RESULTS

Chapter 1: *In vivo* effects of lead acetate and sodium arsenite exposure singly on oxidative stress, antioxidant defenses and immune functions in macrophages from fish *Channa punctatus*

4.1. STUDY OF EFFECTS OF LEAD ACETATE AND SODIUM ARSENITE EXPOSURE (*IN VIVO*) SINGLY ON BIOACCUMULATION, MORPHOLOGICAL ALTERATION AND DNA DAMAGE IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Heavy metal analysis in different organs of untreated and treated fish (ppb/g tissue)

UNTREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
Lead accumulation	**0.02 ± 0.025**	**0.041± 0.02**	**0.045±0.024**	**0.021±0.019**
Arsenic accumulation	**0.031 ± 0.02**	**0.048±0.011**	**0.0255±0.015**	**0.029±0.05**

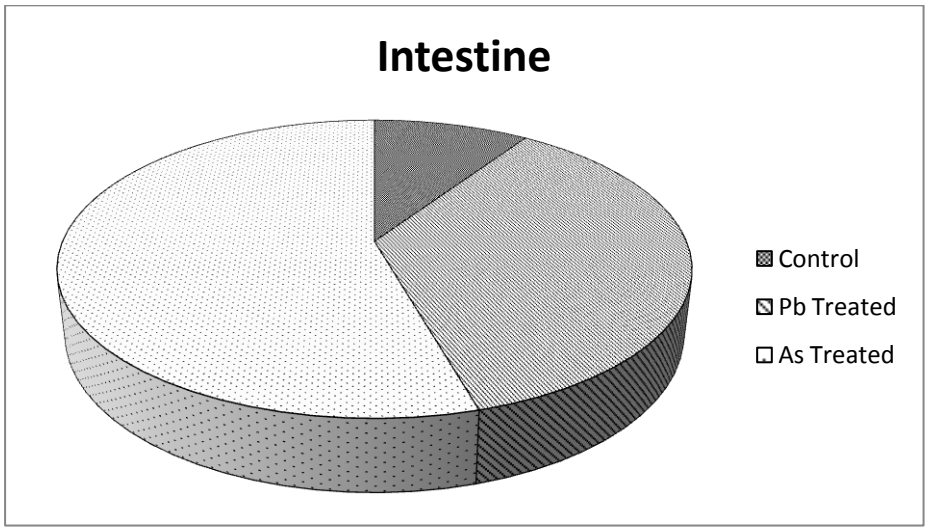
TREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
Lead	**0.33 ± 0.021**	**0.92±0.019**	**0.83 ± 0.021**	**0.43 ± 0.015**
Arsenic	**0.28± 0.015**	**1.06 ± 0.036**	**0.921±0.025**	**0.56 ± 0.025**

Note: The values were statistically significant at $P < 0.05$.

Table 1: Heavy metal analysis in different organs of untreated and treated fish (ppb/g tissue).

i) Effect of lead and arsenic on morphological alteration of macrophages

(a)



(b)

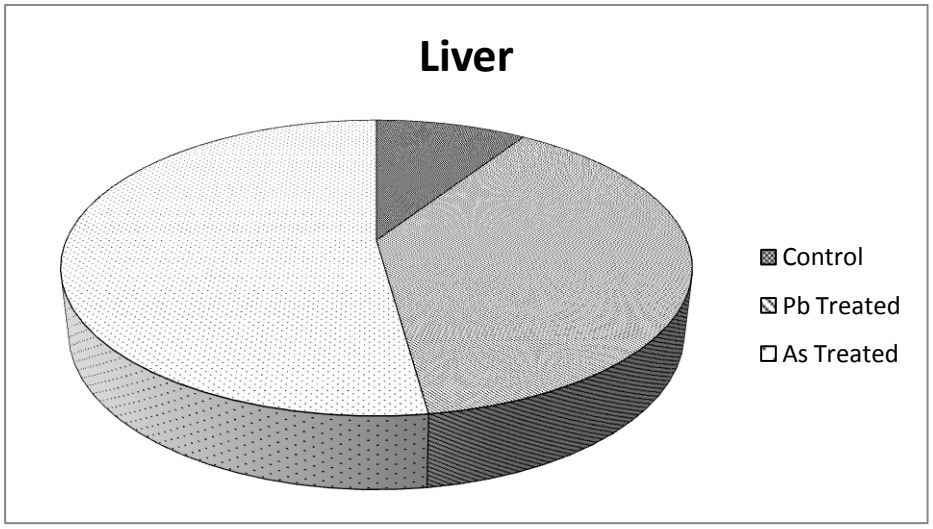
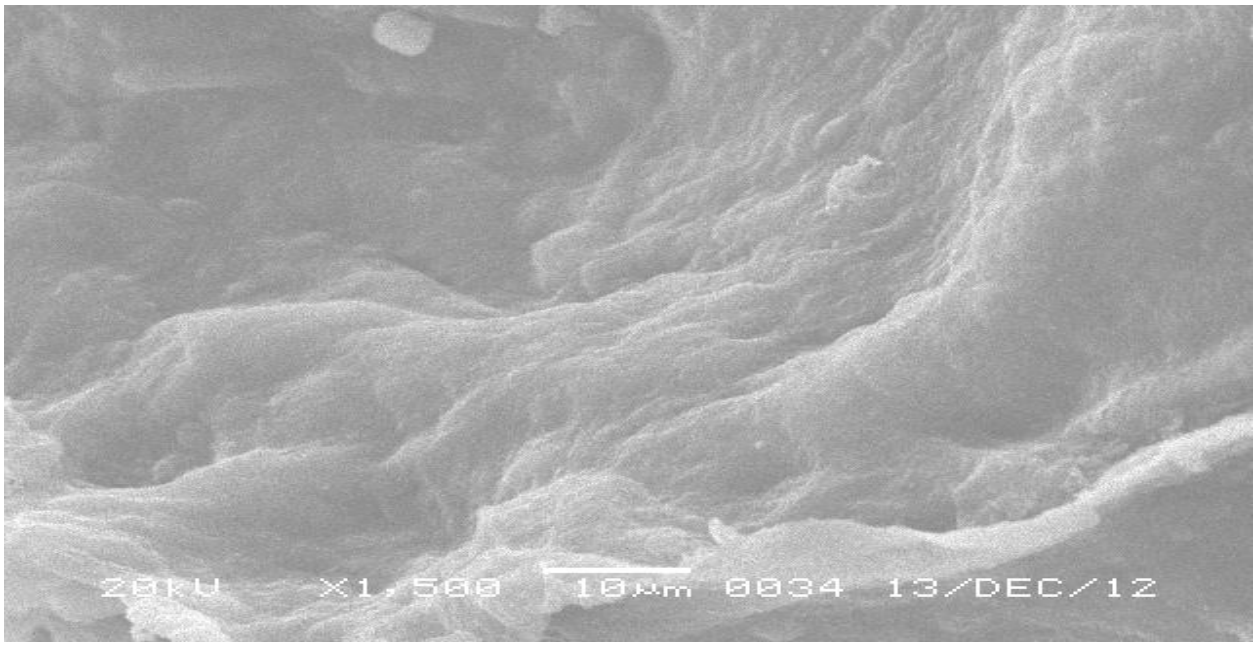


Fig 1: Morphological alteration of (a) intestinal and (b) liver macrophages when treated singly with lead and arsenic

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.05$.

iii) Ultrastructural analysis of tissue by Scanning Electron Microscope

(a)



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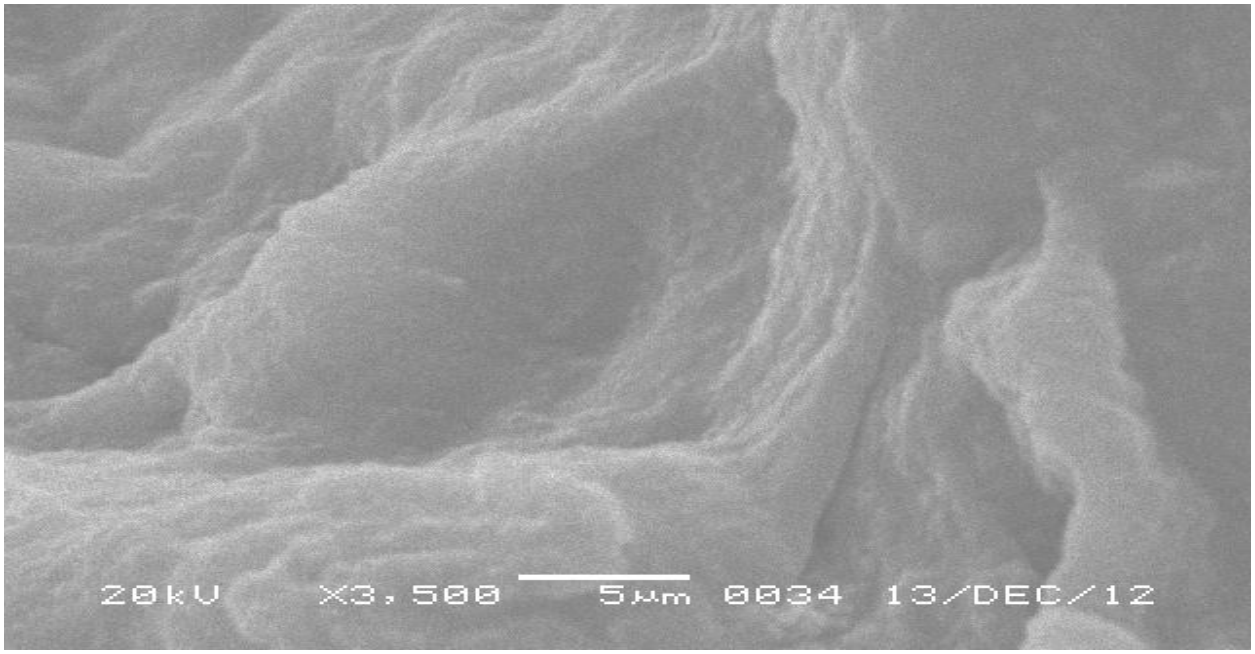
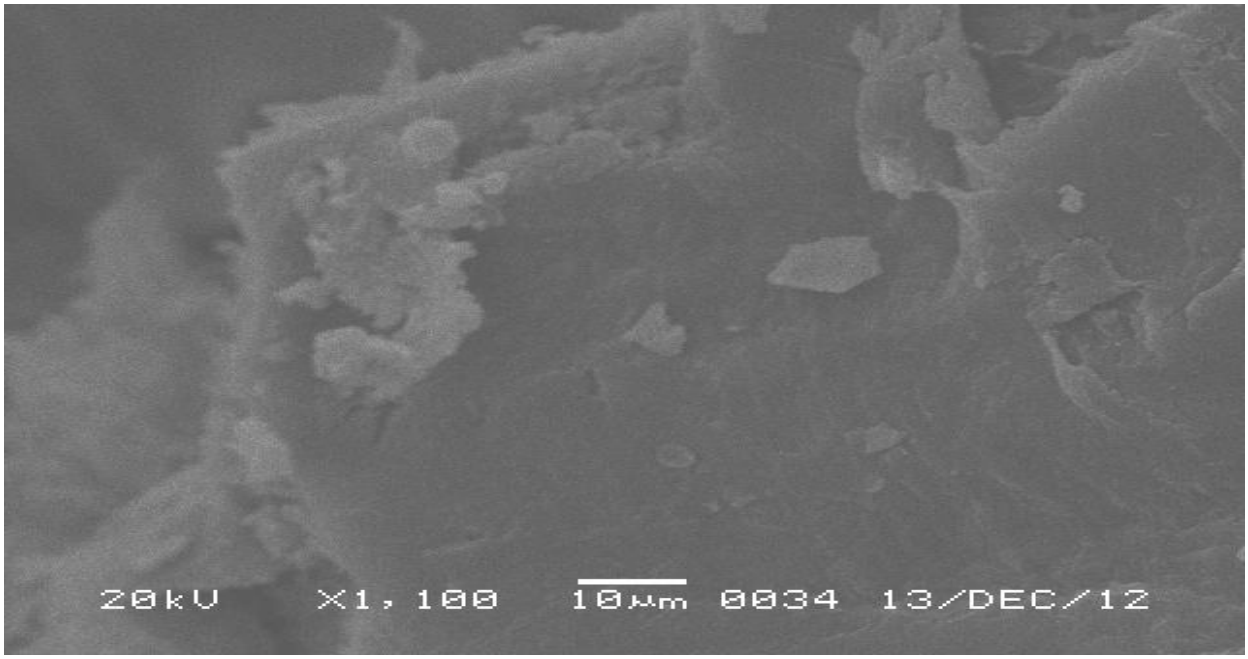


Plate 1(a), (b): Ultramicroscopic photographs of untreated fish showing prominent mucosal foldings and normal epithelium.

(c)



(d)

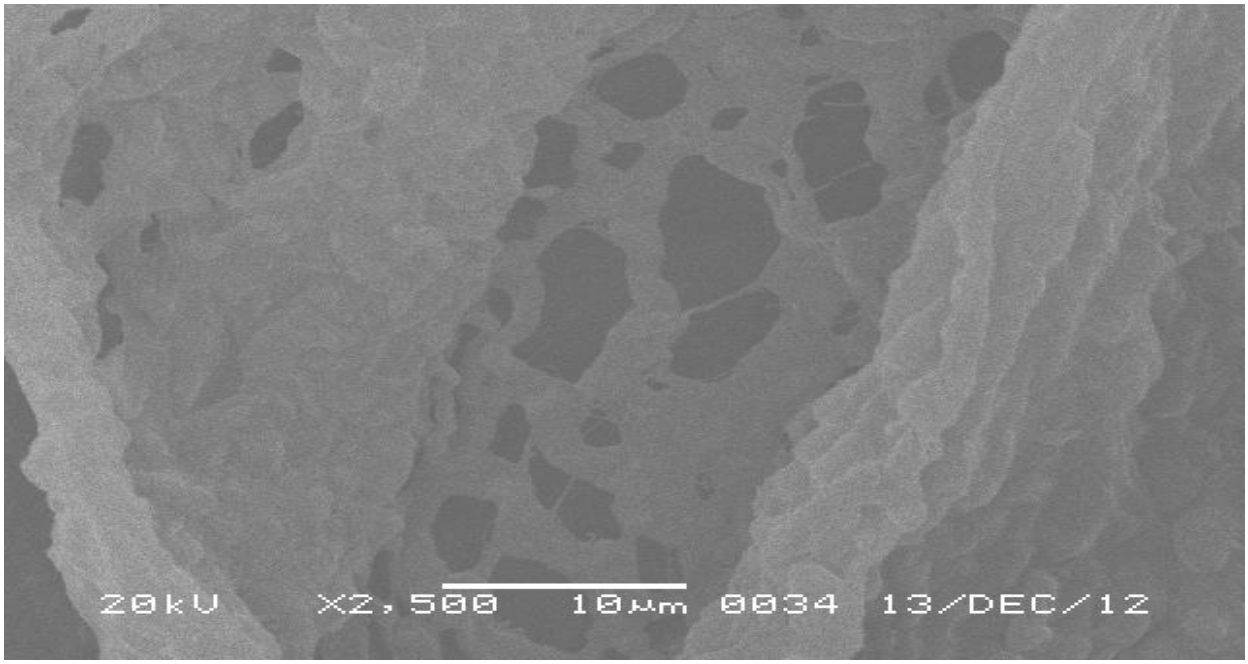
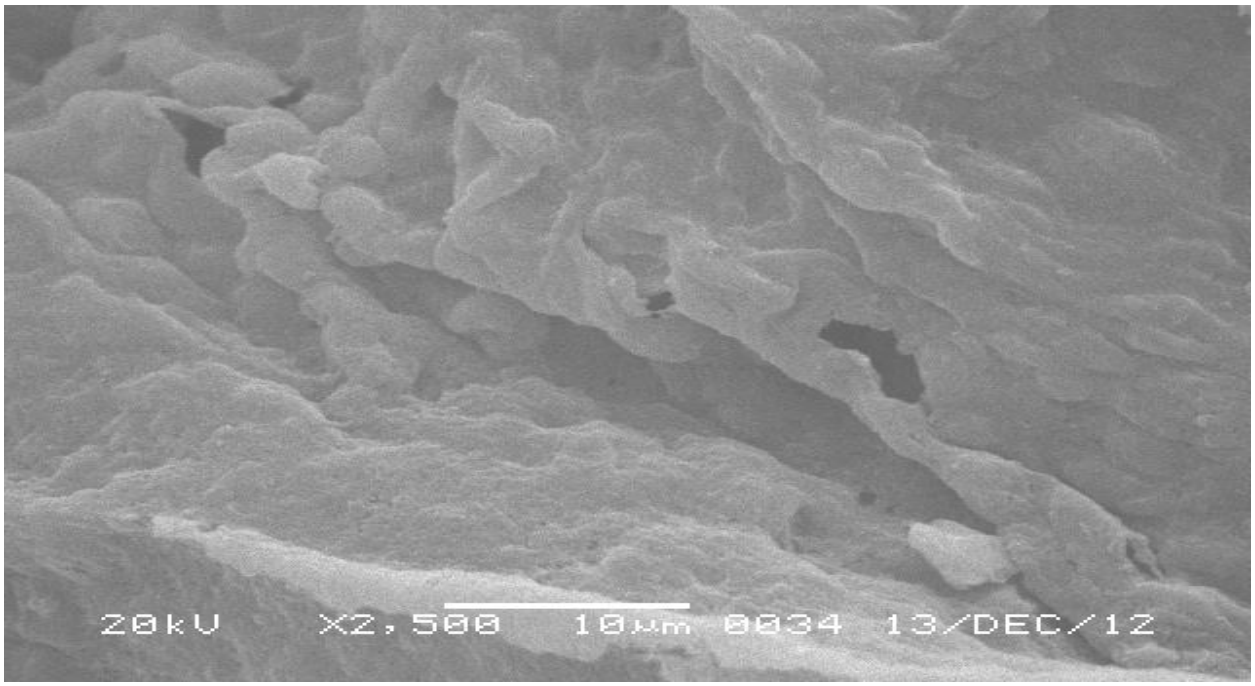


Plate 1(c), (d): Ultramicroscopic photographs of lead treated fish showing debris of mucosal folds, damaged and degenerated epithelium.

(e)



(f)

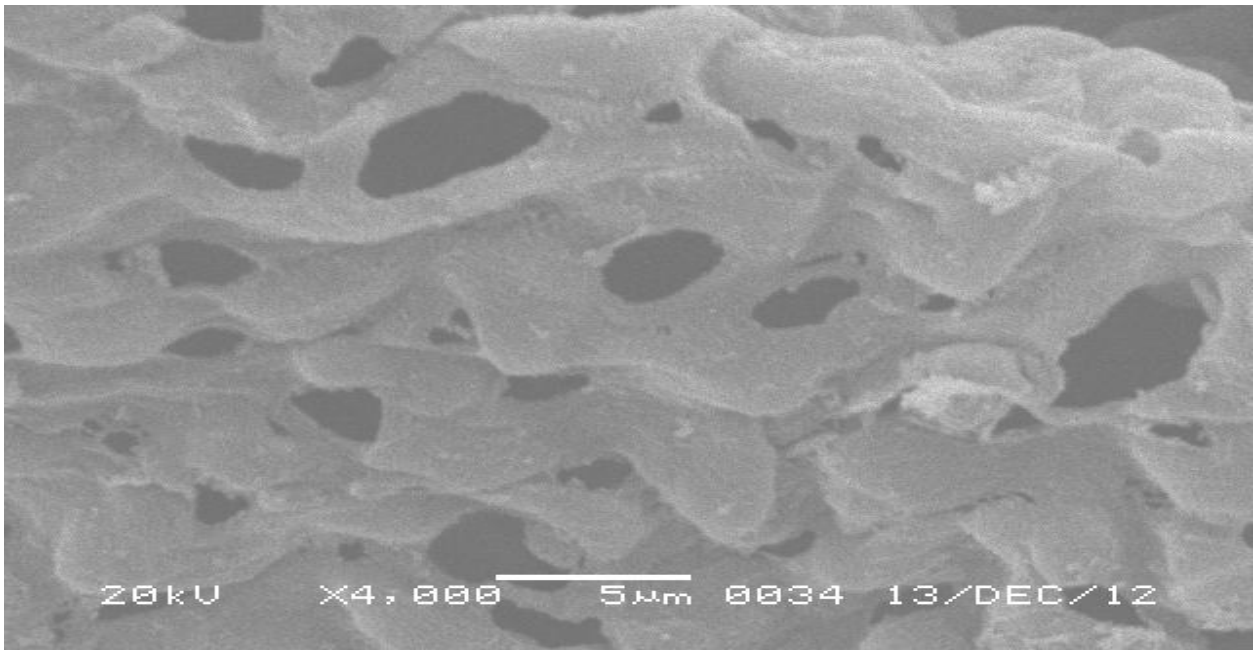


Plate 1(e), (f): Ultramicroscopic photographs of arsenic treated fish showing inflammatory damage in epithelium, disarrangement and fragmentation of mucosal foldings and secretion of mucus.

iv) Percentage of DNA fragmentation in lead and arsenic treated fish

There was a significant increase in DNA fragmentation from 35.83±0.02% (intestine), 36.05±0.03% (liver) in control group to 58±0.04% (intestine), 45.7±0.03% (liver) in lead treated and 74.4±0.032% (intestine), 67.1±0.002% (liver) in arsenic treated group (Fig: 2).

