 and 90% effect) by using the median-effect equation.

Combination index (CI) values were calculated for n number of chemical combination at x% effect by using equation (3).

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\{[D]_j / \sum_1^n [D]\}}{(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}} \quad \text{---------- (3)}$$

$^{n}(CI)_x$ is the combination index for n chemicals at x% inhibition (e.g., cytotoxicity)

$(D_x)_{1-n}$ is the sum of the concentration of n chemicals that exerts x% inhibition in combination

$\{[D_j]/\sum_1^n [D]\}$ is the proportionality of the concentration of each of n chemicals that exerts x% inhibition in combination

$(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the concentration of each chemicals alone that exerts x% inhibition

$f_a$ is the fraction affected by individual chemical/mixture concentration.

**Mixture toxicity prediction by concentration addition, independent action and CI-isobologram method**

The predicted dose response by CI method was compared with classical models of CA and IA.

CA model is used for chemicals which are having similar mode of action.

Concentration addition (CA) = $EC_{xmix} = \sum_{i=0}^{n} \left(\frac{p_i}{EC_{xi}}\right)^{-1}$ ---------- (4)

$EC_{xmix}$ is the effect concentration of the mixture provoking *x*% effect

$EC_{xi}$ is the concentration of component *i* provoking the same effect (*x*%) as the mixture when applied individually

*pi* is the molar concentration ratio of the $i^{th}$ component in the mixture.

For chemicals with different mode of action, interaction is calculated by using equation (5)

Independent action (IA) = $E(C_{mix}) = 1 - \prod_{i=1}^{n} (1-E(C_i))$ ---------- (5)

$C_{mix}$ and $E(C_{mix})$ are the total concentration and total effect of the mixture respectively

$E(C_i)$ is the effect of the $i^{th}$ component with the concentration of *ci* in the mixture.

The predictive dose response for CI method is computed by using equation (6).

$CI = = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{xi} \times CI_{xcomp}}\right)^{-1}$ ---------- (6)

$CI_{xcomp}$ is the computed CI value for each mixtures at different effect level (0.1-97%) from independent experimental toxicity data of the mixtures.[35] Dose response for each mixture was predicted using computed CI values from individual mixture toxicity experiment and different $f_a$ levels.

Quantitative relationship between predicted and observed effect was determiend by index on prediction quality (IPQ).[43] Predicted dose response from three different models CA, IA and

CI was compared with experimental dose response of multi-component mixtures of B[a]P and heavy metal/loid(s).

If the predicted effect is greater than observed value, the prediction quality is determined by using equation (7)

For $EC_{predicted} > EC_{observed}$, $IPQ = \left(\frac{EC_{predicted}}{EC_{observed}}\right) - 1$ ---------- (7)

If the predicted value is less than observed value, the prediction quality is determined by using equation (8)

For $EC_{predicted} < EC_{observed}$, $IPQ = -\left(\frac{EC_{observed}}{EC_{predicted}}\right) + 1$ ---------- (8)

IPQ value of zero indicates exact prediction by reference models, IPQ value <0 indicates overestimation; values>0 indicates underestimation of mixture effects.

**Data analysis**

Computer program CompuSyn developed by Chou and Martin[44] was used for the calculation of dose-effect curve parameters, CI values and $f_a$-CI plot. The details of CI values and corresponding descriptions are presented in Table 1.

## RESULTS

### Individual toxicity of B[a]P and heavy metal/loid(s)

The individual dose response cytotoxicity results showed that both B[a]P and heavy metal/loid(s) were toxic to HepG2 cells and Cd was found to be the most toxic compared to other chemicals with $D_m$ of 2.70 µM followed by B[a]P ($D_m$-37 µM). The $D_m$ concentration for As and Pb is 159 and 217 µM respectively. Dose response curve parameters $D_m$ (concentration for 50% effect on cell viability or $IC_{50}$), m (coefficient of the sigmoidicity of the dose-effect curve), r (linear correlation coefficient of the median-effect plot) for individual chemicals are presented in Table 2.

**Toxicological interactions of multi-component mixtures of B[a]P and heavy metal/loid(s)**

Dose effect curve parameters ($D_m$, m and r) for binary mixtures of heavy metal/loid(s) (As + Cd, Cd+ Pb and As+ Pb), binary mixtures of B[a]P with heavy metal/loid(s) (B[a]P + As, B[a]P + Cd and B[a]P + Pb), ternary mixtures (Cd + As + Pb, B[a]P + As + Cd, B[a]P + AS + Pb and B[a]P + Cd + Pb) and quaternary mixtures (B[a]P + As + Cd+ Pb) and their cytotoxicity to HepG2 cells are summarised in Table 2. Different type of interaction (synergism, antagonism or additivity) between these mixtures and combination index plot ($f_a$-CI plot) are presented in Figure 1. For each mixture, average CI values for four effect level ($IC_{10}$, $IC_{30}$, $IC_{50}$ and $IC_{90}$) are summarised in Table 2.

**Binary mixture toxicity of heavy metal/loid(s)**

All the three heavy metal/loid(s) mixtures (As + Cd, Cd + Pb and As + Pb) displayed synergistic response at 10% level with strong synergism was observed for mixture of As + Pb with CI value of 0.25. The observed synergism was continued up to 30 and 50% level for Cd+ Pb and As + Pb mixtures respectively. Binary mixture of As + Cd showed additive response at 30-50% level. All the mixtures showed antagonistic effect at $IC_{90}$ level and mixture of As + Pb displayed strong antagonism (CI value 3.3).

**Binary mixture toxicity of B[a]P and heavy metal/loid(s)**

Binary mixture of B[a]P + Cd showed synergistic effect up to 50% level and antagonism at 90% level. Mixtures of B[a]P + As, B[a]P + Pb displayed synergism at 10-30% level, additivity at 50% level and antagonism at 90% level. All three mixtures displayed same levels of synergistic response at lower effect levels (10-30% level).

**Ternary and quaternary mixtures toxicity of B[a]P and heavy metal/loid(s)**

Ternary mixtures of B[a]P + As + Cd and B[a]P + Cd + Pb displayed synergism at 10-50% level and antagonism at 90% level. Mixture of B[a]P + As + Pb displayed antagonistic response for entire effect level (10-90%). Ternary mixture of heavy metal/loid(s) showed synergism at 10-50% level and antagonism at 90% level. Among all ternary mixtures, heavy metal/loid(s) mixtures showed strong synergism at lower effect level with CI value of 0.34.

In case of quaternary mixture, synergism was observed up to 50% effect level (strong synergism at 10% level, CI-0.29) and antagonism at 90% level respectively.

**Comparison of mixture effect predicted by CA, IA and CI methods**

The dose response parameters and predicted dose response effect for all three models CA, IA and CI along with experimental data are presented in Table 3 and Figures 2 to 4 respectively. The IPQ value for all three models were less than one for all the mixtures except ternary mixture of B[a]P + As +Pb. This indicates good agreement between observed and predicted effects. An individual comparison of IPQ values for three reference models (CA, IA and CI) showed that prediction power of CI model is superior to classical models of CA and IA (comparatively IPQ value for CI method is less for six out of 11 mixtures compared to CA and IA models). In addition, CI method accurately predicts the synergistic interaction between these mixtures.

## DISCUSSION

In this study, we have determined the multi-component interaction between B[a]P and heavy metal/loid(s) in HepG2 cells by exposing the chemicals mixtures for 24 h and the CI method was used to predict the mixture toxicity. The treatment period of 24 h was selected based on the doubling time of HepG2 cells (24 h) and also as reported by International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity.[45] Cytotoxicity of individual chemical

and mixtures was determined by MTS assay. Various methods including viability assays based on metabolism reductase activity (MTS, XTS, resazurin dye reduction assays), bioluminescent ATP assays and enzyme linked cytotoxicity assays (LDH leakage assays) are used to determine cytotoxicity or cell viability. Each assay is having its own advantages and disadvantages. Eg. Neutral red viability assay is a classic example of a scientifically useful and validated method which is impractical and rarely used. MTS assay has been widely used with great success in toxicity testing. This assay has been well characterized and it is stable, cost-effective, simple to use and suitable for high-throughput testing.[46]

The results showed different kind of interactions between B[a]P and heavy metal/loid(s). Synergism was the predominant response at lower effect level ($IC_{10-30}$) for all mixtures with the exception of ternary mixture of B[a]P + As+ Pb. In case of binary mixtures, all six mixtures showed various degree of synergism (strong to moderate synergism) at these effect levels. The percentage of binary mixtures displaying synergism at $IC_{10}$, $IC_{30}$, and $IC_{50}$ was 100, 83 and 33% respectively. For ternary and quaternary mixtures, the observed synergism at $IC_{10}$ was extended up to $IC_{50}$ level. A total of 75% of ternary mixtures showed synergistic response at $IC_{10-50}$ level.

The observed effect for heavy metal/loid(s) mixtures toxicity i.e. synergism and antagonism at low and higher effect level, respectively is supported by findings of Bae *et al.*[19] *and* Klutse *et al.*[20] Cytotoxic interactions among As, Cd, Cr and Pb was determined by Bae *et al.*[19] using four immortal human keratinocytes and observed similar findings of synergism and antagonism at lower and higher dose of mixtures respectively. Klutse *et al.*[20] studied the interaction between As, Cd, Pb and Hg in MCF cell line and observed that combined toxicity changed from synergistic to additive to antagonistic with increasing concentration of the metal mixtures.

One possible explanation for observed antagonistic response at higher effect level is due to presence of strong defense mechanism in the liver. A low molecular weight protein metallothionein (MT) synthesis was upregulated followed by Cd exposure which protects the liver against Cd toxicity.[47-48] Arsenic also induces MT genes and heat shock protein which may reduce the toxicity of As and of Cd by detoxification.[49-50] Cellular glutathione level plays an important role in resistance to metal toxicity.[51-54] Heavy metal/loid(s) (As, Cd and Pb) are also reported to diminish the toxicity (carcinogenic potential) of B[a]P by decreasing B[a]P mediated induction of its bioactivation by CYP1A1.[23] In addition, Nrf2 (nuclear factor erythroid 2 -related factor 2)-antioxidant response element (ARE) pathway is activated followed by these chemicals exposure. Nrf2-ARE pathway plays a major role in protection of cells from heavy metal/loid(s) and B[a]P induced oxidative-stress.[55-59] In our laboratory, we have used HepG2-Nrf2 reporter system to confirm the activation of Nrf2-ARE pathway by B[a]P and heavy metal/loid(s) (data not shown). Co-exposure of Cd and B[a]P resulted in significant increase in expression of Nrf2-responsive genes and cellular glutathione levels.[60] Combined defense activities like Nrf2-ARE pathway activation, induction of MT and heat shock protein, antioxidant protective effect of glutathione and metabolic inhibition of B[a]P by metal/loid(s) may contribute to protection of cells from mixture toxicity and the observed antagonistic response at higher effect level of mixtures.

The observed interaction between B[a]P and heavy metal/loids especially synergism at different effect level cannot be explained in terms of the known mechanism of action. In few cases these metal/loid(s) were reported to enhance the toxicity of B[a]P. Arsenic was found to have synergistic effect on cytotoxicity of B[a]P in human lung carcinoma cells[61] and also potentiate the genotoxicity of B[a]P.[21,61-62] In case of Cd and B[a]P combination, the available report for their genotoxicity is inconsistent as both synergistic [22,63] and antagonistic effect was reported by various authors.[20,57] There is no detailed report available for these

mixture toxicity especially for higher order mixtures (ternary and quaternary). We have reported the mixture toxicity of multi- component mixtures of B[a]P and heavy metal/loids(s) and showed different nature of interactions between these mixtures.

CA and IA models are traditionally used to predict the mixture toxicity effects of chemicals. Cedergreen et al.,[64] reported that only half of the 158 mixture data was accurately predicted by either CA or IA models. CI method was successfully used to study the interaction between drugs/chemicals for ecotoxicity risk assessment and reported to have improved predictive power compared to classical models.[41-43] We have used CI method to predict the interaction between environmental chemicals using human cell lines and observed that this method is useful to predict the synergistic interaction between B[a]P and heavy metal/loid(s) at lower effect level.

## CONCLUSIONS

Binary, ternary and quaternary mixture toxicity of B[a]P, As, Cd and Pb was determined in HepG2 cells and the CI-isobologram method was used to predict the mixture toxicity. The results showed that the nature of interaction varies according to the effect levels and number of components in the mixtures. This multi-component mixture of B[a]P and heavy metal/loid(s) displayed synergistic and antagonistic interaction at low and higher effect levels respectively. The observed synergistic effect increases with complexity of mixture. Given the complexity of the chemical-biological interaction in the cells, further interaction studies on other endpoints such as oxidative stress, genotoxicity and toxicokinetic interaction can provide a more complete data set for health risk assessment of these mixed contaminants. CI method was found to have improved predictive power of CA and IA models. The predicted synergism among these common environmental contaminants may have important implications on human risk assessment.

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## CONFLICT OF INTEREST

There is no conflict of interest.

## SUPPLEMENTARY INFORMATION

Supplementary information to article is attached.