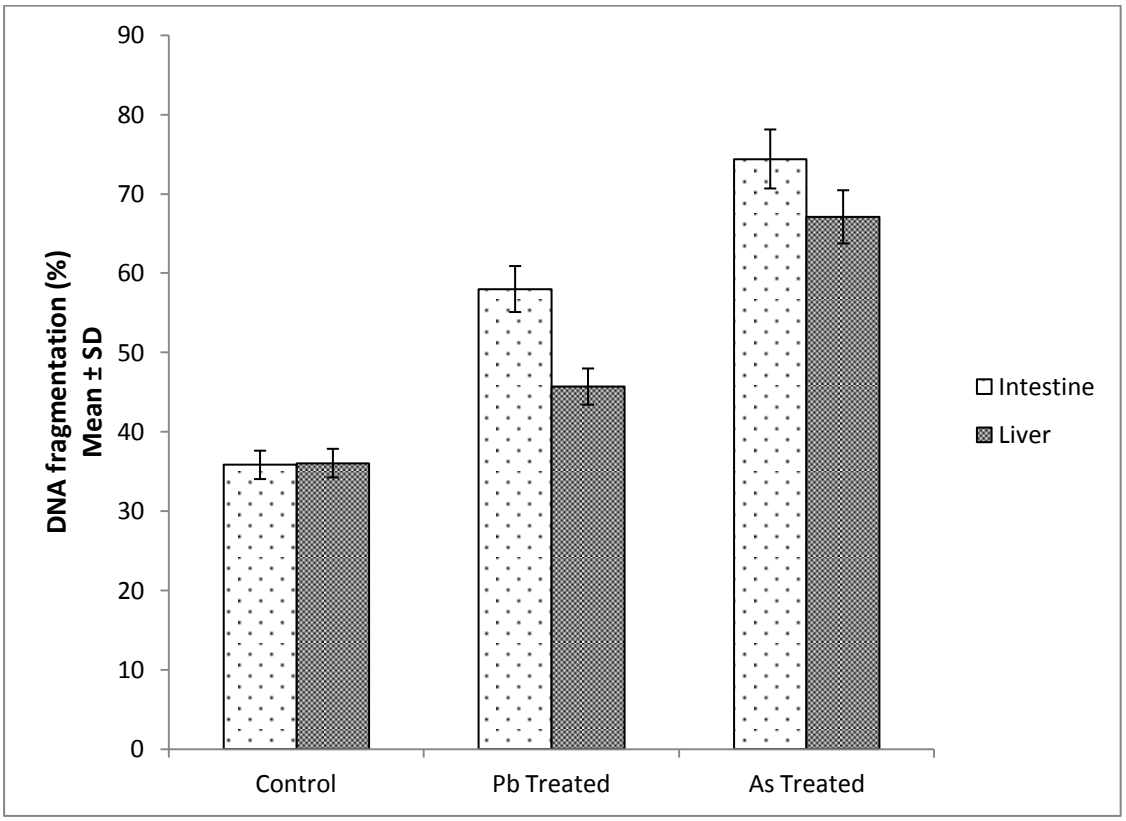


Fig 2: Percentage of DNA fragmentation in lead and arsenic treated fish.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

4.2. STUDY OF EFFECT OF LEAD ACETATE AND SODIUM ARSENITE (*IN VIVO*) SINGLY ON OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Effect of lead and arsenic on respiratory burst activity of fish macrophages

There was a significant increase in respiratory burst activity from 0.521±0.02 (intestine), 0.51 ±0.045 (liver) in control group to 1.148±1.134 (intestine), 1.022±1.122 (liver) in lead treated and 0.977±0.066 (intestine), 0.89±0.034 (liver) in arsenic treated group (Fig: 1).

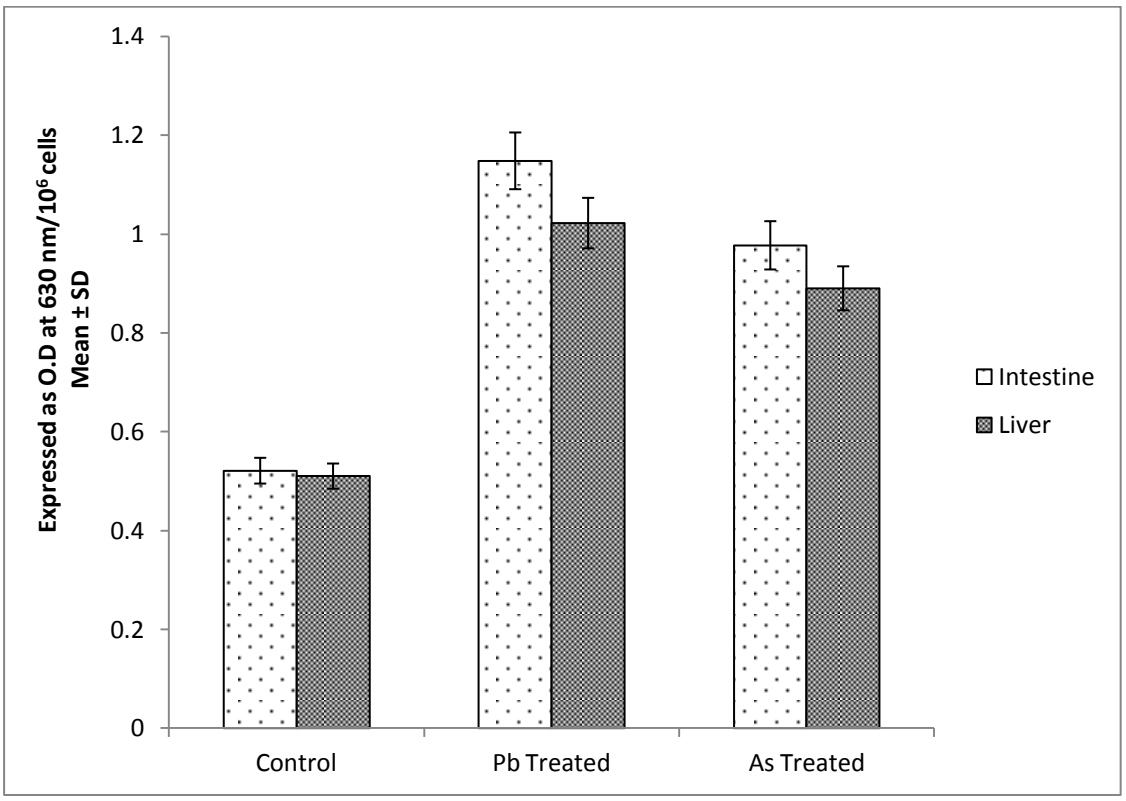


Fig 1: Respiratory burst activity in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) P< 0.05; Pb, As (liver) P< 0.025.

ii) Effect of lead and arsenic on lipid peroxidation of fish macrophages

There was a significant increase in lipid peroxidation from 0.385±1.02 nmoles/hr (intestine), 0.302±1.113 nmoles/hr (liver) in control group to 0.87±1.188nmoles/hr (intestine), 0.753±1.191 nmoles/hr (liver) in lead treated and 0.951±1.22 nmoles/hr (intestine), 0.91±1.11 nmoles/hr (liver) in arsenic treated group (Fig: 2).

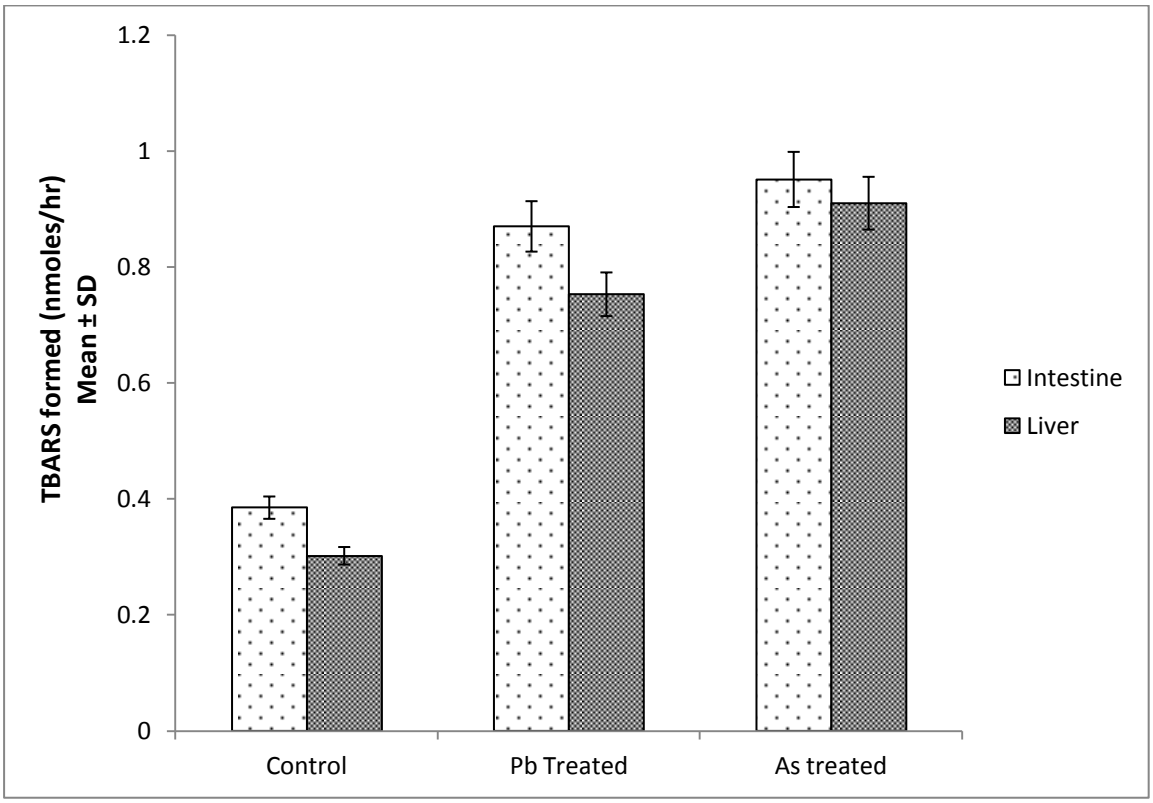


Fig 2: Estimation of lipid peroxidation (LPO) in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) P< 0.025; Pb, As (liver) P< 0.05.

iii) Effect of lead and arsenic on protein carbonylation of fish macrophages

There was a significant increase in protein carbonylation from 0.39±1.22 mg/ml (intestine), 0.51±1.13 mg/ml (liver) in control group to 0.62±1.128 mg/ml (intestine), 0.83±1.155 mg/ml (liver) in lead treated and 0.7±1.92 mg/ml (intestine), 0.89±1.45 mg/ml (liver) in arsenic treated group (Fig: 3).

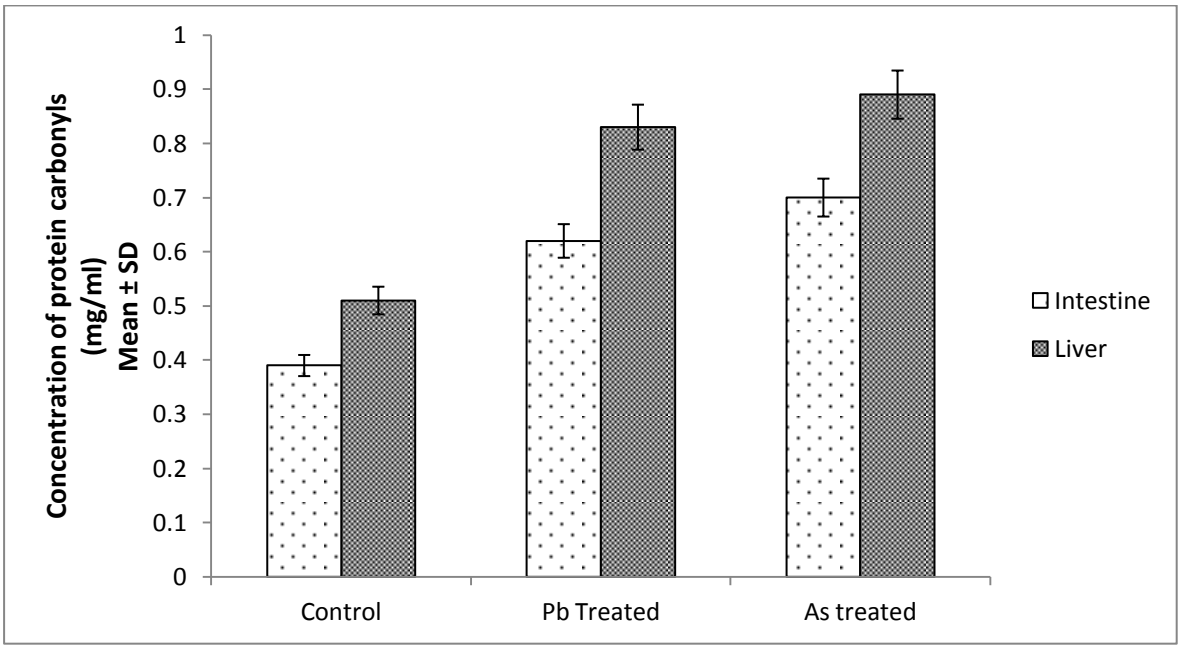


Fig 3: Estimation of protein carbonylation (PC) in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) $P< 0.02$; Pb, As (liver) $P< 0.05$.

iv) Effect of lead and arsenic on catalase (CAT) activity of fish macrophages

There was a significance decrease in catalase activity from 5.764±1.22 U/mg protein (intestine), 6.012±1.82U/mg protein (liver) in control group to 4.496±1.144U/mg protein (intestine), 3.77±1.26 U/mg protein (liver) in lead and 3.929±1.54 U/mg protein (intestine), 4.011±1.69 U/mg protein (liver) in arsenic treated group (Fig: 4).

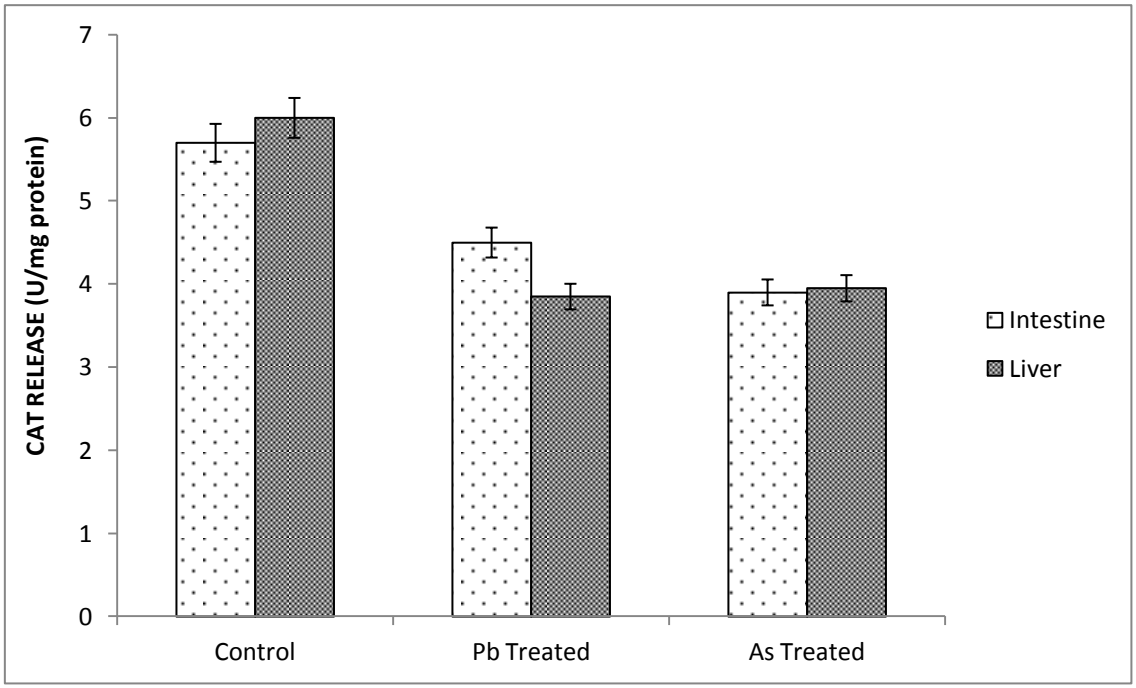


Fig 4: Catalase (CAT) activity in control and heavy metal exposed fish macrophages.

Pb, As (intestine) P< 0.05; Pb, As (liver) P< 0.02.

v) Effect of lead and arsenic on superoxide dismutase (SOD) activity of fish macrophages

There was a significance increase in superoxide dismutase activity from 0.015±1.61 U/mg protein (intestine), 0.021±1.86 U/mg protein (liver) in control group to 0.183±1.127 U/mg protein (intestine), 0.17±1.22 U/mg protein (liver) in lead treated and 0.198±1.77 U/mg protein (intestine), 0.201±0.97 U/mg protein (liver) in arsenic treated group (Fig: 5).

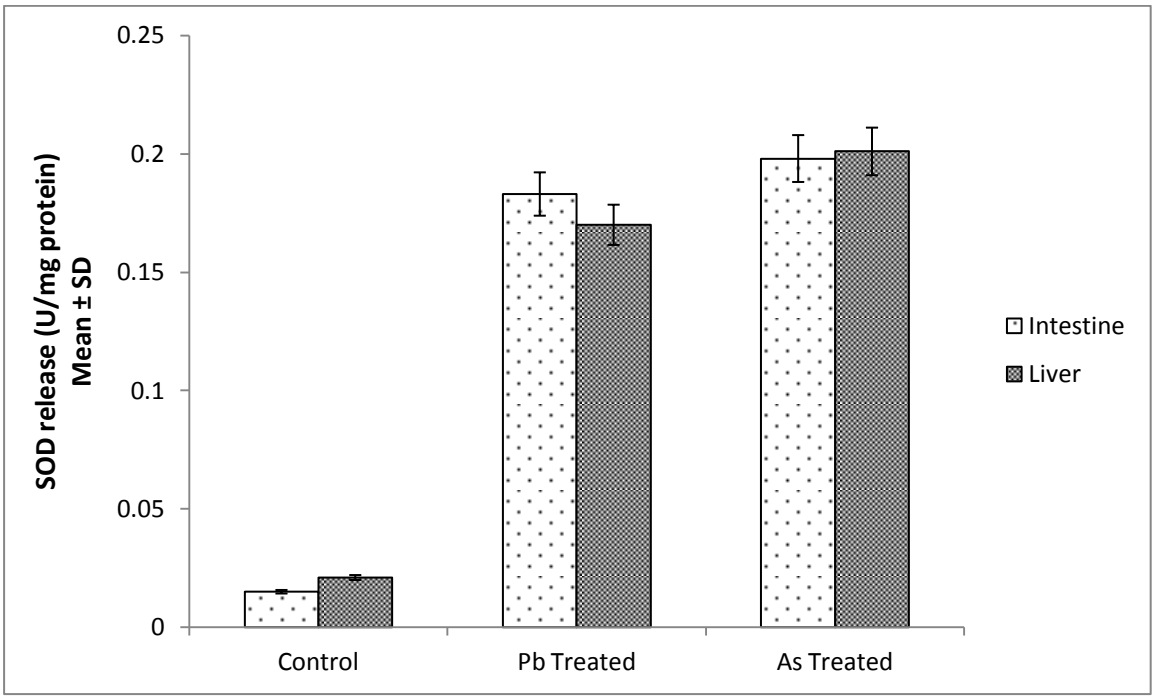


Fig 5: Superoxide dismutase (SOD) activity in control and heavy metal exposed fish macrophages.

As (intestine) P< 0.02; Pb (intestine) P< 0.05; Pb, As (liver) P< 0.01.

vi) Effect of lead and arsenic on glutathione S- transferase (GST) activity of fish macrophages

There was a significance decrease in glutathione S- transferase activity from 28.13±0.06 nmoles/min/mg protein (intestine), 26.9±0.05 nmoles/min/mg protein (liver) in control group to 24.66±0.76 nmoles/min/mg protein (intestine), 24.19±0.03 nmoles/min/mg protein (liver) in lead treated and 20.7±0.07 nmoles/min/mg protein (intestine), 21.03±0.044 nmoles/min/mg protein (liver) in arsenic treated group (Fig: 6).

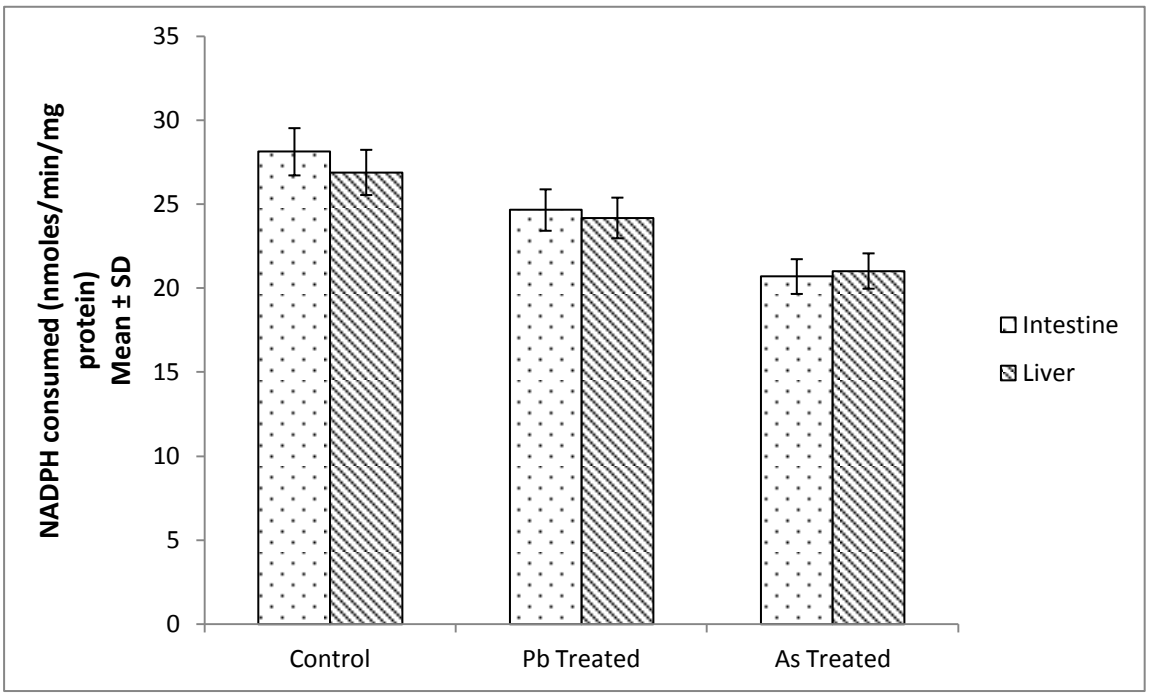


Fig 6: Glutathione S-transferase (GST) activity in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) $P< 0.01$; Pb, As (liver) $P< 0.05$.

vii) Effect of lead and arsenic on glutathione peroxidase (GPx) activity of fish macrophages

There was a significance decrease in glutathione peroxidase activity from 5.8±0.014 nmoles/min/mg protein (intestine), 6.6±0.05 nmoles/min/mg protein (liver) in control group to 4.7±0.03 nmoles/min/mg protein (intestine), 5.3±0.022 nmoles/min/mg protein (liver) in lead treated and 4.2±0.04 nmoles/min/mg protein (intestine), 5.1±0.04 nmoles/min/mg protein in arsenic treated group (Fig: 7).

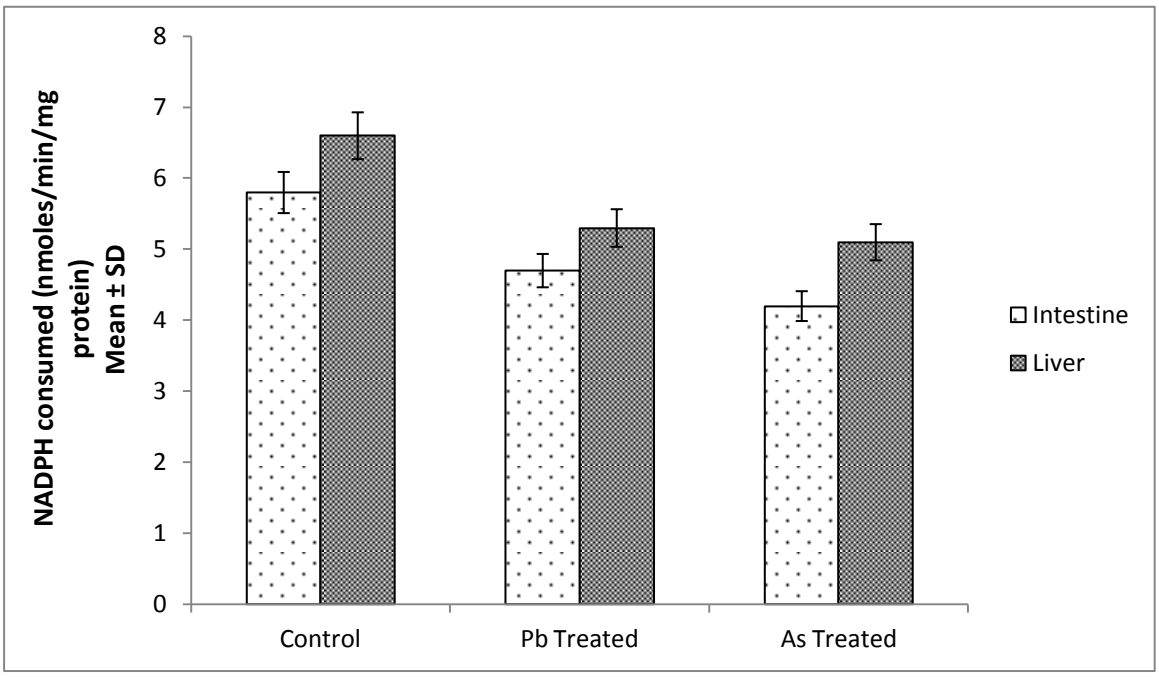


Fig 7: Glutathione peroxidase (GPx) activity in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) $P< 0.01$; Pb, As (liver) $P< 0.025$.

viii) Effect of lead and arsenic on glutathione reductase (GR) activity of fish macrophages

There was a significance decrease in glutathione reductase activity from 3.96±0.02 nmoles/min/mg protein (intestine), 4.51±0.04 nmoles/min/mg protein (liver) in control group to 3.6±0.044 nmoles/min/mg protein (intestine), 4.01±0.051 nmoles/min/mg protein (liver) in lead treated and 3.08±0.042 nmoles/min/mg protein (intestine), 3.82±0.037 nmoles/min/mg protein (liver) in arsenic treated group (Fig: 8).

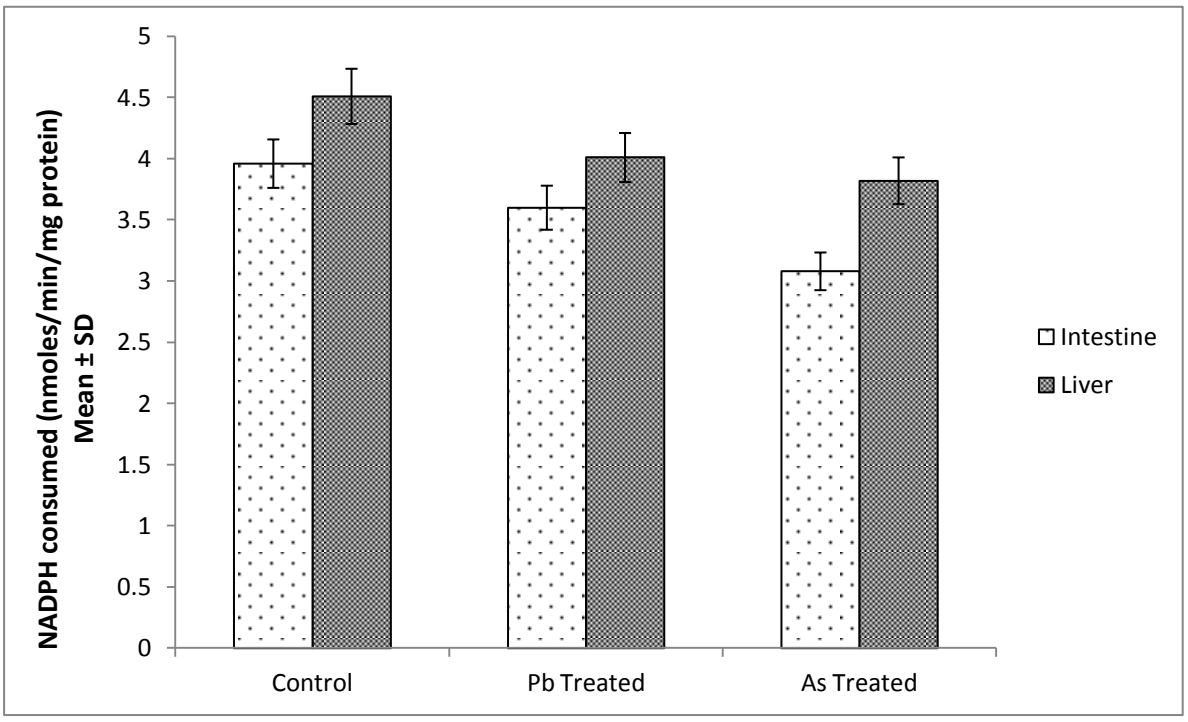


Fig 8: Glutathione reductase (GR) activity in macrophages of fish treated with lead and arsenic. Pb, As (intestine) P< 0.025; Pb, As (liver) P< 0.05.

ix) Effect of lead and arsenic on reduced glutathione (GSH) activity of fish macrophages

There was a significance decrease in reduced glutathione activity from 0.823±1.18 nmoles/min/mg protein (intestine), 0.766±1.11 nmoles/min/mg protein (liver) in control group to 0.555±1.06 nmoles/min/mg protein (intestine), 0.442±1.03 nmoles/min/mg protein (liver) in lead treated and 0.531±1.13 nmoles/min/mg protein (intestine), 0.413±1.12 nmoles/min/mg protein (liver) in arsenic treated group (Fig: 9).

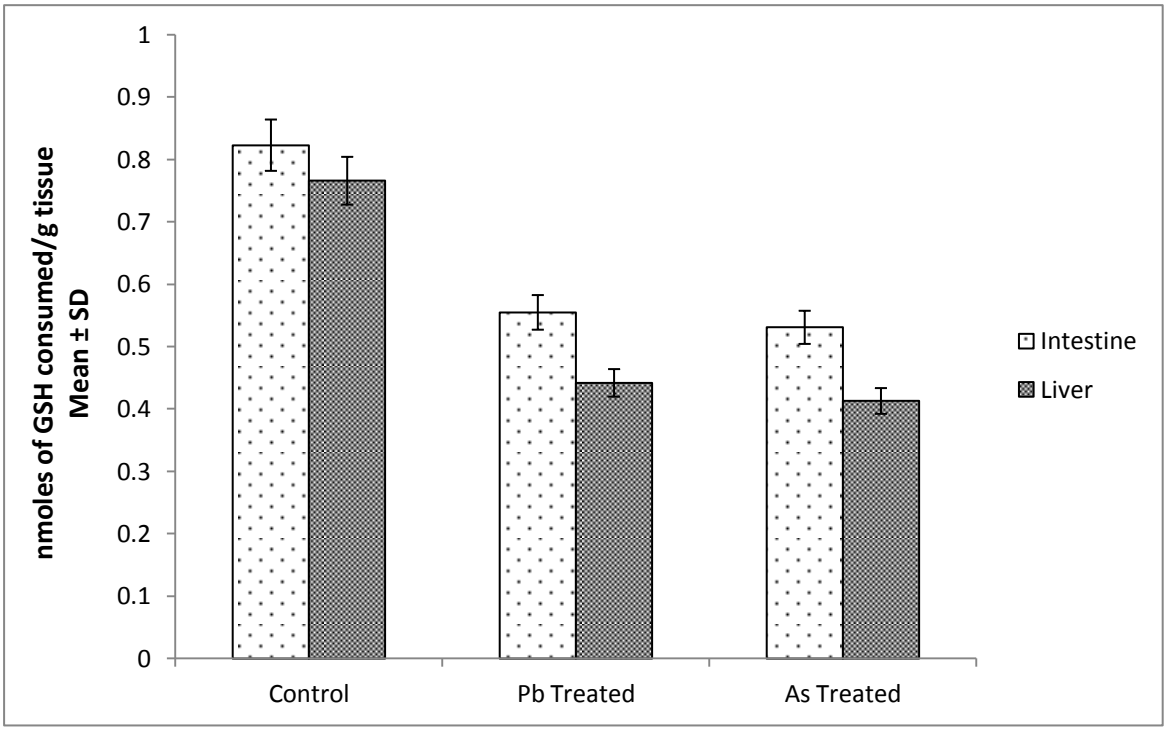


Fig 9: Reduced glutathione (GSH) consumed in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

4.3. STUDY OF EFFECT OF LEAD ACETATE AND SODIUM ARSENITE (*IN VIVO*) SINGLY ON INNATE IMMUNE RESPONSES IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Effect of lead and arsenic on phagocytic activity of fish macrophages

There was a significant decrease in phagocytic activity from 21666.66±1.78 %(intestine), 21822.51±1.83 % (liver) in control group to 10667±1.44 % (intestine), 18264.43±1.55 % (liver) in lead treated and 10322.12±1.65 % (intestine), 17331.44±1.66 % (liver) in arsenic treated group (Fig: 1).

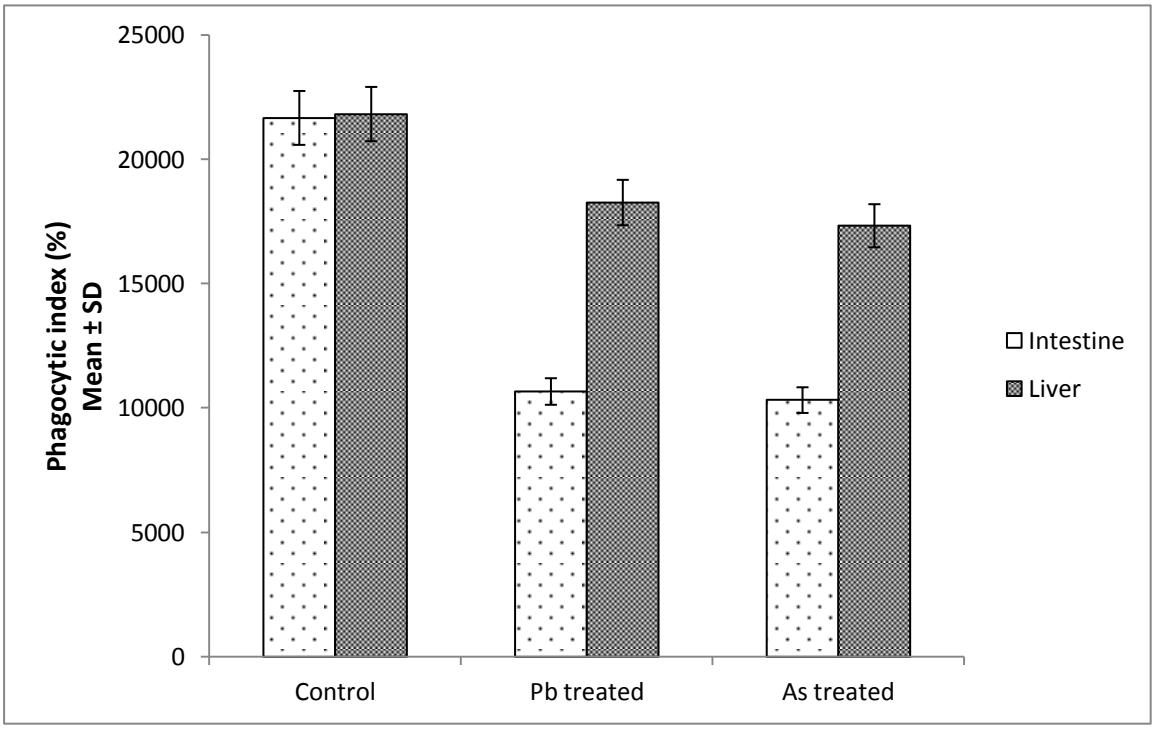


Fig 1: Phagocytic activity in macrophages of fish treated with lead and arsenic. As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

ii) Effect of lead and arsenic on intracellular killing activity of fish macrophages

There was a significant increase in bacterial viability (%) at different time interval in both lead and arsenic treated group as compared to the control group (Fig: 2).

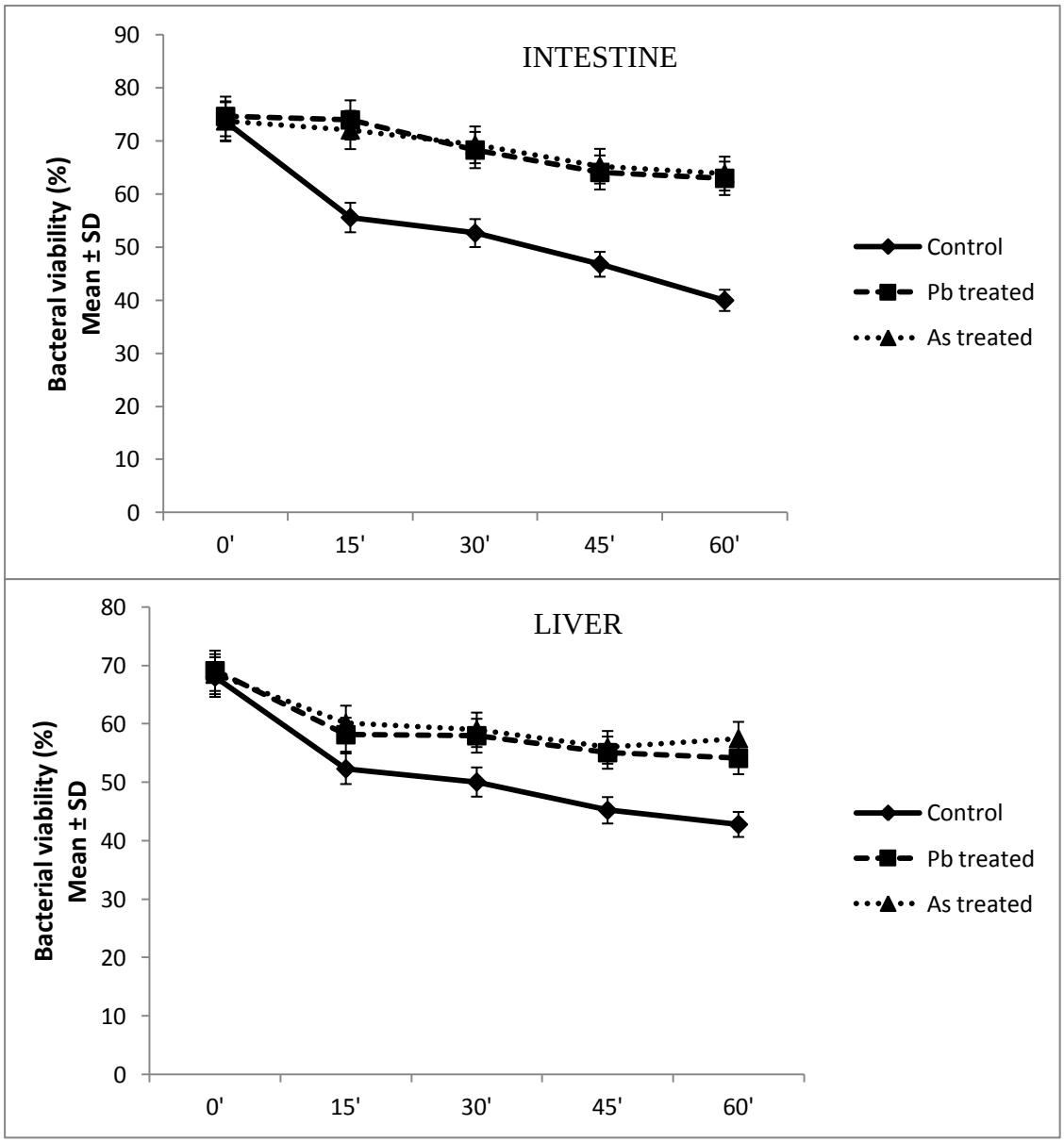


Fig 2: Intracellular killing activity in macrophages of fish treated with lead and arsenic. Intestine- Pb ($P<0.05$); As ($P<0.005$) Liver- Pb ($P<0.01$); As ($P<0.025$)

iii) Effect of lead and arsenic on chemotactic migration of fish macrophages

There was a significant decrease in chemotactic migration at different interval of time in both lead and arsenic treated group as compared to the control (Fig: 3).