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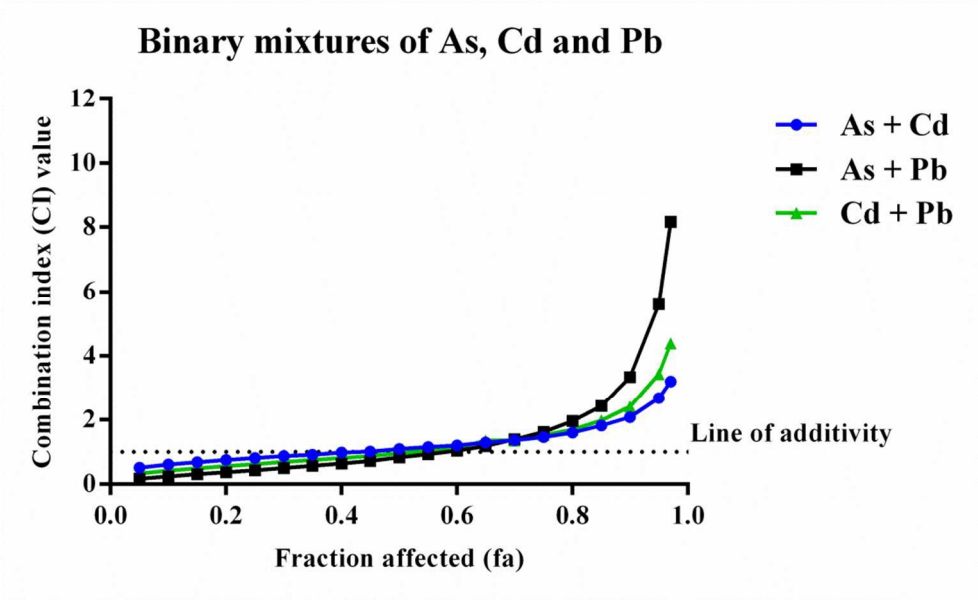
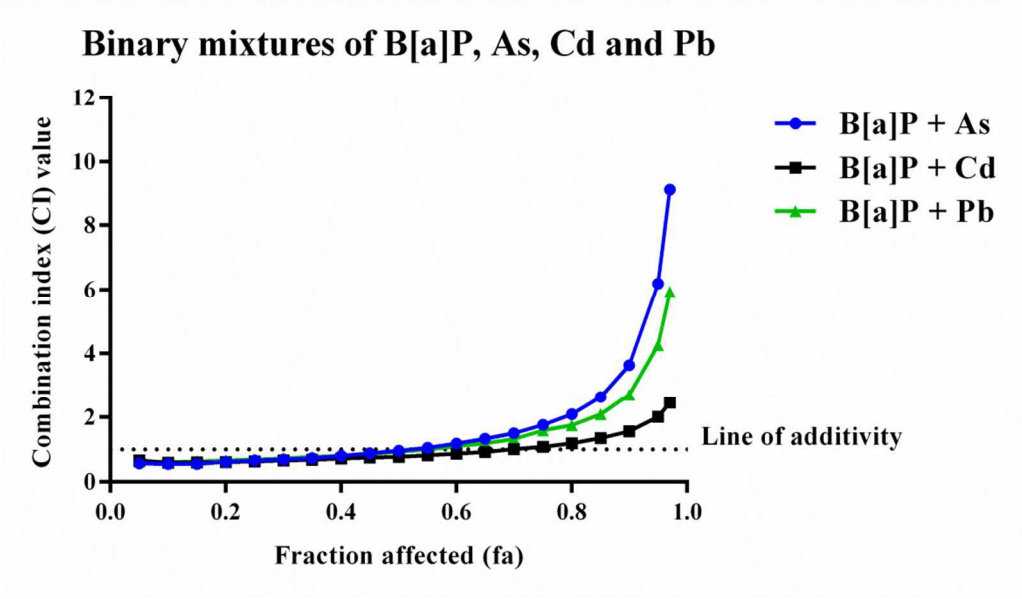
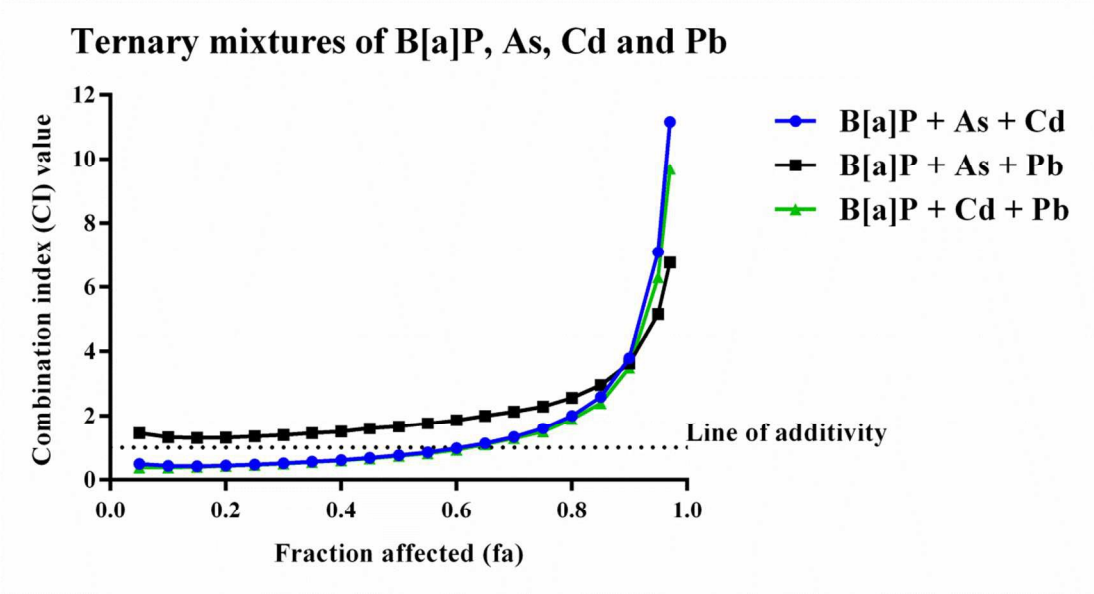