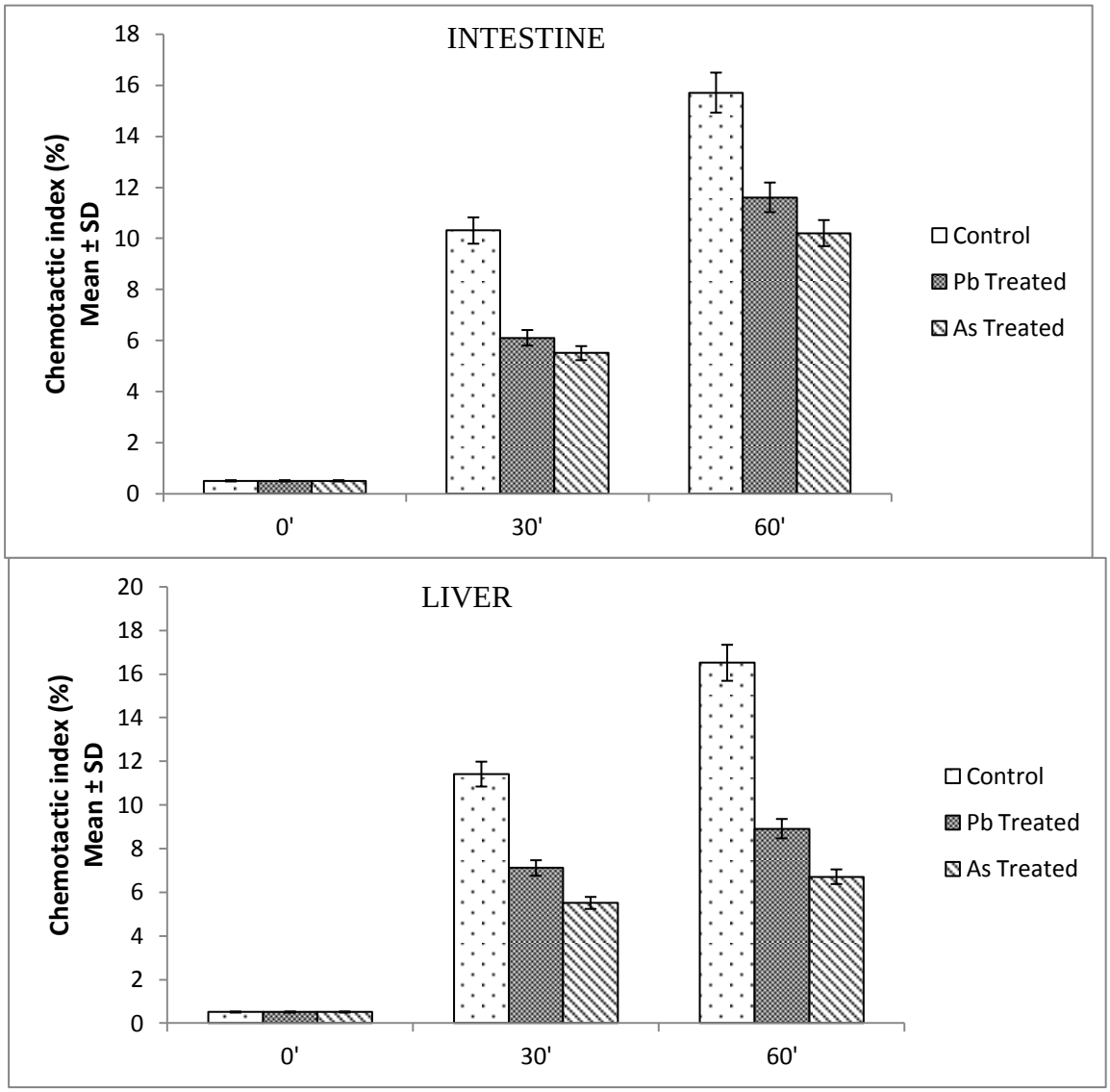


Fig 3: Chemotactic index in macrophages of fish treated with lead and arsenic.

Intestine- Pb ($P<0.04$); As ($P<0.025$) Liver- Pb ($P<0.02$); As ($P<0.001$).

iv) Effect of lead and arsenic on the *in vitro* cell adhesion property of fish macrophages

There was a significant decrease in the cell adhesion property of cells at different time interval in both lead and arsenic treated group as compared to the control (Fig: 4).

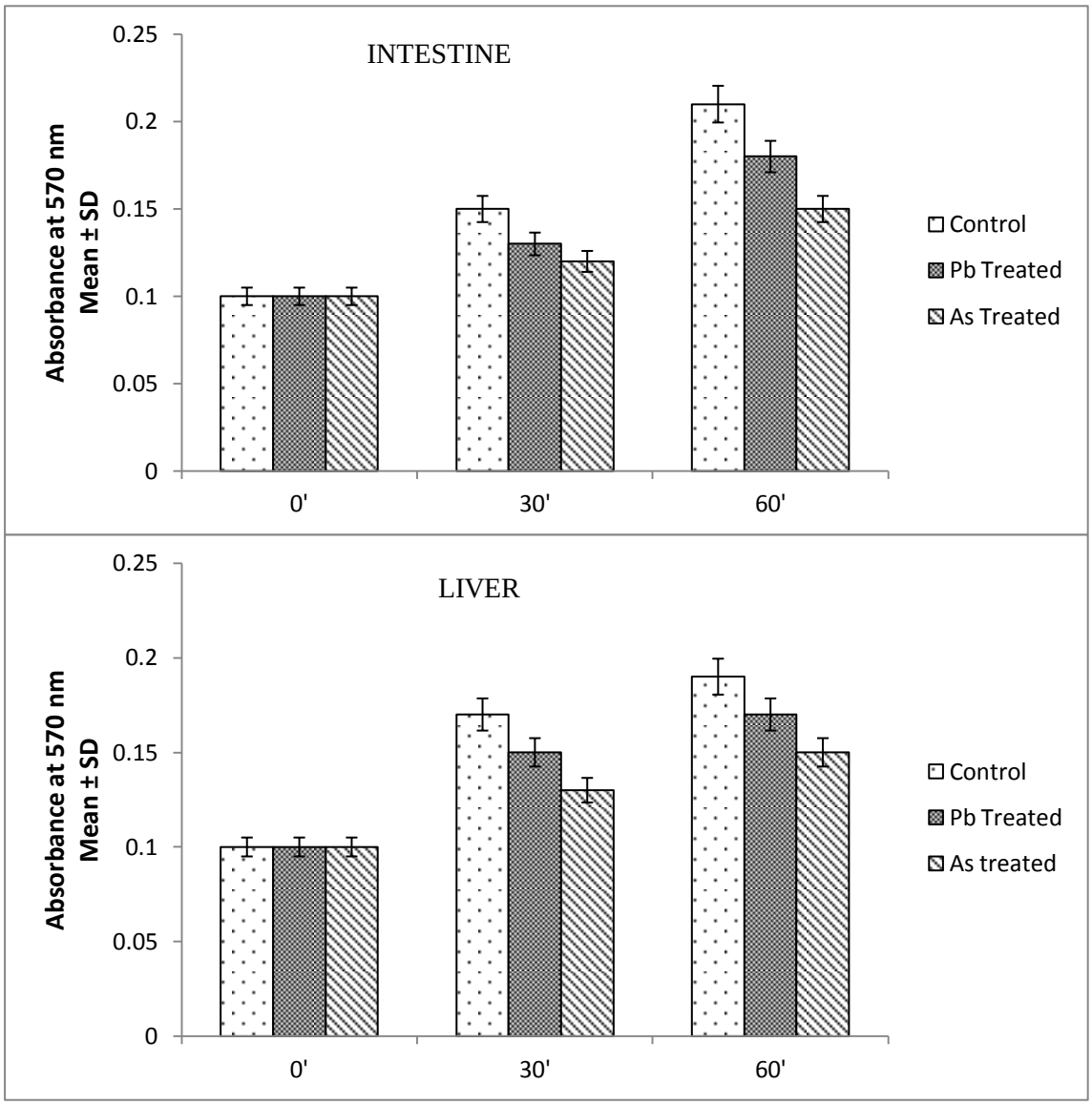


Fig 4: *In vitro* cell adhesion in macrophages of fish treated with lead and arsenic.

Intestine- Pb ($P<0.025$); As ($P<0.004$) Liver- Pb ($P<0.01$); As ($P<0.025$)

v) Effect of lead and arsenic on nitric oxide (NO) release of fish macrophages

There was a significant decrease in nitric oxide release from 50.02±0.06µg/100 ml (intestine), 48.61±0.03 µg/100 ml (liver) in control group to 4.61±0.041µg/100 ml (intestine), 5.18±0.051 µg/100 ml (liver) in lead treated and 5.76±0.038 µg/100 ml (intestine), 4.83±0.05 µg/100 ml (liver) in arsenic treated group (Fig: 5).

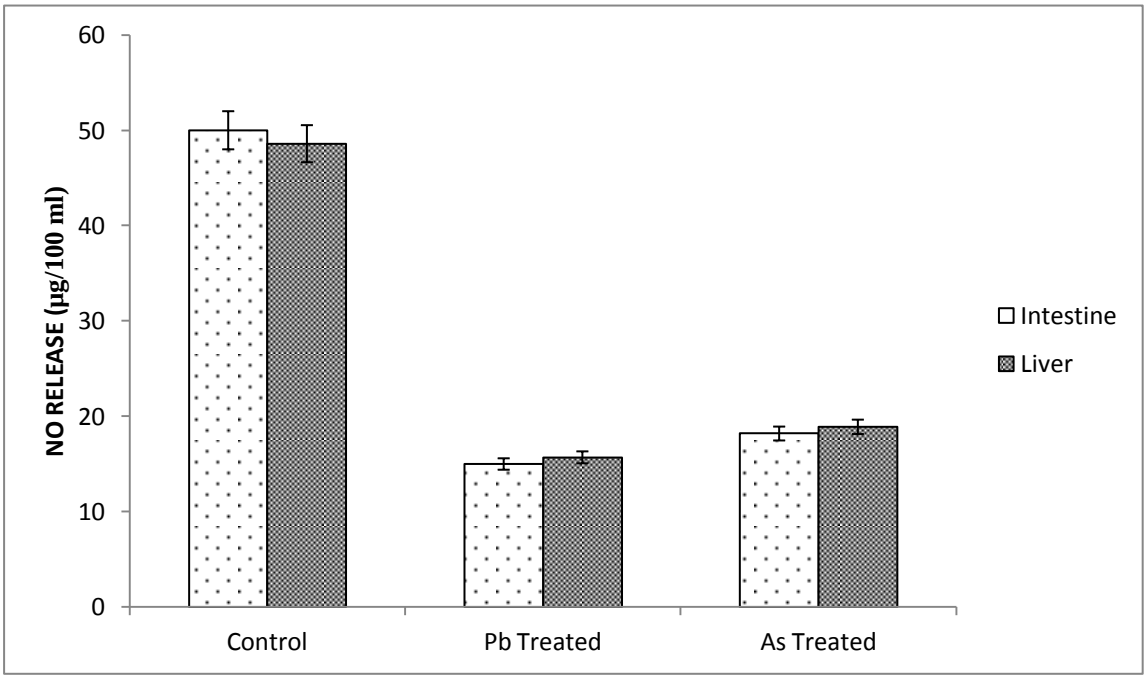


Fig 5: Nitric oxide release in macrophages of fish treated with lead and arsenic.

Liver- Pb ($P< 0.01$) As ($P<0.02$); Intestine- Pb, As ($P< 0.05$).

vi) Effect of lead and arsenic on myeloperoxidase (MPO) release of fish macrophages

There was a significant decrease in myeloperoxidase release from 43.51±0.04% (intestine), 46.01±0.06 % (liver) in control group to 24.42±1.134 % (intestine), 31.06±0.04 % (liver) in lead treated and 21.29±0.02 % (intestine), 26.4±0.06 % (liver) in arsenic treated group (Fig: 6).

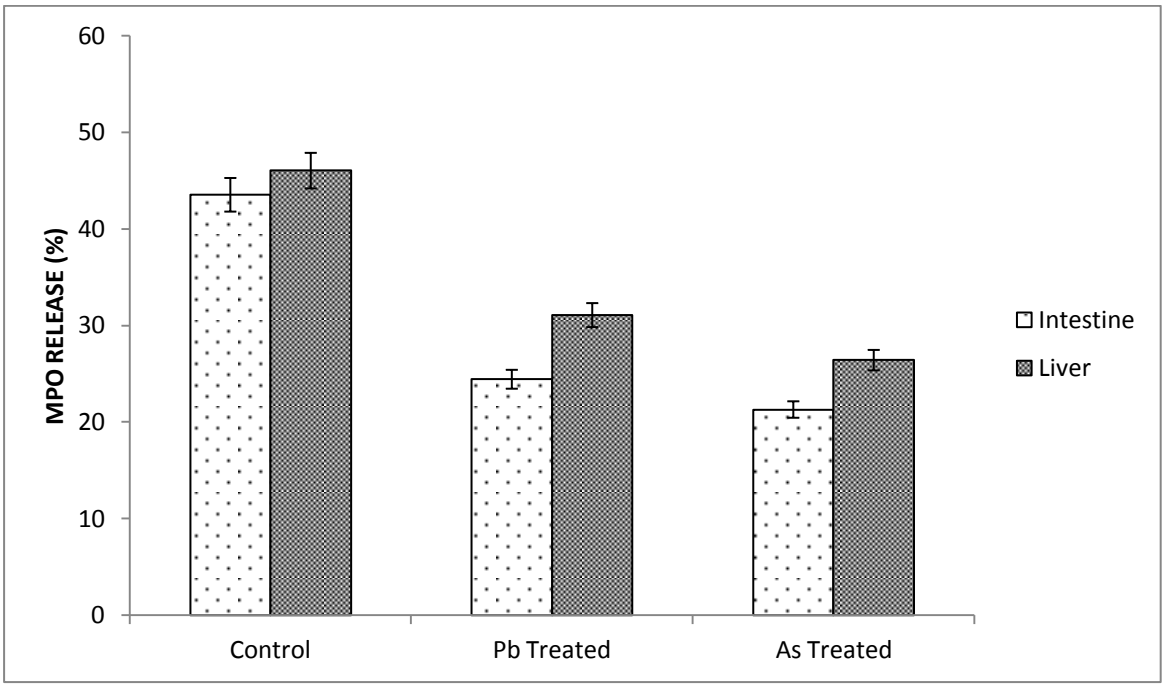


Fig 6: Myeloperoxidase release in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) P<0.02; Pb (liver) P< 0.05; As (liver) P< 0.04.

vii) Effect of lead and arsenic on TNF-α release of fish macrophages

TNF-α release both from cell lysate and plasma was significantly decreased in lead treated and arsenic treated group as compared to the control group (Fig: 7).

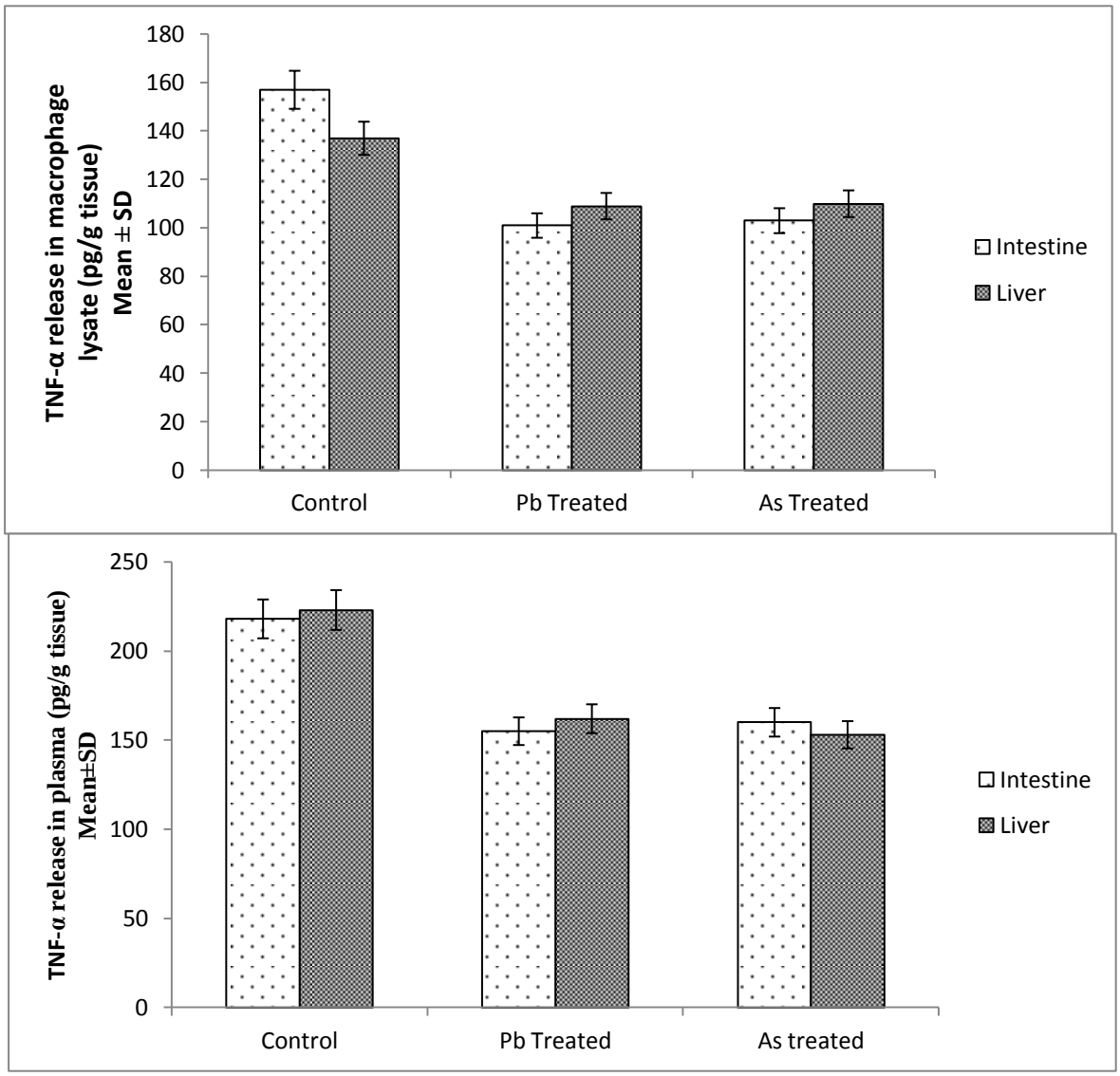


Fig 7: (a) TNF-α released from cell lysate of lead and arsenic treated group (b) TNF-α released from plasma of lead and arsenic treated group.

As (liver) $P<0.02$; Pb, As (intestine) $P< 0.05$; Pb (liver) $P< 0.01$.

viii) Effect of lead and arsenic on IL- 1b release of fish macrophages

There was a significant decrease in IL-1b release from 0.074±0.06 pg/g tissue (intestine), 0.078±0.02 pg/g tissue (liver) in control group to 0.068±0.092 pg/g tissue (intestine), 0.069±0.06 pg/g tissue (liver) in lead treated and 0.057±0.044 pg/g tissue (intestine), 0.05±0.034 pg/g tissue (liver) in arsenic treated group (Fig: 8).

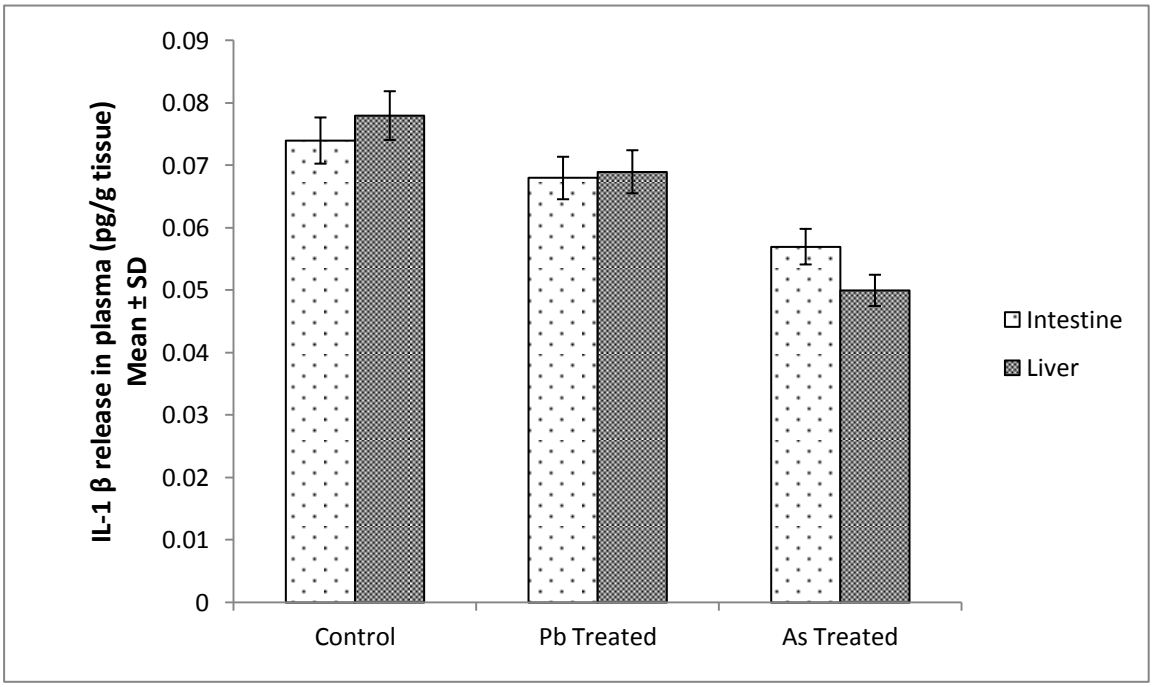


Fig 8: IL-1b released from plasma of lead and arsenic treated group.

As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

Chapter 2: *In vitro* study of effect of lead acetate and sodium arsenite on oxidative stress, antioxidant defenses and immune functions in macrophages from fish *Channa punctatus*

4.4. STUDY OF EFFECT OF LEAD ACETATE AND SODIUM ARSENITE (*IN VITRO*) SINGLY ON OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Effect of lead and arsenic on respiratory burst activity of fish macrophages

There was a significant increase in respiratory burst activity from 0.14±1.63 (intestine), 0.16±1.38 (liver) in control group to 0.42±1.23 (intestine), 0.44±1.31 (liver) in lead treated and 0.48±1.11 (intestine), 0.47±1.19 (liver) in arsenic treated group (Fig:1).

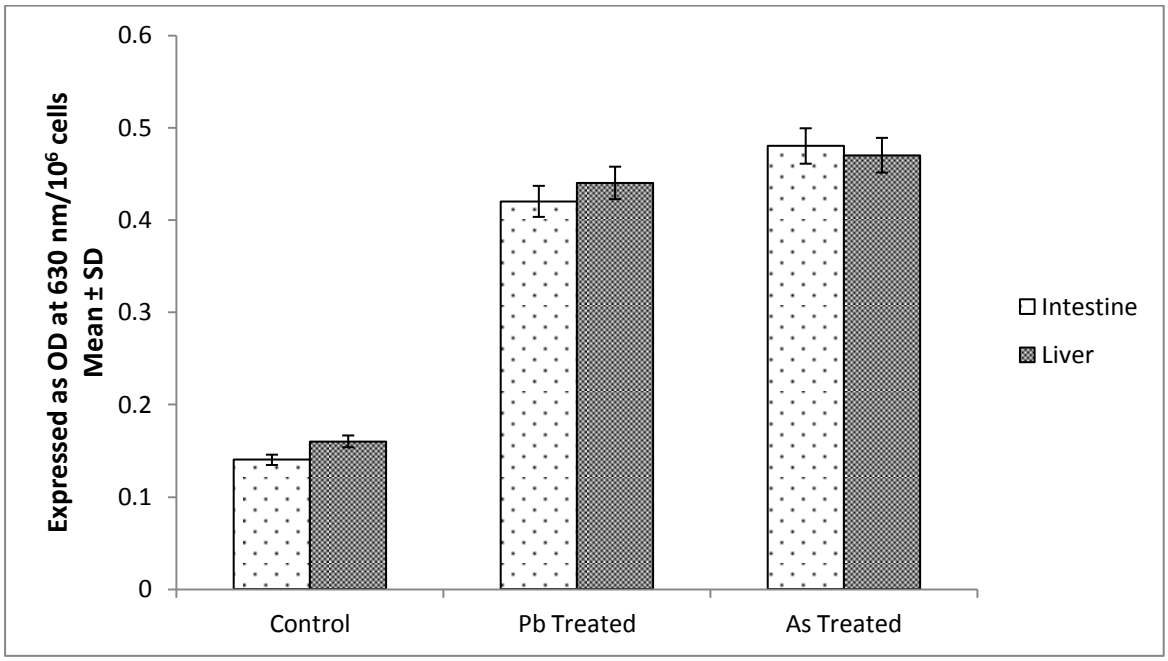


Fig 1: Respiratory burst activity in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) P< 0.025; Pb, As (liver) P< 0.005.

ii) Effect of lead and arsenic on protein carbonylation of fish macrophages

There was a significant increase in protein carbonylation from 0.08±0.02 mg/ml (intestine), 0.07±0.02 mg/ml (liver) in control group to 0.13±0.134 mg/ml (intestine), 0.14 mg/ml (liver) in lead treated and 0.15±0.055 mg/ml (intestine), 0.17±0.11 mg/ml (liver) in arsenic treated group (Fig:2).

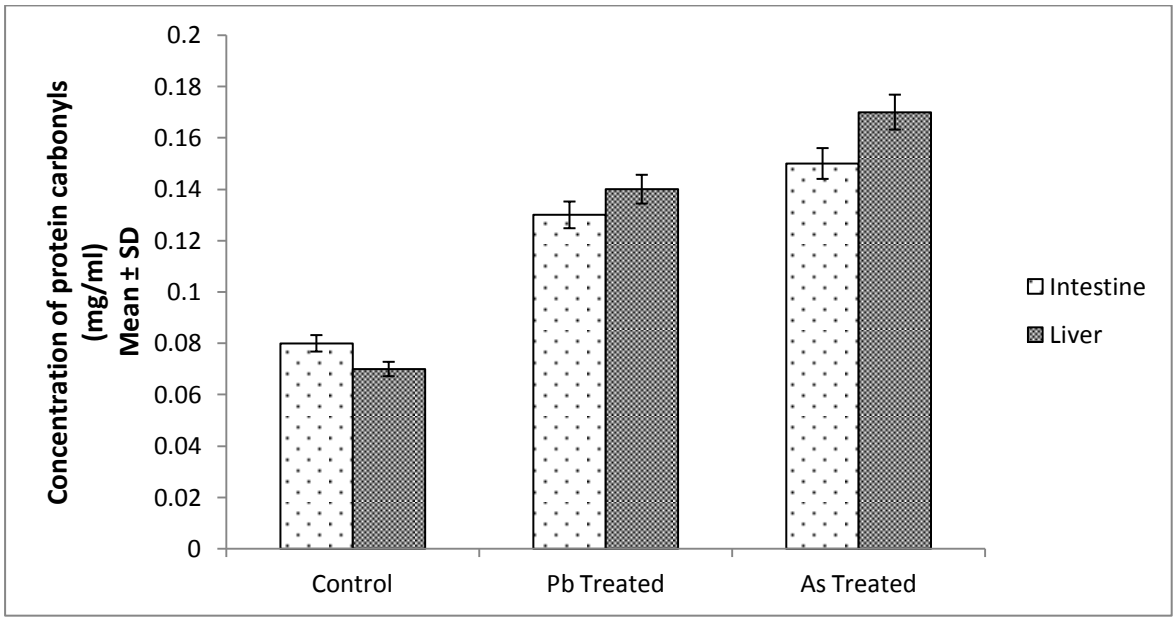


Fig 2: Estimation of protein carbonylation (PC) in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) $P< 0.04$; Pb, As (liver) $P< 0.01$.

iii) Percentage of DNA fragmentation in lead and arsenic treated fish macrophages

There was a significant increase in DNA fragmentation from 0.01±0.12% (intestine), 0.012±0.13% (liver) in control group to 0.03±0.18% (intestine), 0.036±0.13% (liver) in lead treated and 0.044±0.03% (intestine), 0.041±0.002% (liver) in arsenic treated group (Fig: 3).

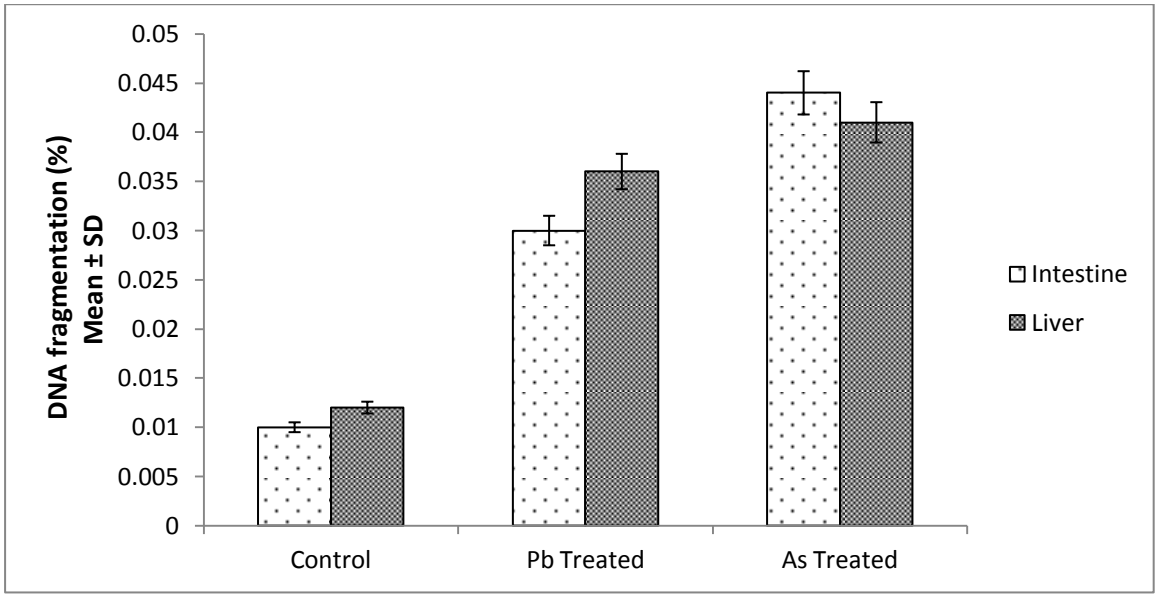


Fig 3: Percentage of DNA fragmentation in lead and arsenic treated fish.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

iv) Effect of lead and arsenic on catalase (CAT) activity of fish macrophages

There was a significance decrease in catalase activity from 0.24±1.37 U/mg protein (intestine), 0.22±1.82 U/mg protein (liver) in control group to 0.16±1.184 U/mg protein (intestine), 0.17±1.36 U/mg protein (liver) in lead treated and 0.13±1.55 U/mg protein (intestine), 0.11±1.99 U/mg protein (liver) in arsenic treated group (Fig:4).

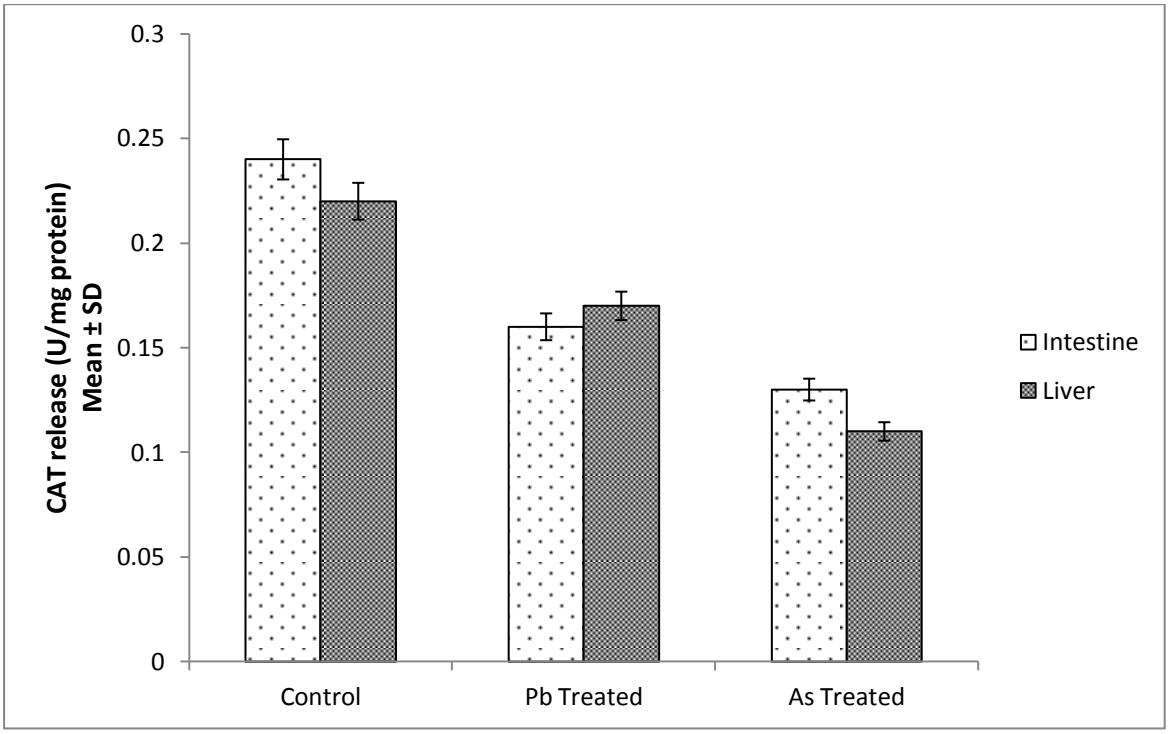


Fig 4: Catalase (CAT) activity in control and heavy metal exposed fish macrophages.

Pb, As (intestine) P< 0.01; Pb, As (liver) P< 0.025.

v) Effect of lead and arsenic on superoxide dismutase (SOD) activity of fish macrophages

There was a significance increase in superoxide dismutase activity from 0.03±1.26 U/mg protein (intestine), 0.02±1.86 U/mg protein (liver) in control group to 0.08±1.17 U/mg protein (intestine), 0.09±1.92 U/mg protein (liver) in lead treated and 0.07±1.52 U/mg protein (intestine), 0.1±1.37 U/mg protein (liver) in arsenic treated group (Fig:5).

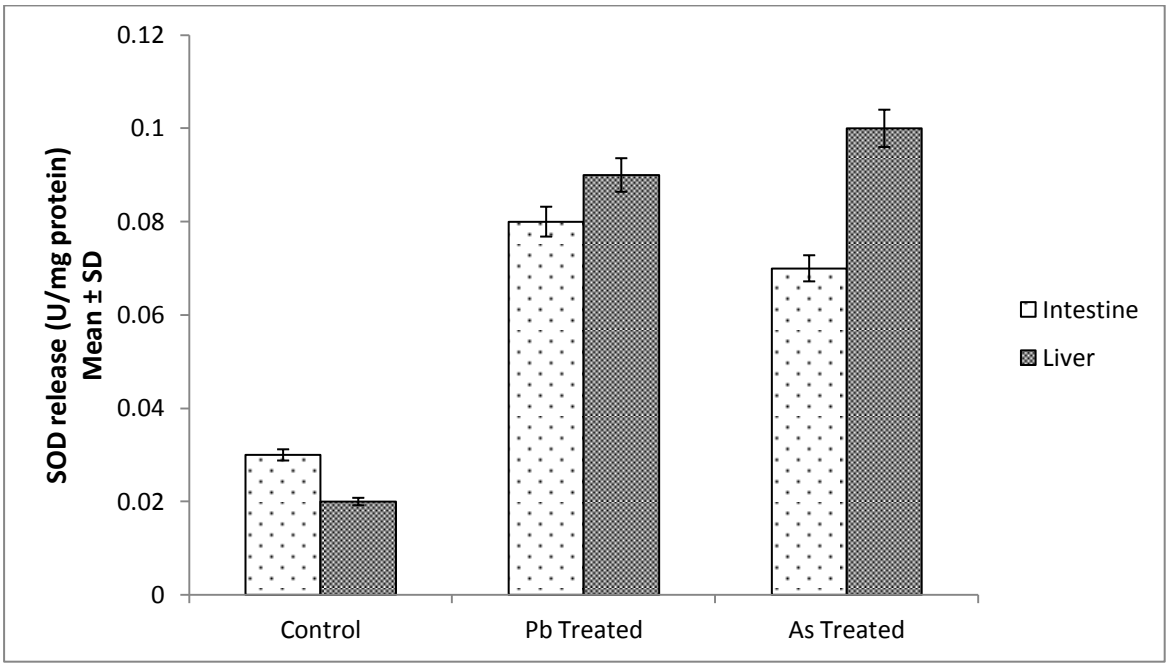


Fig 5: Superoxide dismutase (SOD) activity in control and heavy metal exposed fish macrophages.

As (intestine) P< 0.02; Pb (intestine) P< 0.02; Pb, As (liver) P< 0.04.

vi) Effect of lead and arsenic on reduced glutathione (GSH) activity of fish macrophages

There was a significance decrease in reduced glutathione activity from 0.05±1.13 nmoles/min/mg protein (intestine), 0.041±1.1 nmoles/min/mg protein (liver) in control group to 0.022±1.12 nmoles/min/mg protein (intestine), 0.025±1.33 nmoles/min/mg protein (liver) in lead treated and 0.02±1.18 nmoles/min/mg protein (intestine), 0.019±1.22 nmoles/min/mg protein (liver) in arsenic treated group (Fig:6).

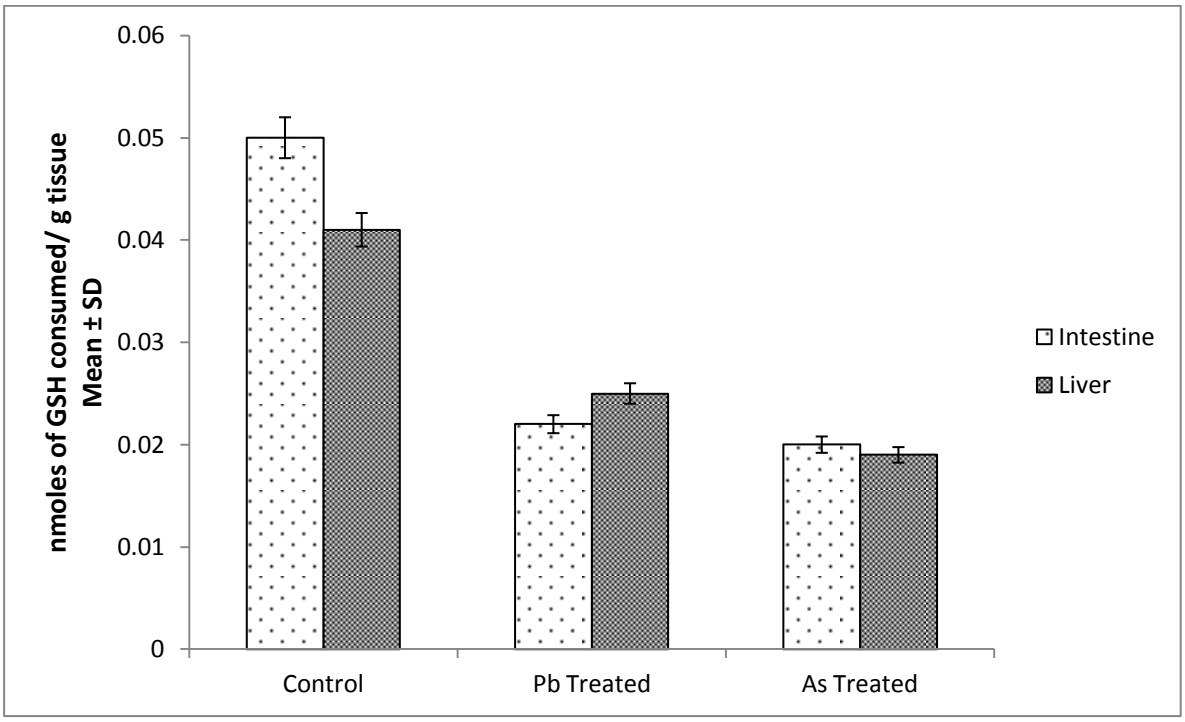


Fig 6: Reduced glutathione (GSH) consumed in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) $P< 0.25$; Pb, As (liver) $P< 0.001$.