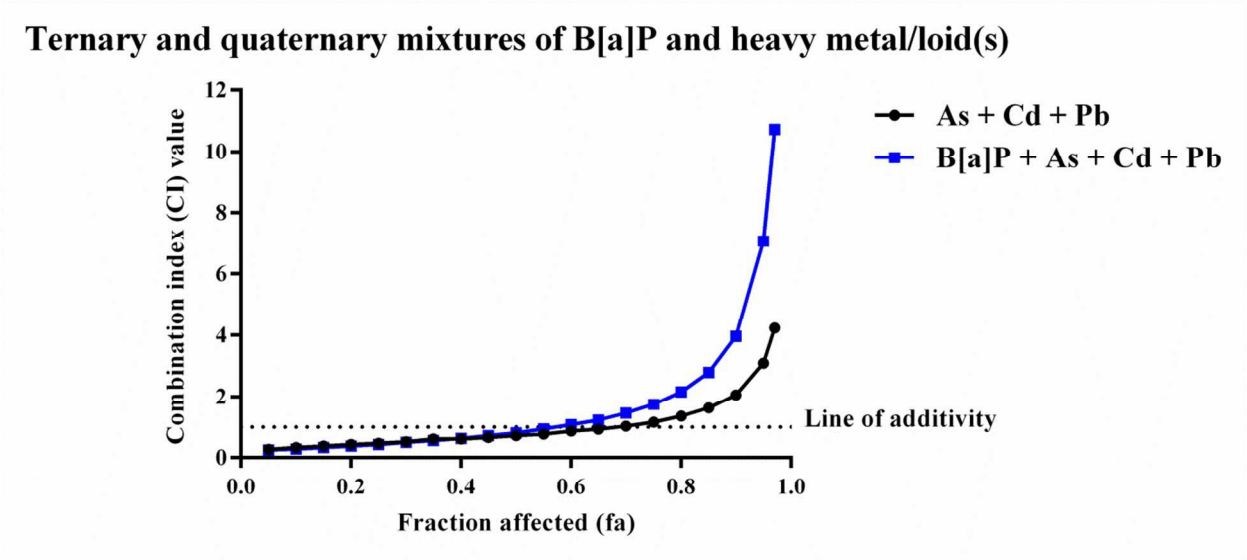
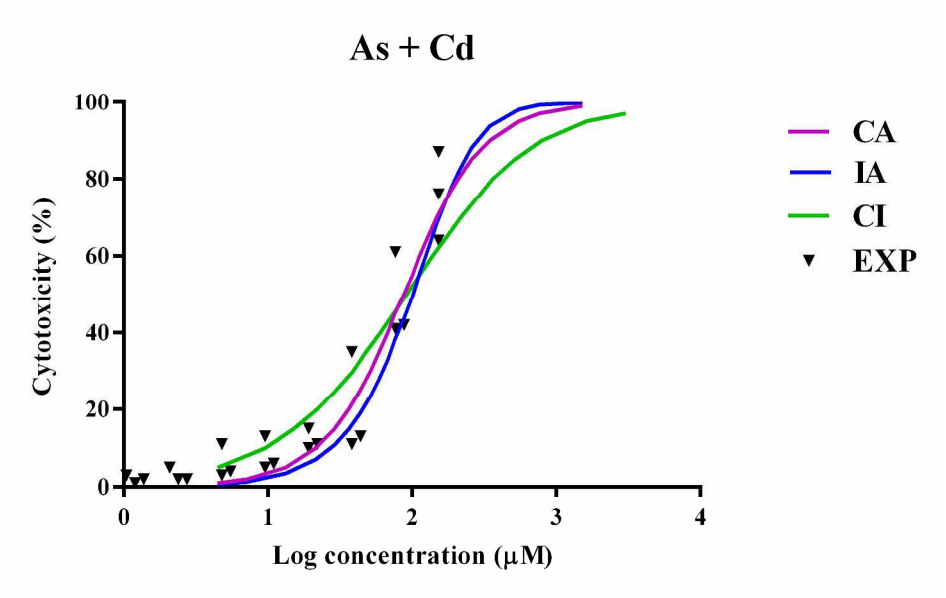
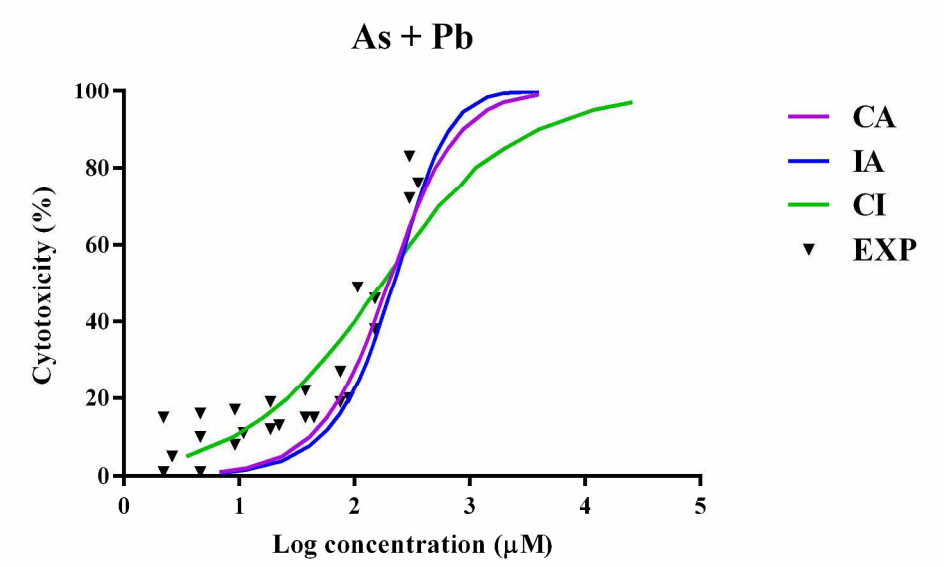




**Figure 1. Combination index plot ($f_a$–CI plot) for multi-component mixtures of benzo[a]pyrene (B[a]P) and heavy metal/loid(s) (arsenic (As), cadmium (Cd) and lead (Pb) in HePG2 cells. CI values are plotted as a function of cell viability ($f_a$). CI < 1, = 1 and > 1 indicates synergism, additive and antagonism respectively.**



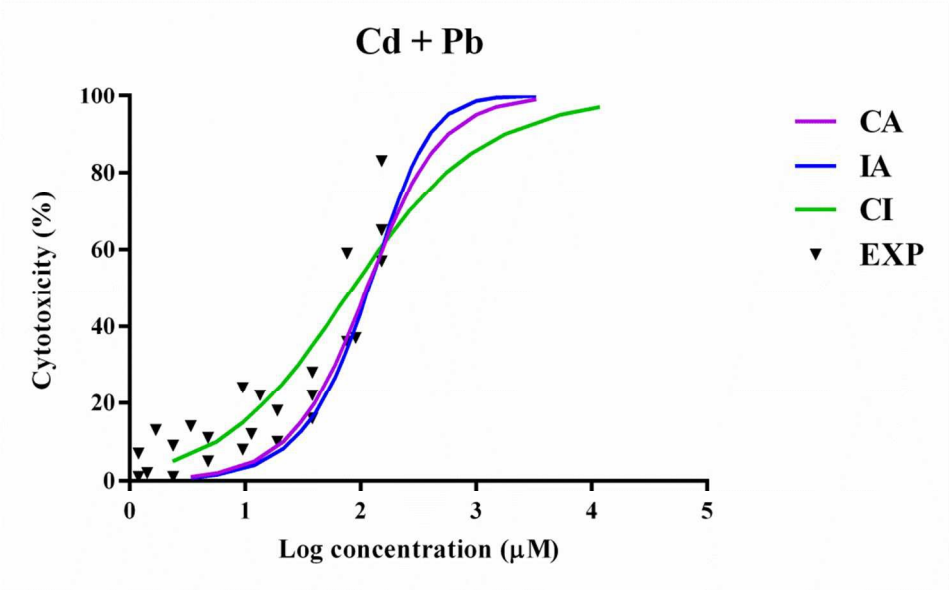
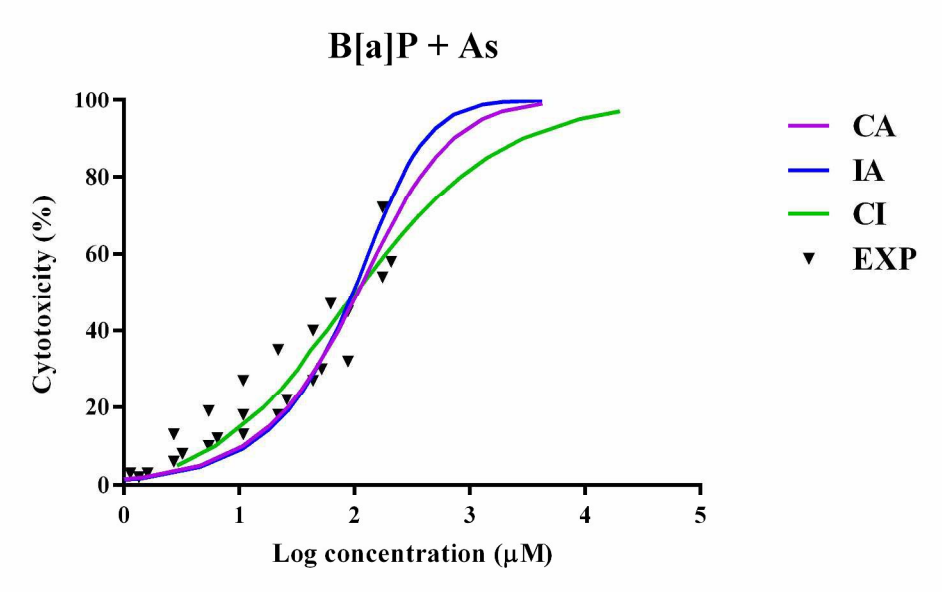
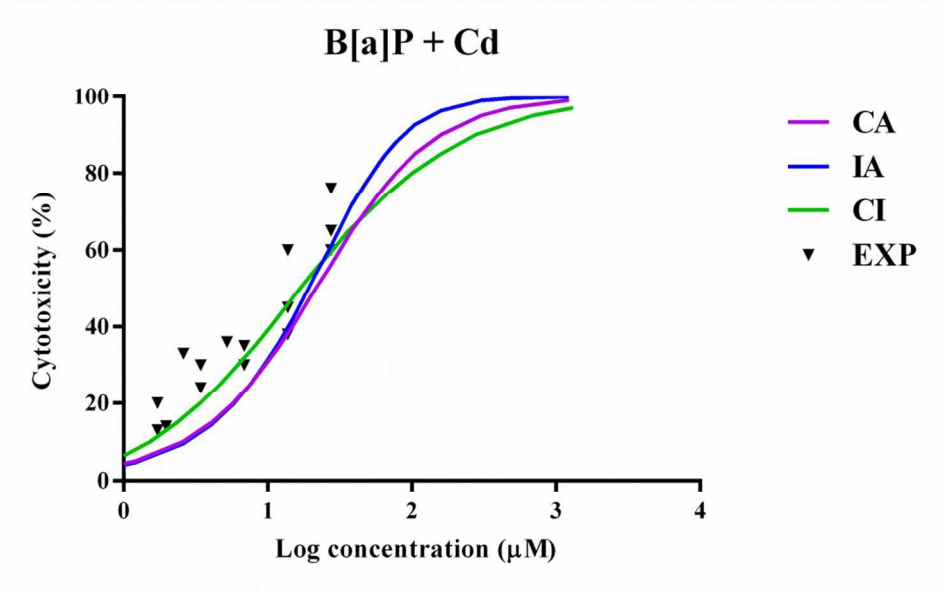



**Figure 2: Comparison of dose-response effect of binary mixtures of heavy metal/loid(s) (As + Cd, As + Pb and Cd + Pb) predicted by concentration addition (CA), independent action (IA), combination-index (CI) methods and observed experimental toxicity effect (EXP) in HepG2 cells. Experimental values are expressed as mean values from three independent experiments. As –arsenic; Cd -cadmium and Pb-lead.**