4.5. STUDY OF EFFECT OF LEAD ACETATE AND SODIUM ARSENITE (*IN VITRO*) SINGLY ON INNATE IMMUNE RESPONSES IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Effect of lead and arsenic on phagocytic activity of fish macrophages

There was a significant decrease in phagocytic activity from 2163.11±1.22 % (intestine), 2122.11±1.33 % (liver) in control group to 1088.62±1.27 % (intestine), 1065.43±1.55 % (liver) in lead treated and 1035.12±1.99 % (intestine), 1043.44±1.96 % (liver) in arsenic treated group (Fig:1).

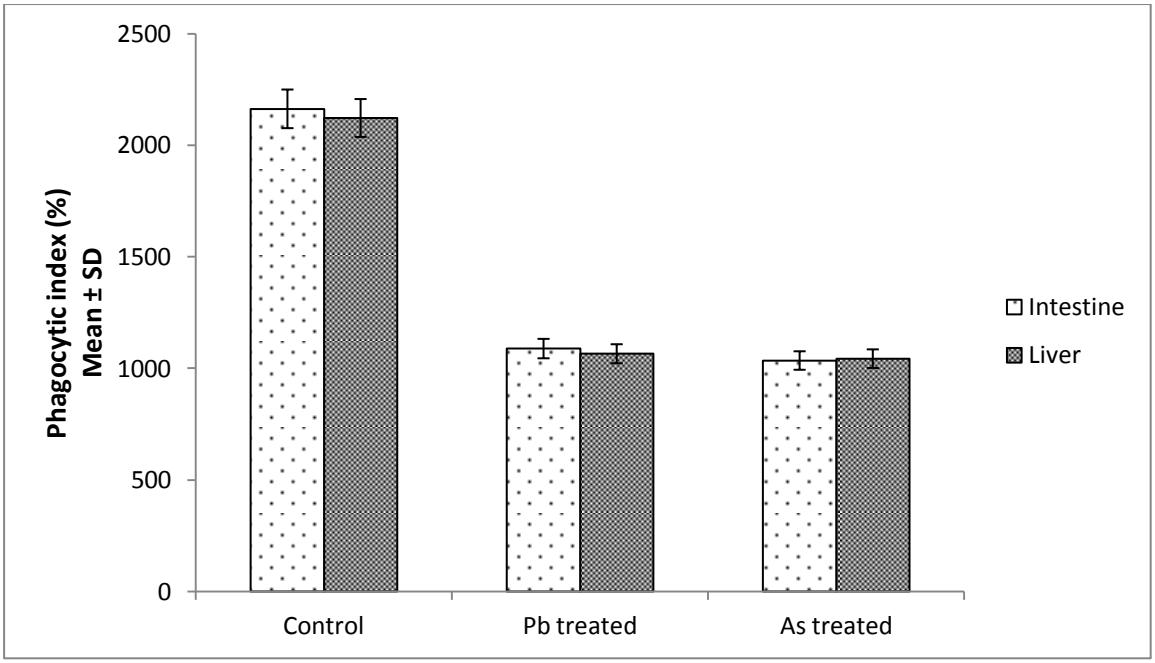


Fig 1: Phagocytic activity in macrophages of fish treated with lead and arsenic. As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

ii) Effect of lead and arsenic on nitric oxide (NO) release of fish macrophages

There was a significant decrease in nitric oxide release from 0.03±0.11µg/100 ml (intestine), 0.031±0.03 µg/100 ml (liver) in control group to 0.021±0.04 µg/100 ml (intestine), 0.02±0.053 µg/100 ml (liver) in lead treated and 0019±0.018 µg/100 ml (intestine), 0.018±0.15 µg/100 ml (liver) in arsenic treated group (Fig:2).

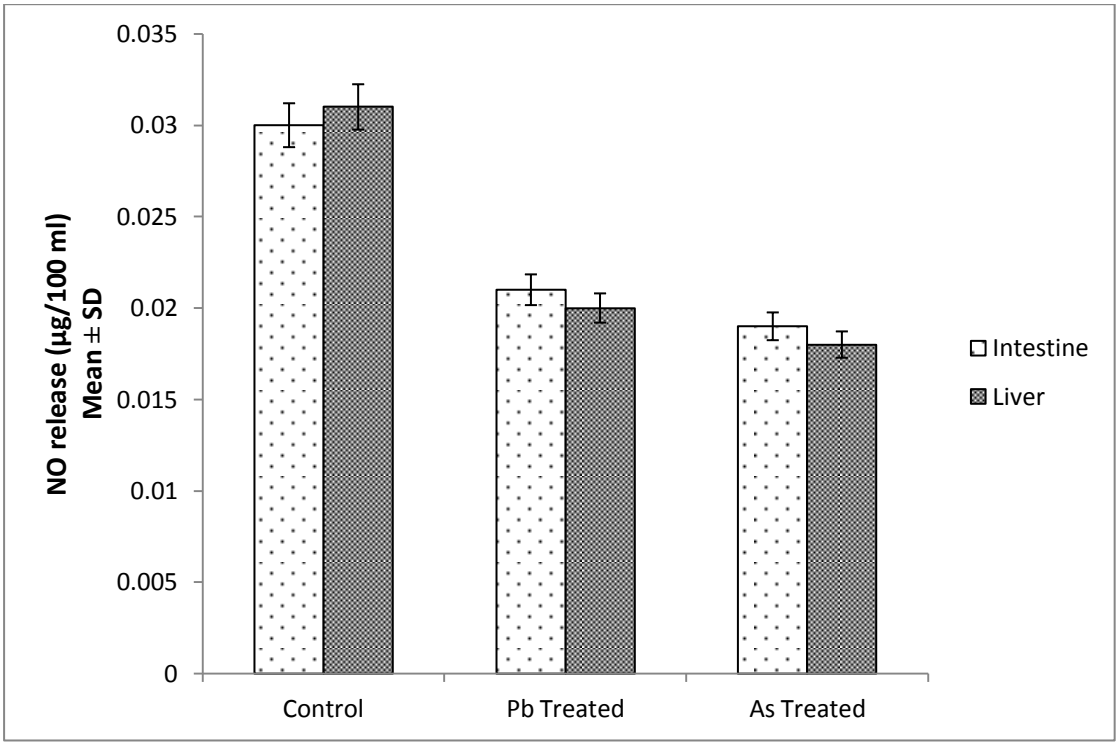


Fig 2: Nitric oxide release in macrophages of fish treated with lead and arsenic.

As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

iii) Effect of lead and arsenic on myeloperoxidase (MPO) release of fish macrophages

There was a significant decrease in myeloperoxidase release from 0.2±0.14 % (intestine), 0.22±0.16 % (liver) in control group to 0.15±1.14 % (intestine), 0.14±0.77 % (liver) in lead treated and 0.11±0.52 % (intestine), 0.13±0.66 % (liver) in arsenic treated group (Fig:3).

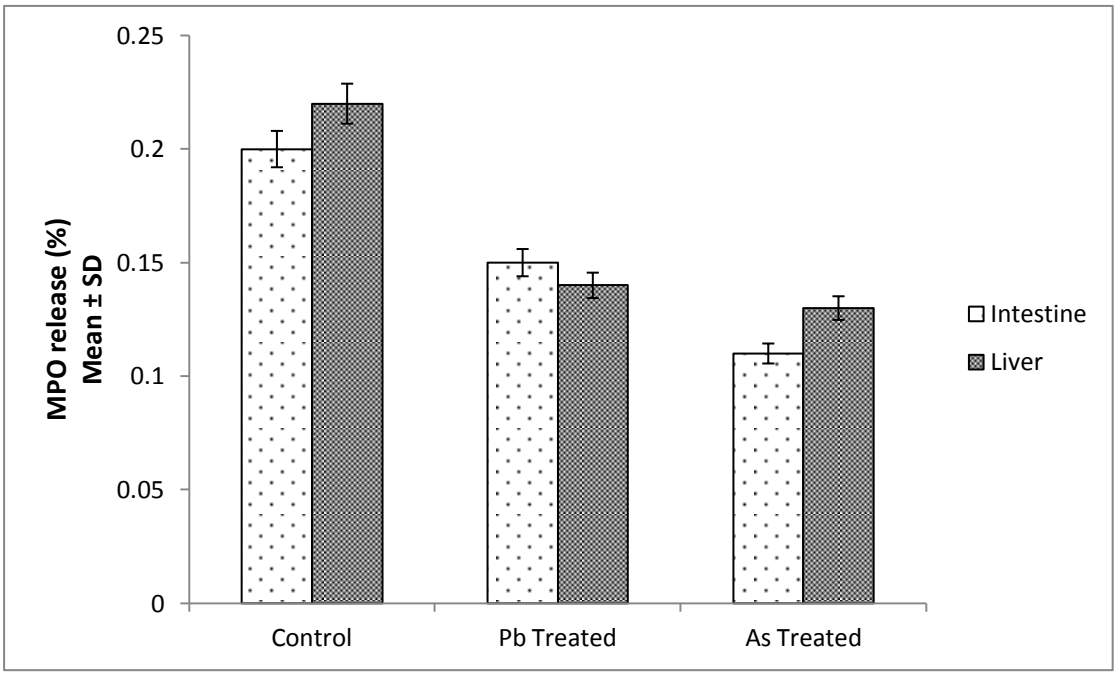


Fig 3: Myeloperoxidase release in macrophages of fish treated with lead and arsenic.

Pb, As (intestine) P<0.02; Pb (liver) P< 0.05; As (liver) P< 0.04.

iv) Effect of lead and arsenic on TNF-αrelease of fish macrophages

TNF-α release both from cell lysate and plasma was significantly decreased in lead treated and arsenic treated group as compared to the control group (Fig:4).

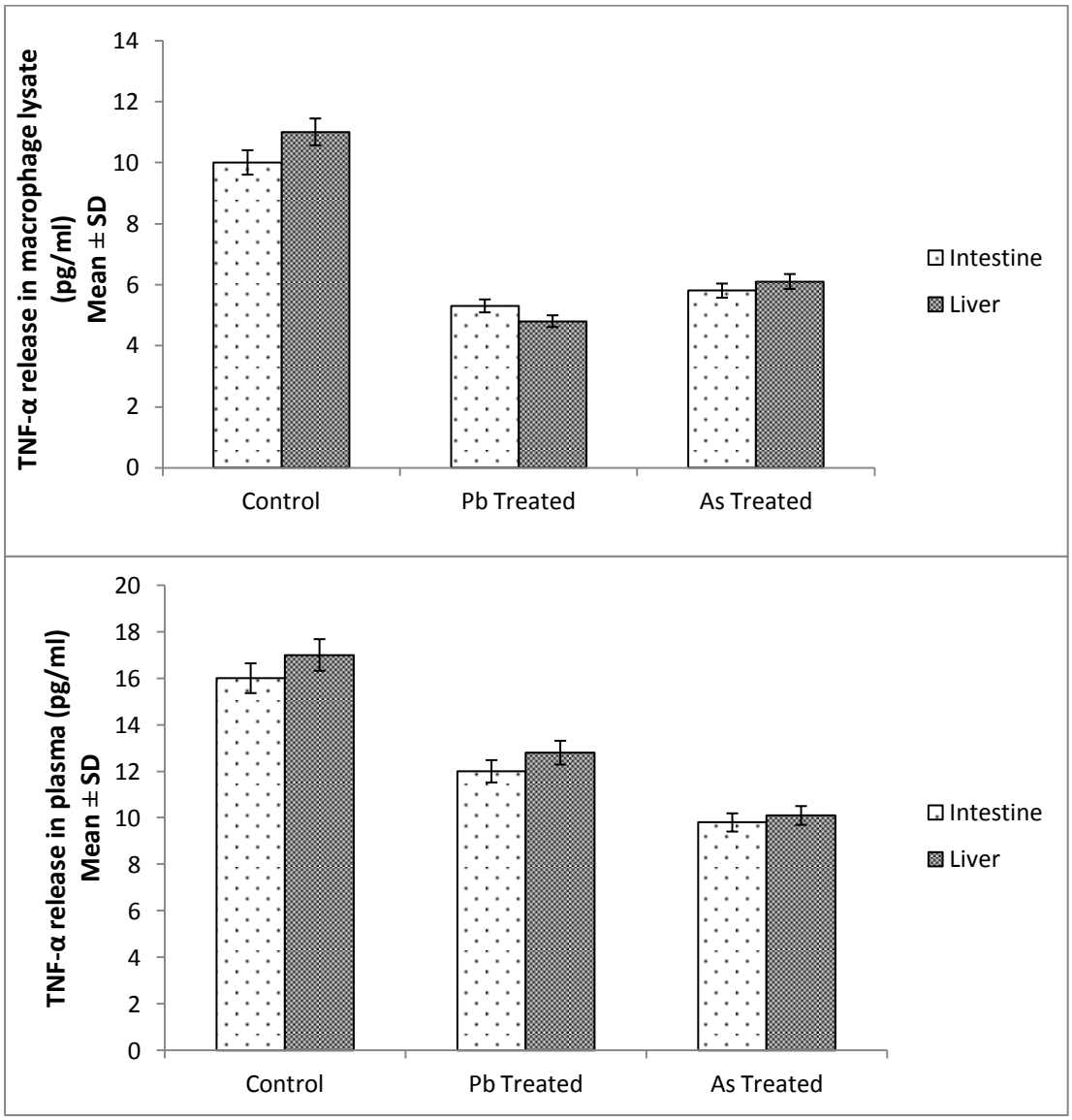


Fig 4: (a) TNF-α released from cell lysate of lead and arsenic treated group (b) TNF-α released from plasma of lead and arsenic treated group.

As (liver) $P<0.02$; Pb, As (intestine) $P< 0.05$; Pb (liver) $P< 0.01$.

v) Effect of lead and arsenic on IL- 1b release of fish macrophages

There was a significant decrease in IL-1b release from 0.074±0.06 pg/g tissue (intestine), 0.078±0.02 pg/g tissue (liver) in control group to 0.068±0.092 pg/g tissue (intestine), 0.069±0.06 pg/g tissue (liver) in lead treated and 0.057±0.044 pg/g tissue (intestine), 0.05±0.034 pg/g tissue (liver) in arsenic treated group (Fig:5).

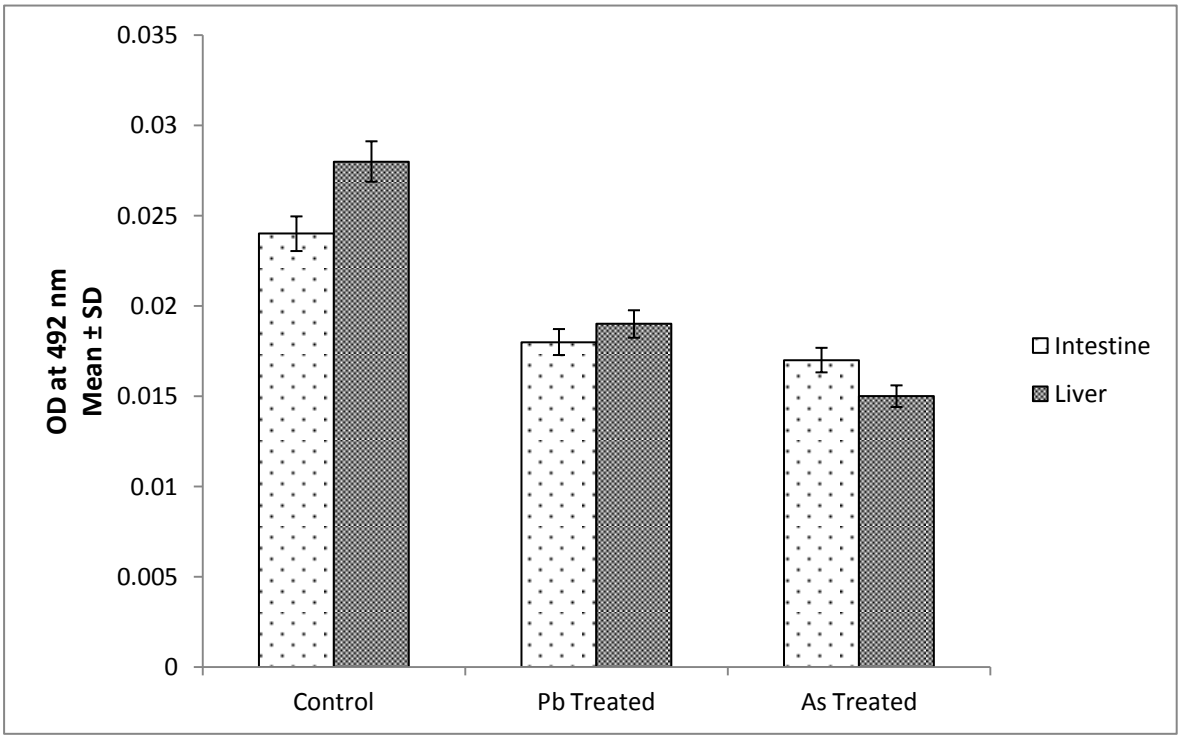


Fig 5: IL-1b released from plasma of lead and arsenic treated group.

As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

Chapter 3: *In vivo* study of lead acetate and sodium arsenite exposure simultaneously on intestinal and liver macrophages of fish *Channa punctatus*

4.6. EFFECTS FROM COMBINED LEAD ACETATE AND SODIUM ARSENITE (*IN VIVO*) EXPOSURE ON BIOACCUMULATION, MORPHOLOGICAL ALTERATION AND DNA DAMAGE IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Heavy metal analysis in different organs of untreated and treated multi-metal group (ppm/g tissue)

UNTREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
Lead accumulation	**0.02 ± 0.025**	**0.041± 0.02**	**0.045±0.024**	**0.021±0.019**
Arsenic accumulation	**0.031 ± 0.02**	**0.048±0.011**	**0.0255±0.015**	**0.029±0.05**

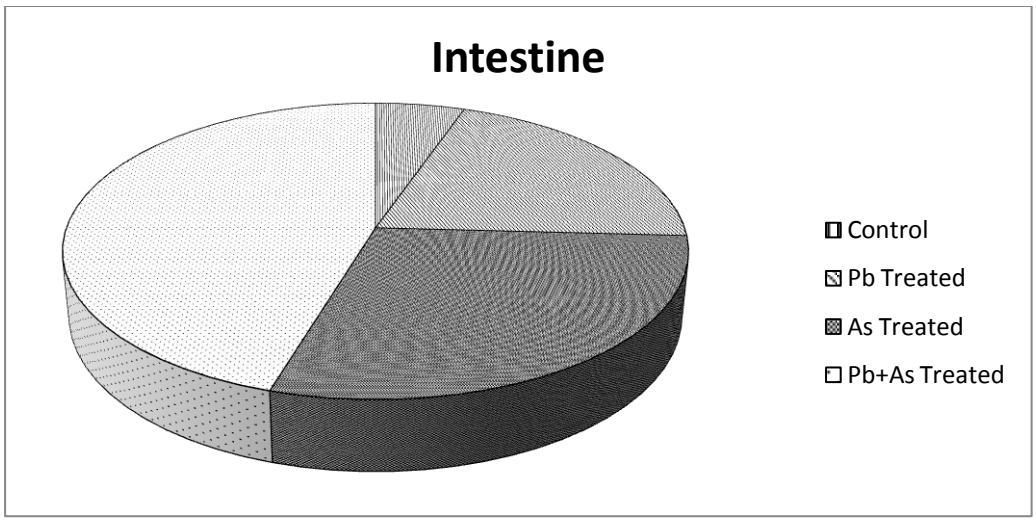
TREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
Lead	**0.33 ± 0.021**	**0.92±0.019**	**0.83 ± 0.021**	**0.43 ± 0.015**
Arsenic	**0.28± 0.015**	**1.06 ± 0.036**	**0.921±0.025**	**0.56 ± 0.025**

Note: The values were statistically significant at $P < 0.005$.