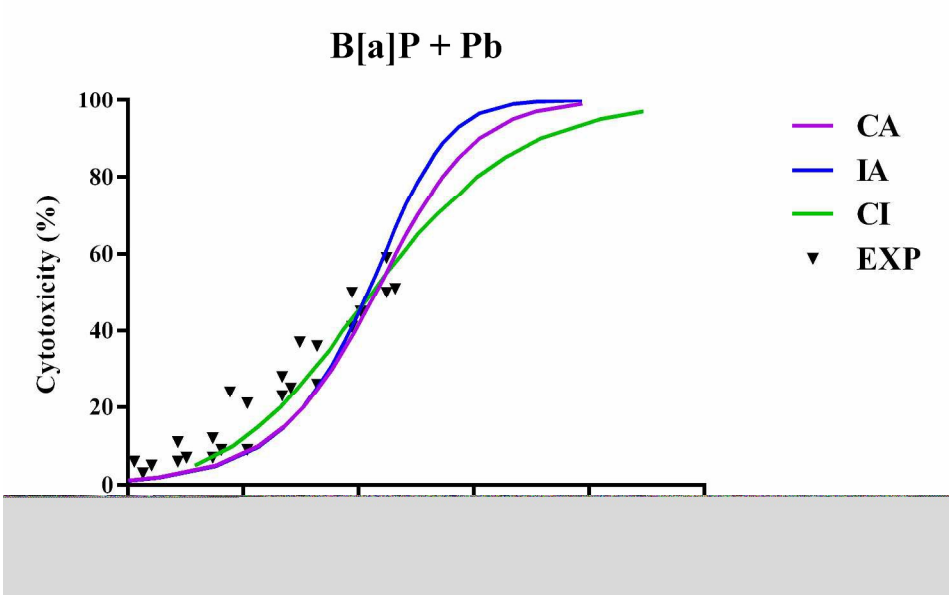
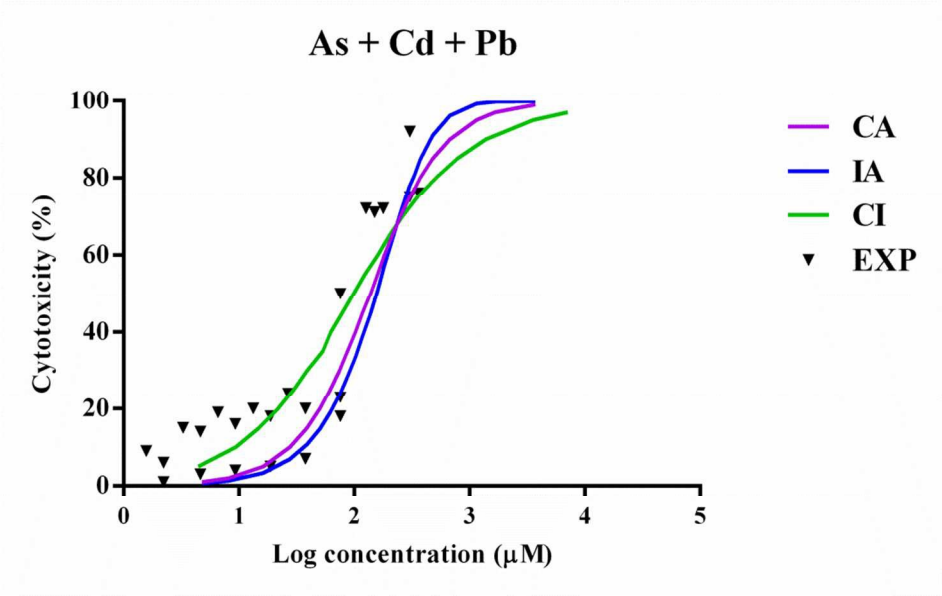
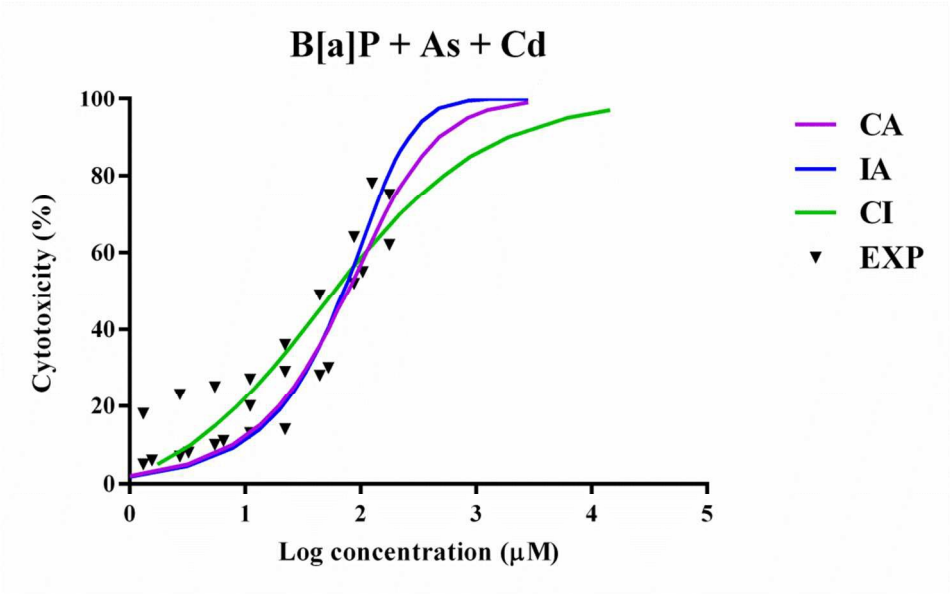
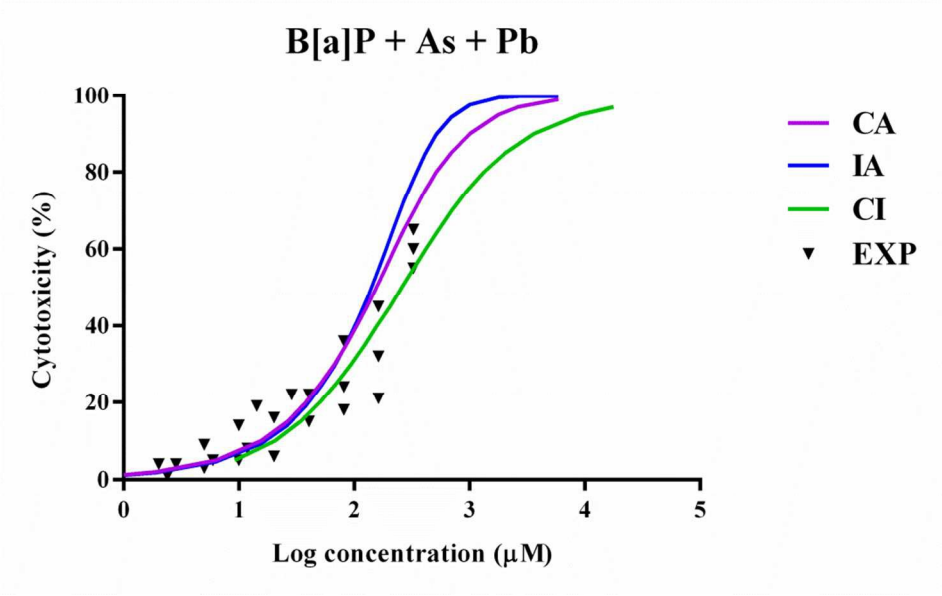
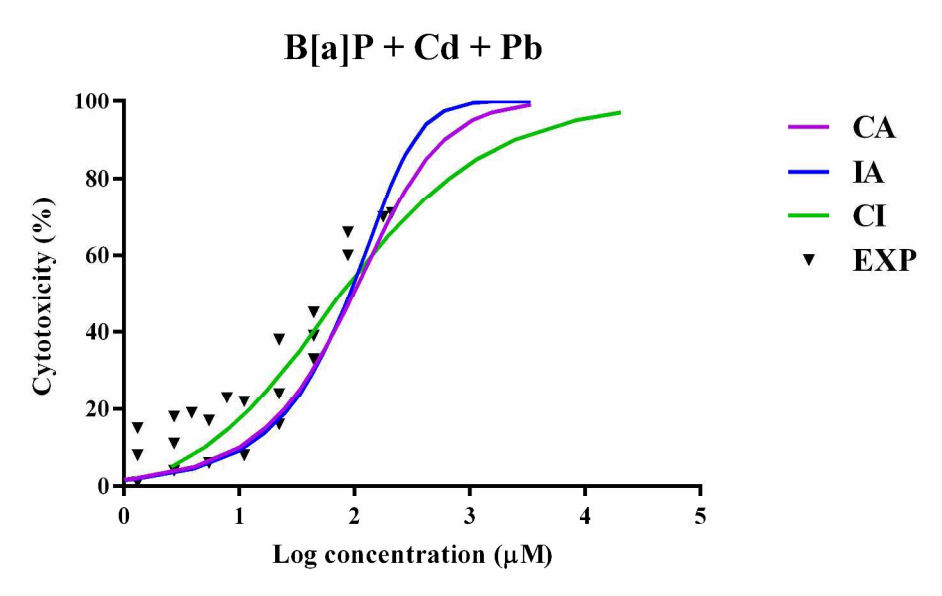