Table 1: Heavy metal analysis in different organs of untreated group and group with simultaneous treatment of lead and arsenic (ppm/g tissue).

i) Morphological alteration of macrophages in multi- metal treated group

(a)



(b)

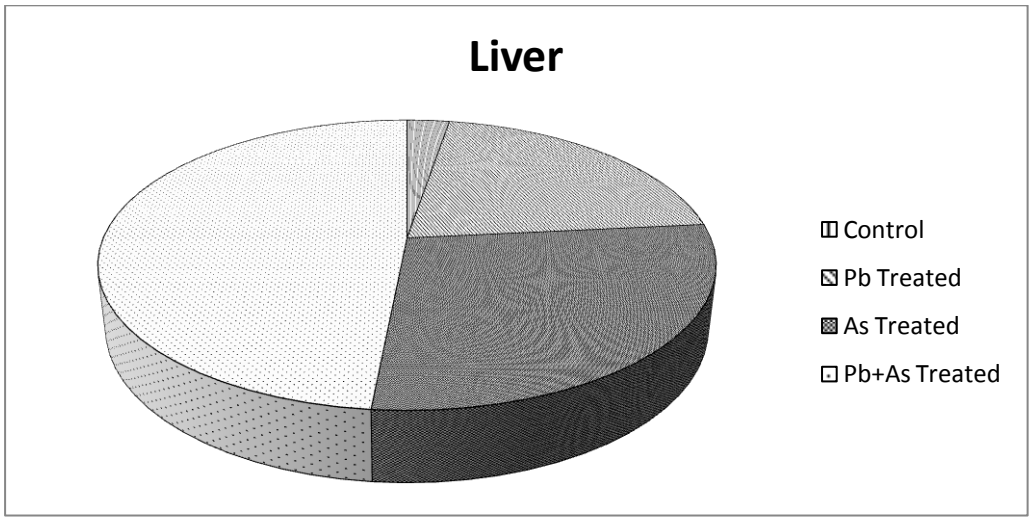
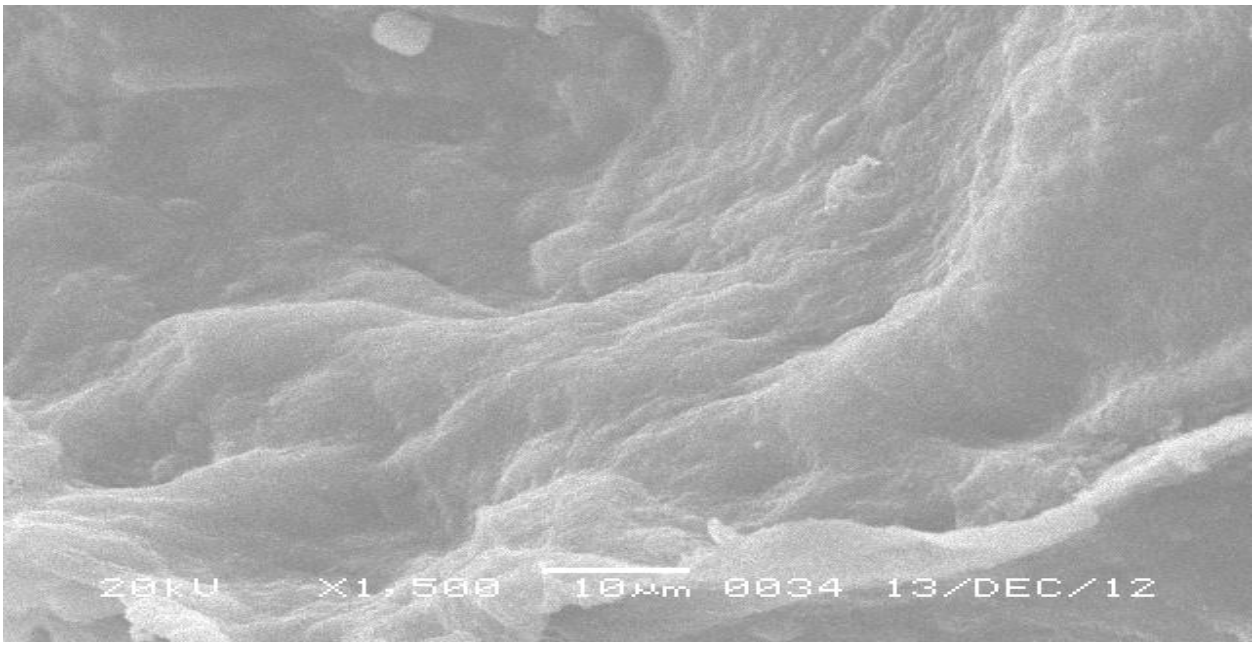


Fig 1: Combined effect of lead and arsenic on the morphological alteration of (a) intestinal and (b) liver macrophages. Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.05$.

ii) Ultrastructural analysis of tissue by Scanning Electron Microscope

(a)



(b)

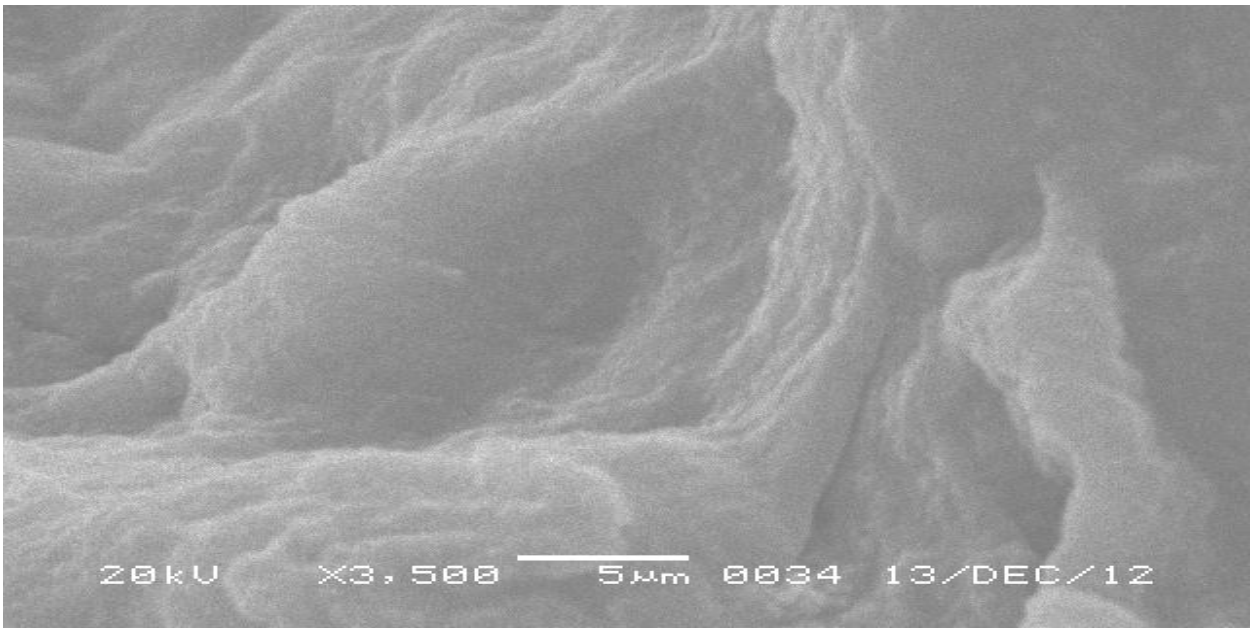
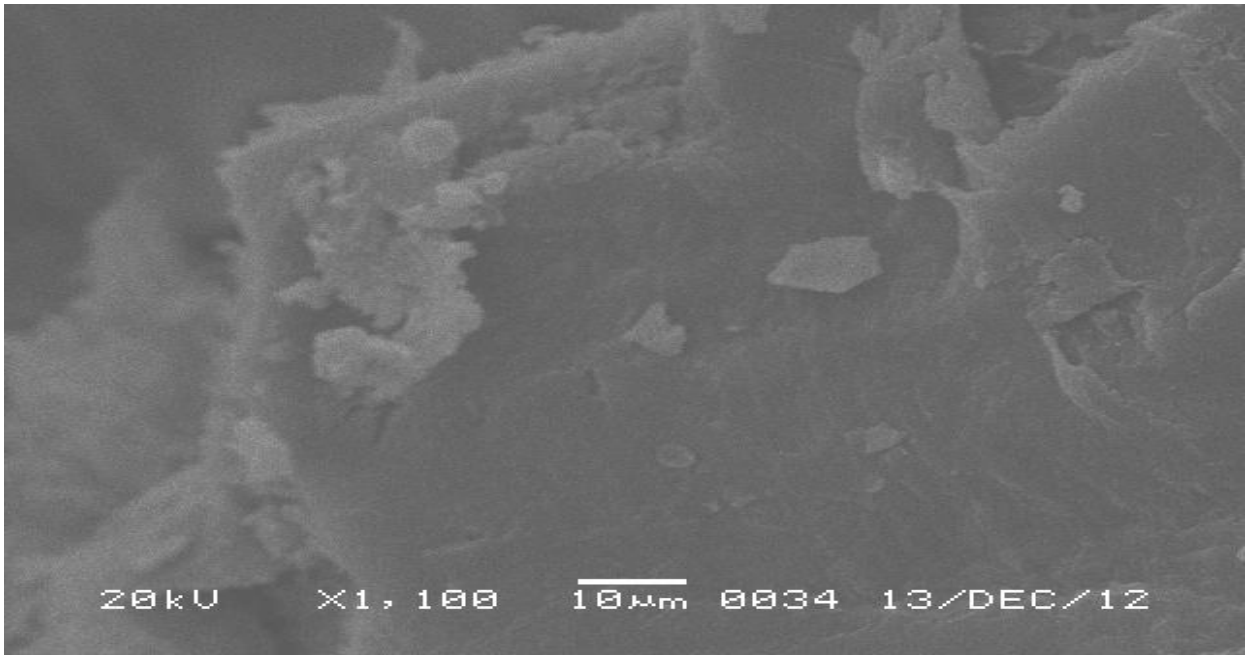


Plate 1(a), (b): Ultramicroscopic photographs of untreated fish showing prominent mucosal foldings and normal epithelium.

(c)



(d)

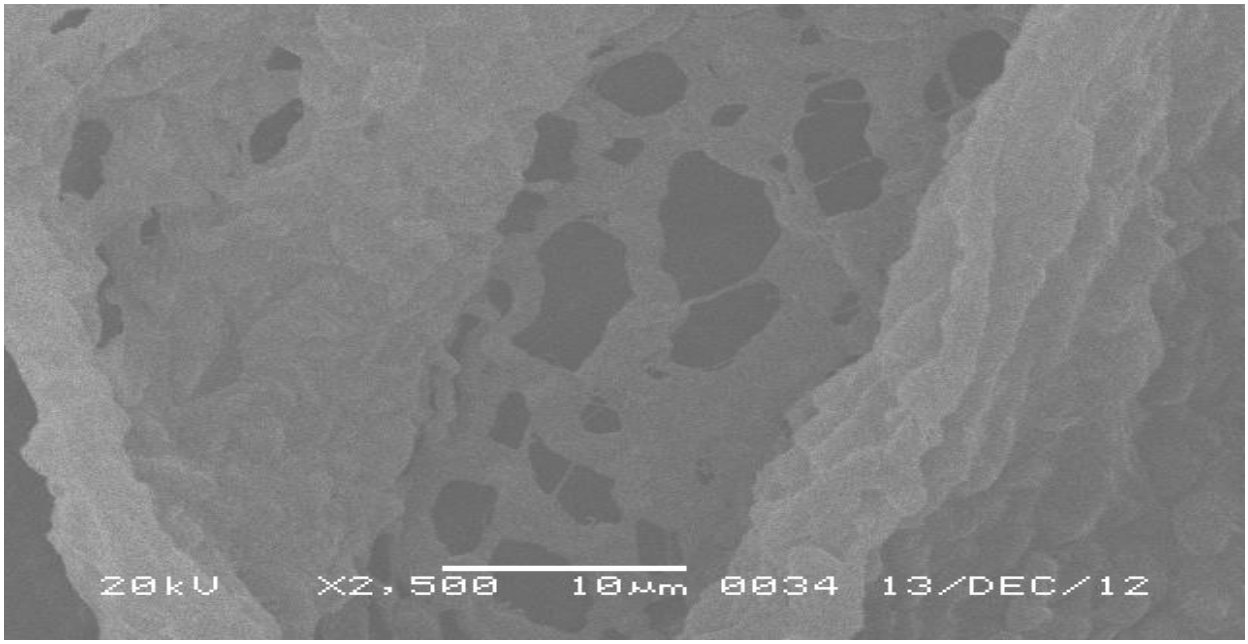
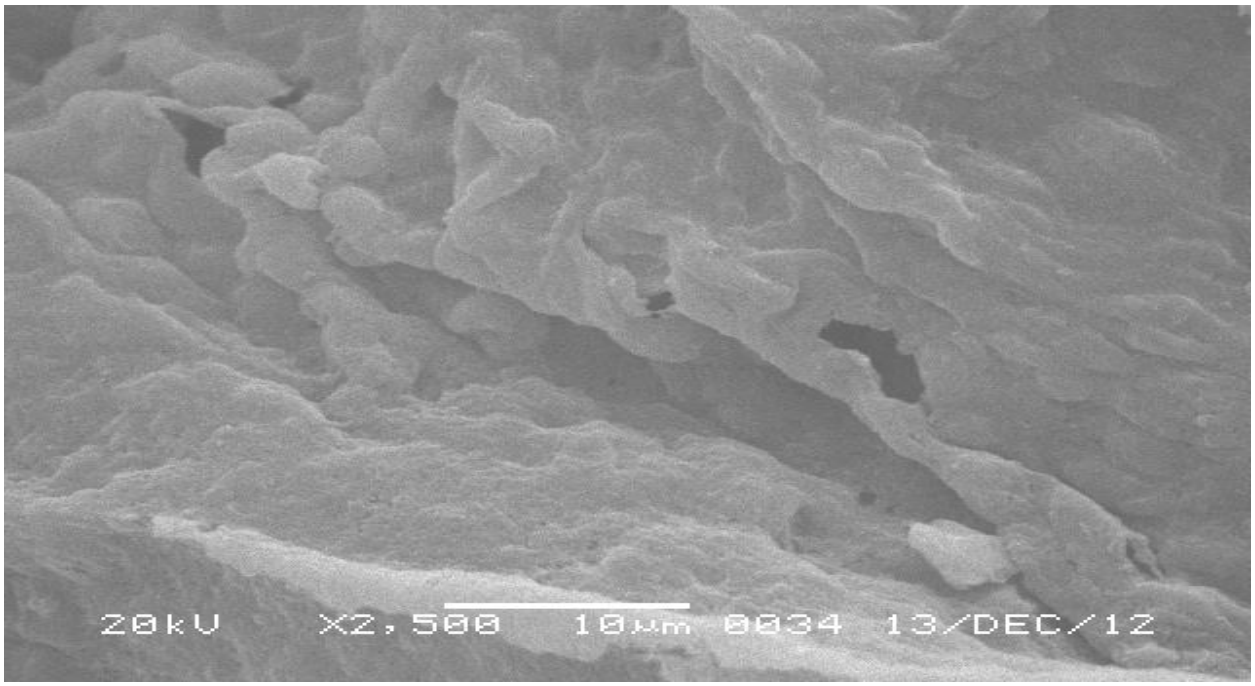


Plate 1(c), (d): Ultramicroscopic photographs of lead treated fish showing debris of mucosal folds, damaged and degenerated epithelium.

(e)



(f)

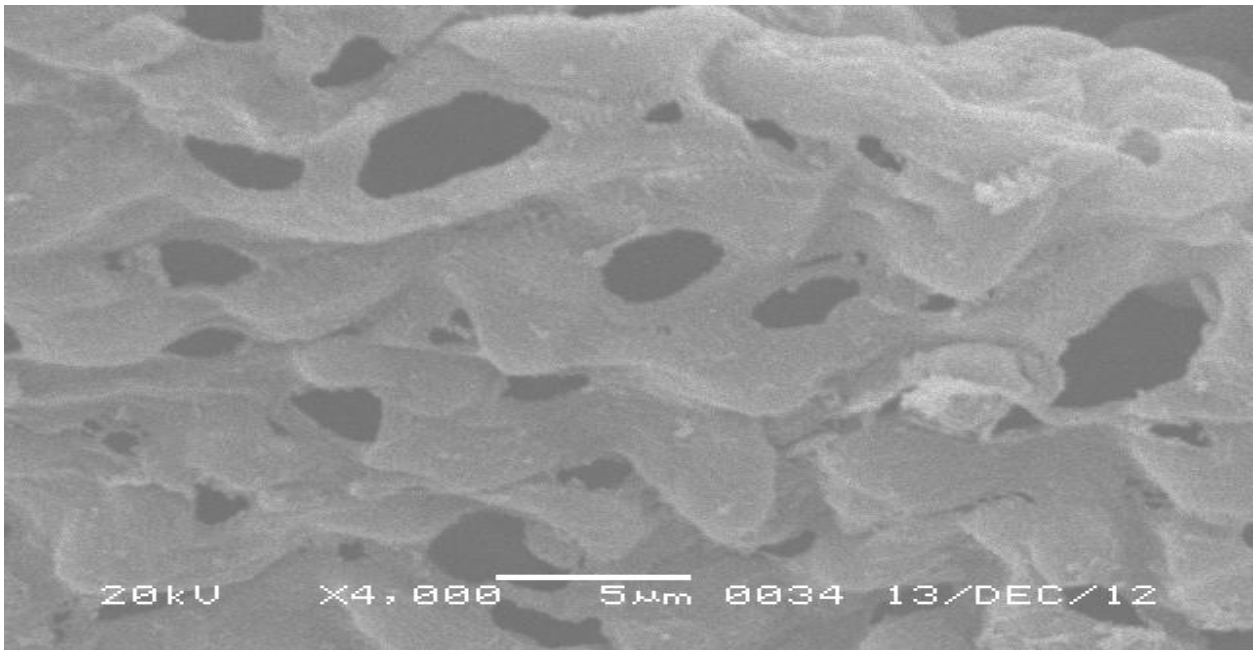
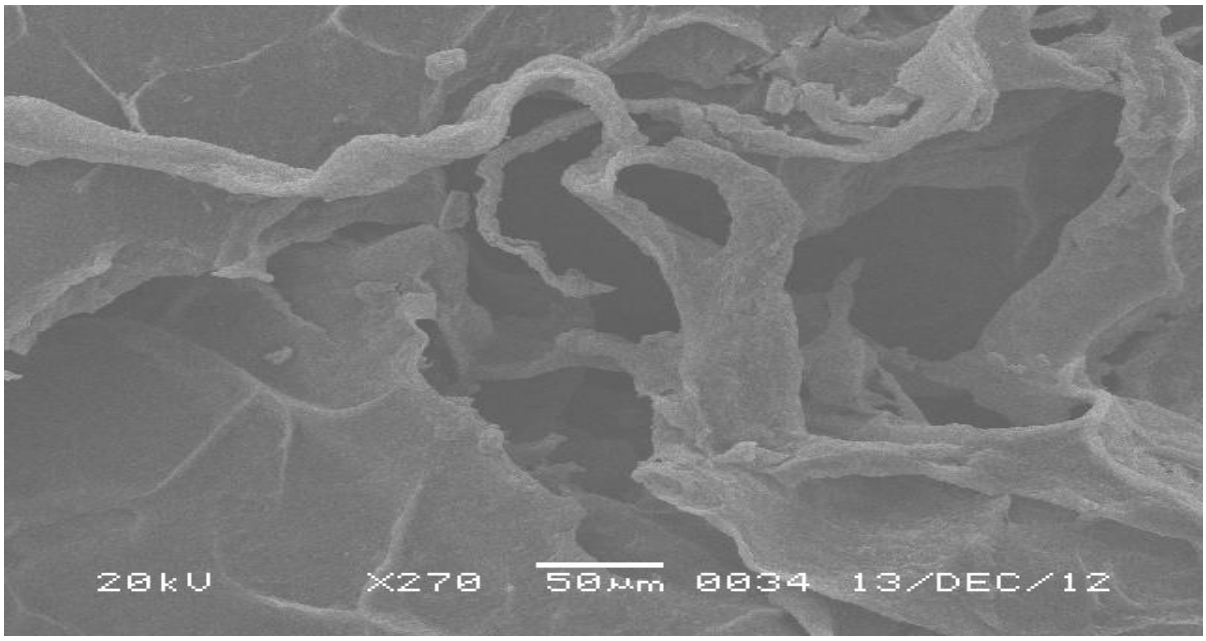


Plate 1(e), (f): Ultramicroscopic photographs of arsenic treated fish showing severe damage in epithelium, disarrangement and fragmentation of mucosal foldings and secretion of mucus.