**Figure 3: Comparison of dose-response effect of binary mixtures of B[a]P and heavy metal/loid(s) (B[a]P + As, B[a]P + Cd and B[a]P + Pb) predicted by concentration addition (CA), independent action (IA), combination-index (CI) methods and observed experimental toxicity effect (EXP) in HepG2 cells. Experimental values are expressed as mean values from three independent experiments. B[a]P – benzo[a]pyrene; As –arsenic; Cd -cadmium and Pb-lead.**





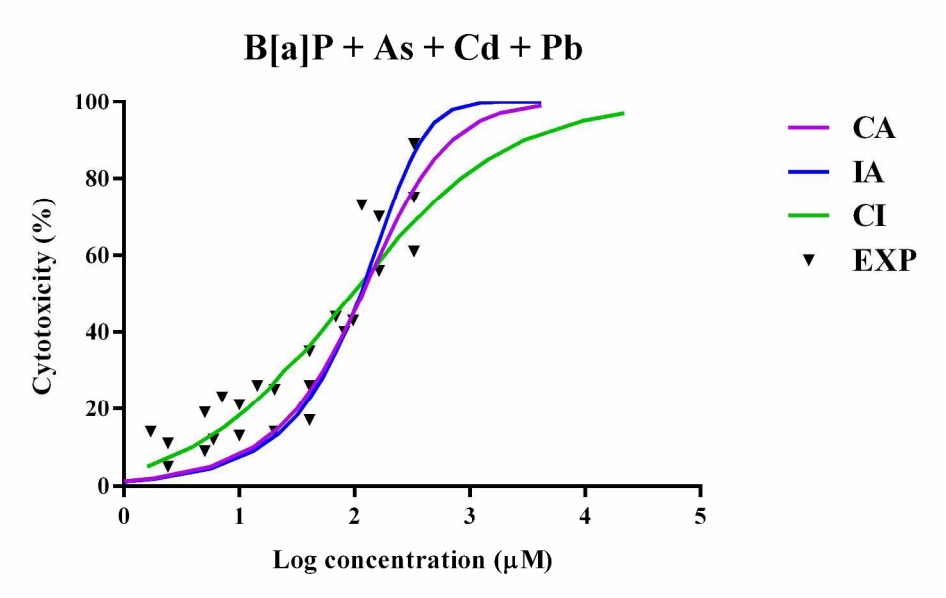



**Figure 4: Comparison of dose-response effect of multi-component mixtures of B[a]P and heavy metal/loid(s) predicted by concentration addition (CA), independent action (IA), combination-index (CI) methods and observed experimental toxicity effect (EXP) in HepG2 cells. Experimental values are expressed as mean values from three independent experiments. B[a]P – benzo[a]pyrene; As –arsenic; Cd -cadmium and Pb-lead.**

**Table 1: Description for combination index values**

| Combination index (CI) range | Description |
|---|---|
| <0.1 | Very strong synergism |
| 0.1-0.3 | Strong synergism |
| 0.3-0.7 | synergism |
| 0.7-0.85 | Moderate synergism |
| 0.85-0.90 | Slight synergism |
| 0.90-1.10 | Nearly additive |
| 1.10-1.20 | Slight antagonism |
| 1.20-1.45 | Moderate antagonism |
| 1.45-3.3 | antagonism |
| 3.3-10 | Strong antagonism |
| >10 | Very strong antagonism |

**Table 2: Dose response parameters and combination index (CI) values for multi-component mixtures of B[a]P and heavy metal/loid(s) in HepG2 cells**

| | | | | CI values for different effect levels and description | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chemical combination** | **$D_m$ (µM)** | **m** | **r** | **$IC_{10}$** | | **$IC_{30}$** | | **$IC_{50}$** | | **$IC_{90}$** | |
| As | 159 | 1.5 ± 0.25 | 0.94 | --- | | --- | | --- | | --- | |
| Cd | 2.70 | 1.4 ± 0.10 | 0.97 | --- | | --- | | --- | | --- | |
| Pb | 217 | 1.2 ± 0.32 | 0.96 | --- | | --- | | --- | | --- | |
| B[a]P | 37.0 | 0.9 ± 0.11 | 0.97 | --- | | --- | | --- | | --- | |
| **Binary mixtures- heavy metal/loid(s) mixtures** | | | | | | | | | | | |
| As + Cd | 87.7 | 1.0 ± 0.17 | 0.97 | 0.62 ± 0.27 | SYN | 0.88 ± 0.36 | ADD | 1.1 ± 0.36 | ADD | 2.1 ± 0.43 | ANT |
| As + Pb | 170.0 | 0.78 ± 0.30 | 0.92 | 0.25 ± 0.19 | SYN | 0.51 ± 0.22 | SYN | 0.83 ± 0.23 | SYN | 3.3 ± 0.92 | ANT |
| Cd + Pb | 93.2 | 0.91 ± 0.07 | 0.94 | 0.43 ± 0.17 | SYN | 0.69 ± 0.35 | SYN | 0.97 ± 0.55 | ADD | 2.40 ± 1.5 | ANT |
| **Binary mixtures- B[a]P + heavy metal/loid(s) mixtures** | | | | | | | | | | | |
| B[a]P + As | 109.5 | 0.73 ± 0.07 | 0.97 | 0.55 ± 0.48 | SYN | 0.70 ± 0.36 | SYN | 0.97 ± 0.38 | ADD | 3.6 ± 1.8 | ANT |
| B[a]P + Cd | 13.7 | 0.88 ± 0.02 | 0.95 | 0.60 ± 0.30 | SYN | 0.66 ± 0.25 | SYN | 0.78 ± 0.37 | SYN | 1.6 ± 0.90 | ANT |
| B[a]P + Pb | 132.7 | 0.68 ± 0.05 | 0.97 | 0.64 ± 0.30 | SYN | 0.73 ± 0.13 | SYN | 0.93 ± 0.26 | ADD | 2.7 ± 0.57 | ANT |
| **Ternary mixtures of B[a]P and heavy metal/loid(s)** | | | | | | | | | | | |
| As+ Cd + Pb | 101.7 | 0.93 ± 0.24 | 0.92 | 0.34 ± 0.29 | SYN | 0.52 ± 0.28 | SYN | 0.72 ± 0.22 | SYN | 2.1 ± 0.81 | ANT |
| B[a]P + As + Cd | 73.9 | 0.67 ± 0.12 | 0.98 | 0.43 ± 0.39 | SYN | 0.52 ± 0.26 | SYN | 0.77 ± 0.26 | SYN | 3.8 ± 1.5 | ANT |
| B[a]P + As + Pb | 305 | 0.74 ± 0.14 | 0.97 | 1.33 ± 0.76 | ANT | 1.40 ± 0.62 | ANT | 1.67 ± 0.64 | ANT | 3.6 ± 1.0 | ANT |
| B[a]P + Cd + Pb | 67.0 | 0.74 ± 0.23 | 0.97 | 0.53 ± 0.52 | SYN | 0.59 ± 0.36 | SYN | 0.78 ± 0.20 | SYN | 3.3 ± 1.5 | ANT |
| **Quaternary mixtures of B[a]P and heavy metal/loid(s)** | | | | | | | | | | | |
| B[a]P + As + Cd + As | 68.0 | 0.7 ± 0.13 | 0.95 | 0.29 ± 0.19 | SYN | 0.50 ± 0.25 | SYN | 0.81 ± 0.35 | SYN | 4.0 ± 1.4 | ANT |

Values are mean or mean ± SD for Dm, m and CI values; Values are from three independent experiments for each mixture. $D_m$- concentration for 50% effect on cell viability; m- coefficient of the sigmoidicity of the dose-effect curve; r-linear correlation coefficient of the median-effect plot; CI-Combination index; IC- Inhibitory concentration; ADD-additivity; ANT-antagonism; SYN-synergism.

**Table 3: Comparison of experimental data and dose response effect predicted by concentration addition (CA), independent action (IA) models and combination index- isobologram (CI) method.**

| | | | | Prediction by CA, IA and CI method | | | | | | | | IPQ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experimental data | | | CA | | | IA | | | CI | | | CA | IA | CI |
| | $D_m$ | m | r | $D_m$ | m | r | $D_m$ | m | r | $D_m$ | m | r | | | |
| **Binary mixtures- heavy metal/loid(s) mixtures** | | | | | | | | | | | | | | | |
| As + Cd | 87.7 | 1.0 ± 0.17 | 0.97 | 84.25 | 1.4 | 0.99 | 73.3 | 1.8 | 0.99 | 102.4 | 0.91 | 0.99 | -0.04 | -0.20 | 0.17 |
| As + Pb | 170.0 | 0.78 ± 0.30 | 0.92 | 212 | 1.4 | 0.99 | 174.0 | 1.6 | 0.99 | 182 | 0.74 | 0.91 | 0.24 | 0.02 | 0.07 |
| Cd+ Pb | 93.2 | 0.91 ± 0.07 | 0.94 | 119.3 | 1.2 | 0.99 | 96 | 1.5 | 0.99 | 110 | 0.86 | 0.99 | 0.28 | 0.03 | 0.18 |
| **Binary mixtures- B[a]P + heavy metal/loid(s) mixtures** | | | | | | | | | | | | | | | |
| B[a]P + As | 109.5 | 0.73 ± 0.07 | 0.97 | 86.0 | 1.0 | 0.98 | 57.5 | 1.2 | 0.95 | 102.2 | 0.70 | 0.99 | -0.27 | -0.9 | -0.07 |
| B[a]P + Cd | 13.7 | 0.88 ± 0.02 | 0.95 | 18.4 | 1.0 | 0.99 | 13.6 | 1.3 | 0.97 | 16.1 | 0.85 | 0.99 | 0.34 | -0.007 | 0.18 |
| B[a]P+ Pb | 132.7 | 0.68 ± 0.05 | 0.97 | 116 | 0.89 | 0.99 | 76.3 | 1.1 | 0.97 | 135.3 | 0.68 | 0.99 | -0.14 | -0.74 | 0.02 |
| **Ternary mixtures of B[a]P and heavy metal/loid(s)** | | | | | | | | | | | | | | | |
| Cd+ Pb+ As | 101.7 | 0.93 ± 0.24 | 0.92 | 135.3 | 1.4 | 0.99 | 117 | 1.8 | 0.97 | 100.3 | 0.85 | 0.99 | 0.33 | 0.15 | -0.01 |
| B[a]P +As + Cd | 73.9 | 0.67 ± 0.12 | 0.98 | 62.3 | 0.92 | 0.98 | 40.1 | 1.2 | 0.95 | 86.8 | 0.63 | 0.99 | -0.19 | -0.84 | 0.13 |
| B[a]P + As + Pb | 305 | 0.74 ± 0.14 | 0.97 | 119 | 0.96 | 0.98 | 77.3 | 1.3 | 0.96 | 254 | 0.84 | 0.99 | -1.6 | -2.9 | -0.20 |
| B[a]P + Cd + Pb | 67.0 | 0.74 ± 0.23 | 0.97 | 75.0 | 0.91 | 0.98 | 47.3 | 1.3 | 0.95 | 63.3 | 0.7 | 0.99 | 0.12 | -0.42 | 0.06 |
| **Quaternary mixtures of B[a]P and heavy metal/loid(s)** | | | | | | | | | | | | | | | |
| B[a]P + As + Cd + Pb | 68.0 | 0.7 ± 0.13 | 0.95 | 86.5 | 1.0 | 0.98 | 53.0 | 1.4 | 0.96 | 46 | 0.78 | 0.99 | 0.27 | -0.28 | -0.48 |

Values are mean or mean ± SD for experimental data; For CA, IA and CI- values are mean; $D_m$- concentration for 50% effect on cell viability; m- coefficient of the sigmoidicity of the dose-effect curve; r-linear correlation coefficient of the median-effect plot; CA- concentration addition; IA- Independent action; CI- Combination index- isobologram; IPQ- index on prediction quality. IPQ= 0 indicates perfect agreement between observed and predicted effect; IPQ < 0-overestimation; IPQ >0-underestimation.

**Table of contents**

**Highlights:**

Multi-component mixtures of B[a]P and heavy metal/loid(s) toxicity was determined in HepG2 cells. Concentration dependent synergism, additive or antagonism was predicted by combination-index isobologram method.