(g)



(h)

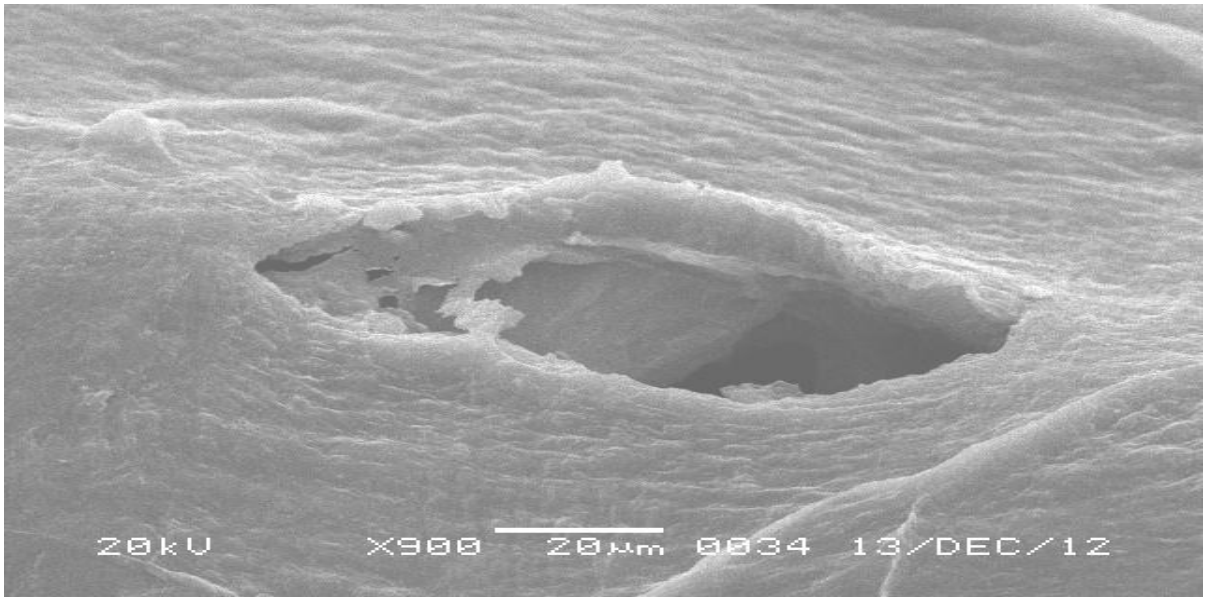


Plate 1(g), (h): Ultramicroscopic photographs of multi- metal treated fish showing large areas of disarranged and degenerated mucosal folds and severe damage in epithelium.

iv) Percentage of DNA fragmentation in multi- metal treated group

There was a significant increase in DNA fragmentation from 35.83±0.21% (intestine), 36.05±0.34% (liver) in control group to 92±0.24% (intestine), 89.2±0.22% (liver) in multi-metal treated group (Fig: 2).

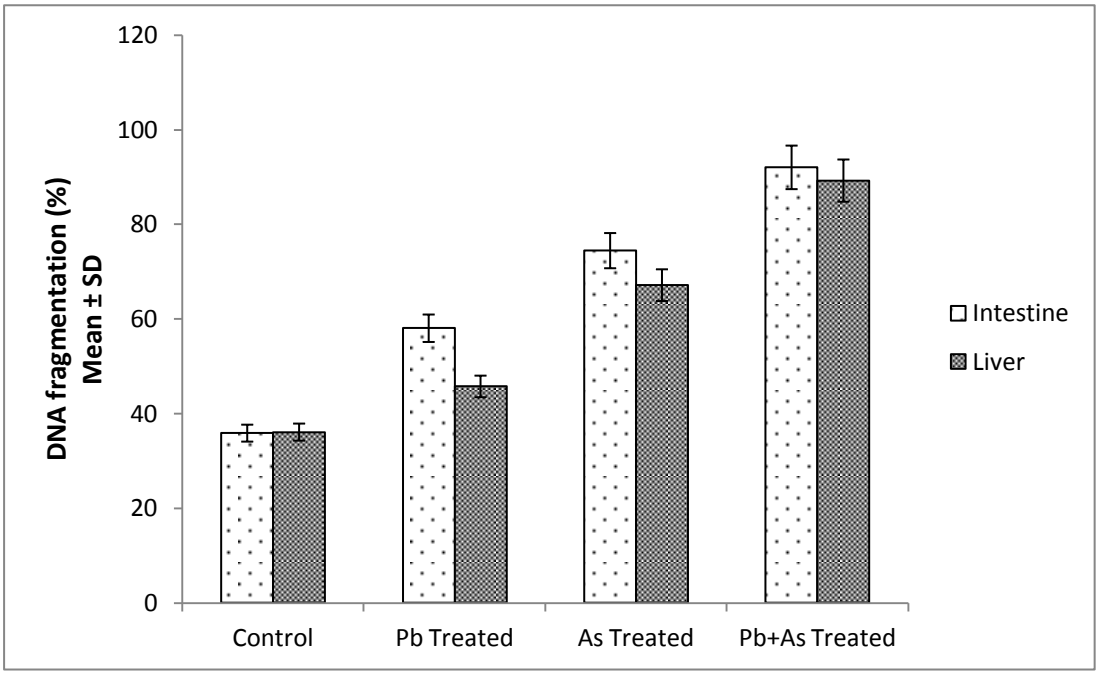


Fig 2: Interactive effect of lead and arsenic on percentage of DNA fragmentation in fish macrophages

Pb, As (intestine) P< 0.02; Pb, As (liver) P< 0.05.

4.7. STUDY OF EFFECT OF LEAD ACETATE AND SODIUM ARSENITE EXPOSURE (*IN VIVO*) SIMULTANEOUSLY ON OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Effect of multi- metal exposure on respiratory burst activity of fish macrophages

There was a significant increase in respiratory burst activity from 0.521±0.02 (intestine), 0.51±0.22 (liver) in control group to 1.2±1.16 (intestine), 1.24±1.11 (liver) in multi-metal treated group (Fig: 1).

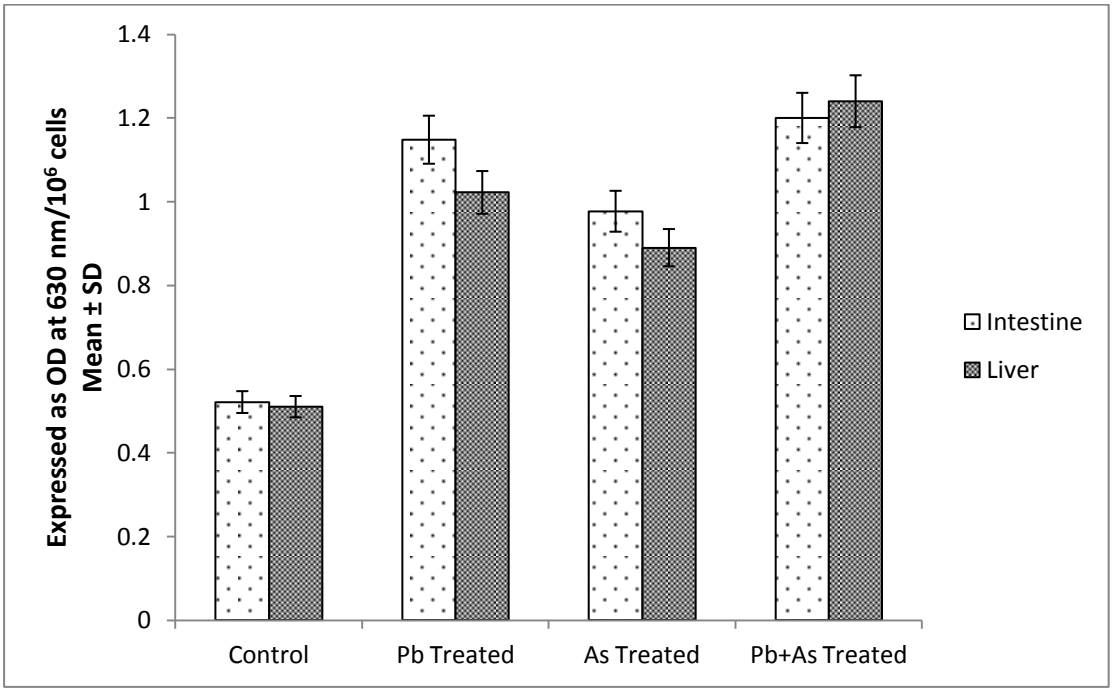


Fig 1: Respiratory burst activity in macrophages of fish treated simultaneously with lead and arsenic.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.025$.

ii) Effect of multi- metal exposure on lipid peroxidation of fish macrophages

There was a significant increase in lipid peroxidation from 0.385±0.18nmoles/hr (intestine), 0.302±0.22 nmoles/hr (liver) in control group to 1.04±1.41 nmoles/hr (intestine), 1.21±0.31 nmoles/hr (liver) in multi-metal treated group (Fig: 2).

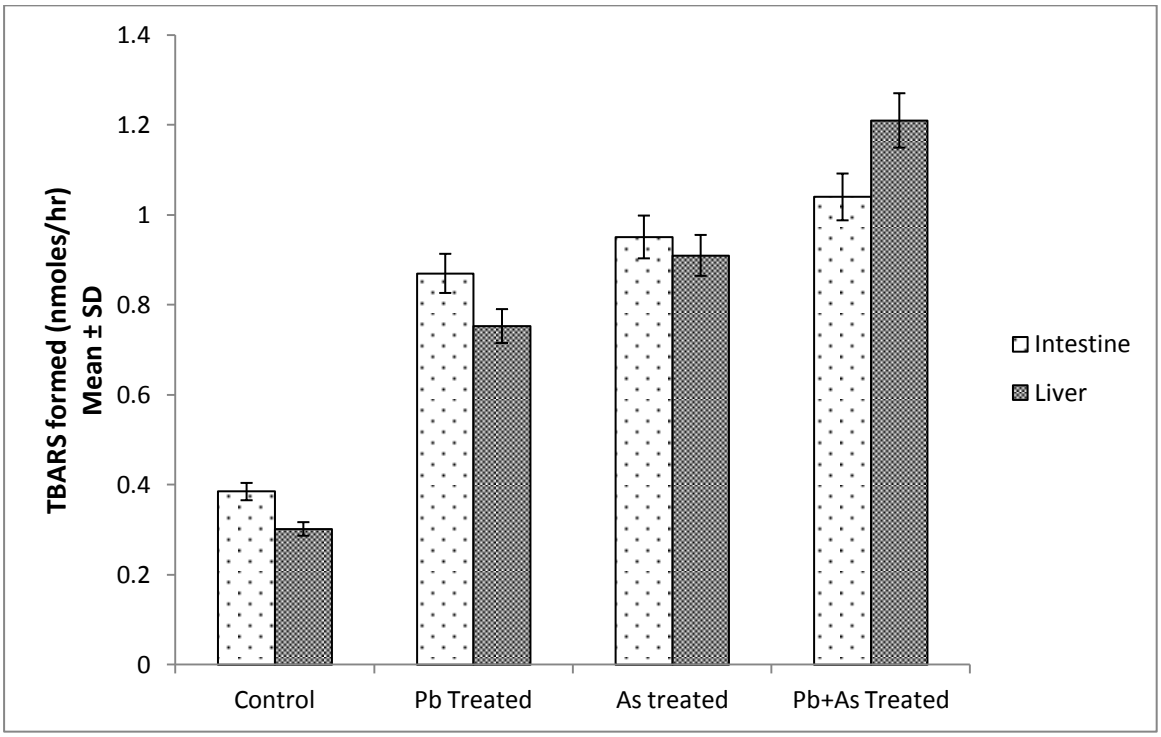


Fig 2: Estimation of lipid peroxidation (LPO) in multi- metal exposed fish macrophages.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

iii) Effect of multi- metal exposure on protein carbonylation of fish macrophages

There was a significant increase in protein carbonylation from 0.39±0.21mg/ml (intestine), 0.51±0.51 mg/ml (liver) in control group to 0.76±0.25 mg/ml (intestine), 0.79±0.22 mg/ml (liver) in multi-metal treated group (Fig: 3).

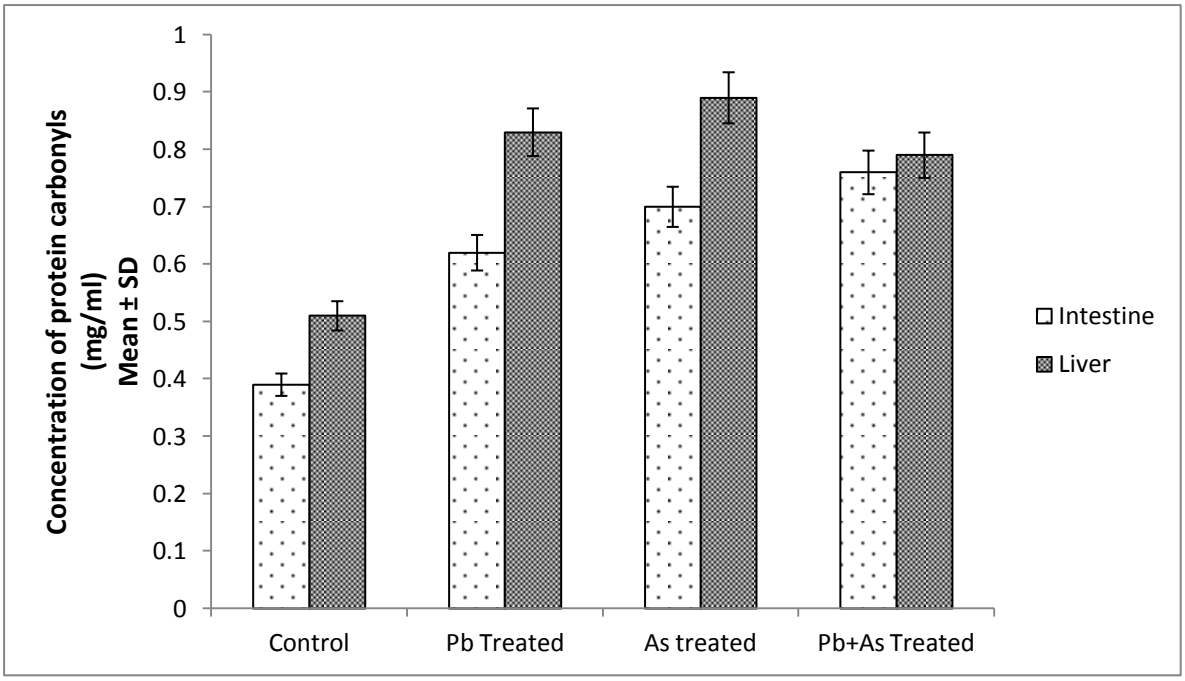


Fig 3: Estimation of protein carbonylation (PC) in multi- metalexposed fish macrophages.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

iv) Effect of multi- metal exposure on catalase (CAT) activity of fish macrophages

There was a significance decrease in catalase activity from 5.76±0.29U/mg protein (intestine), 6.012±0.33 U/mg protein (liver) in control group to 3.014±0.25 U/mg protein (intestine), 3.119±0.21 U/mg protein (liver) in multi-metal treated group (Fig: 4).

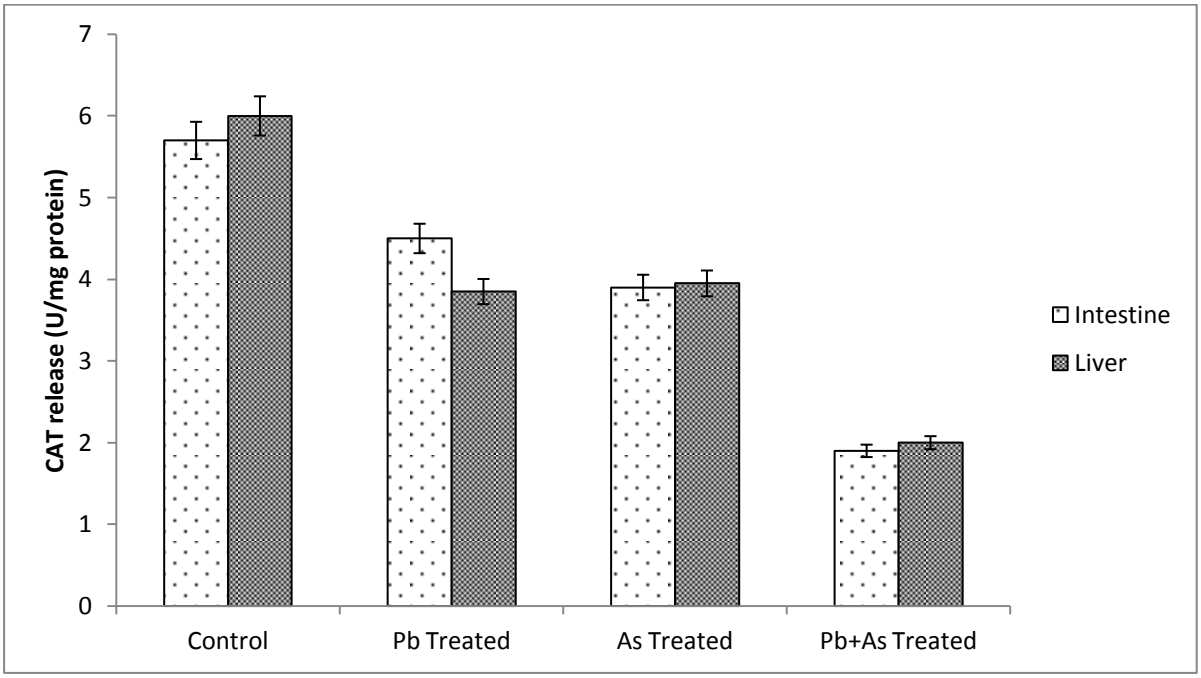


Fig 4: Catalase (CAT) released from control and multi-metal exposed fish macrophages.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.02$.

v) Effect of multi- metal exposure on superoxide dismutase (SOD) activity of fish macrophages

There was a significance decrease in superoxide dismutase activity from 0.015±0.11 U/mg protein (intestine), 0.021±0.42 U/mg protein (liver) in control group to 0.22±0.21 U/mg protein (intestine), 0.25±0.36 U/mg protein (liver) in multi-metal treated group (Fig: 5).

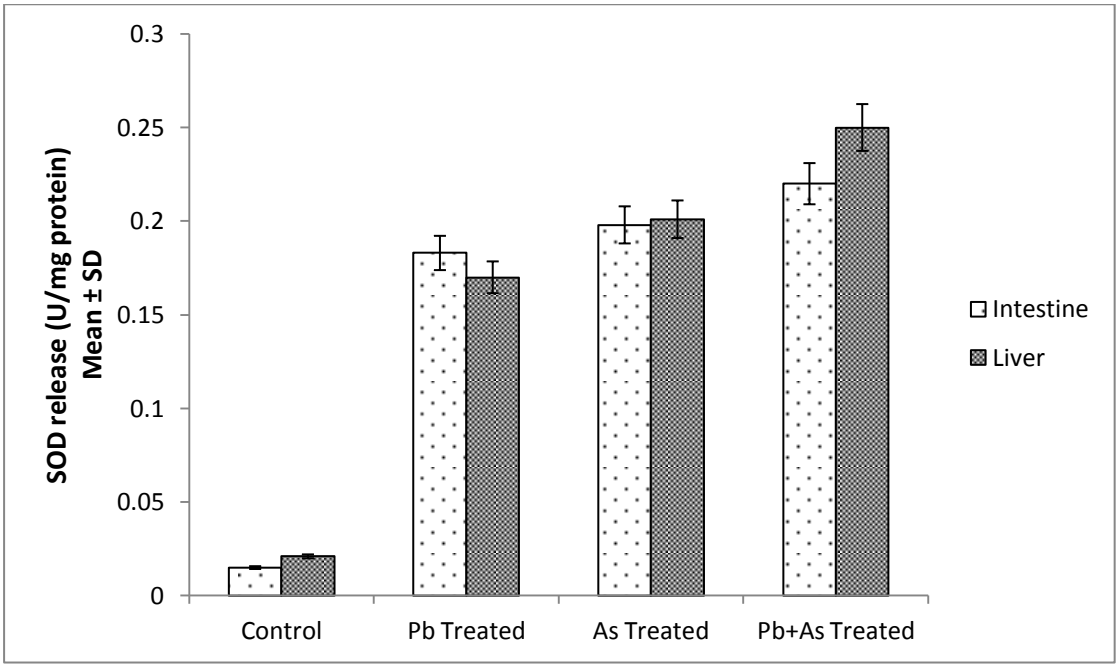


Fig 5: Superoxide dismutase (SOD) released from control and multi-metal exposed fish macrophages.

As (intestine) $P< 0.02$; Pb (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

vi) Effect of multi- metal exposure on glutathione S- transferase (GST) activity of fish macrophages

There was a significance decrease in glutathione S- transferase activity from 28.13±0.12 nmoles/min/mg protein (intestine), 26.9±0.23 nmoles/min/mg protein (liver) in control group to 18.11±1.13 nmoles/min/mg protein (intestine),17.03±1.21 nmoles/min/mg protein (liver) in multi-metal treated group (Fig: 6).

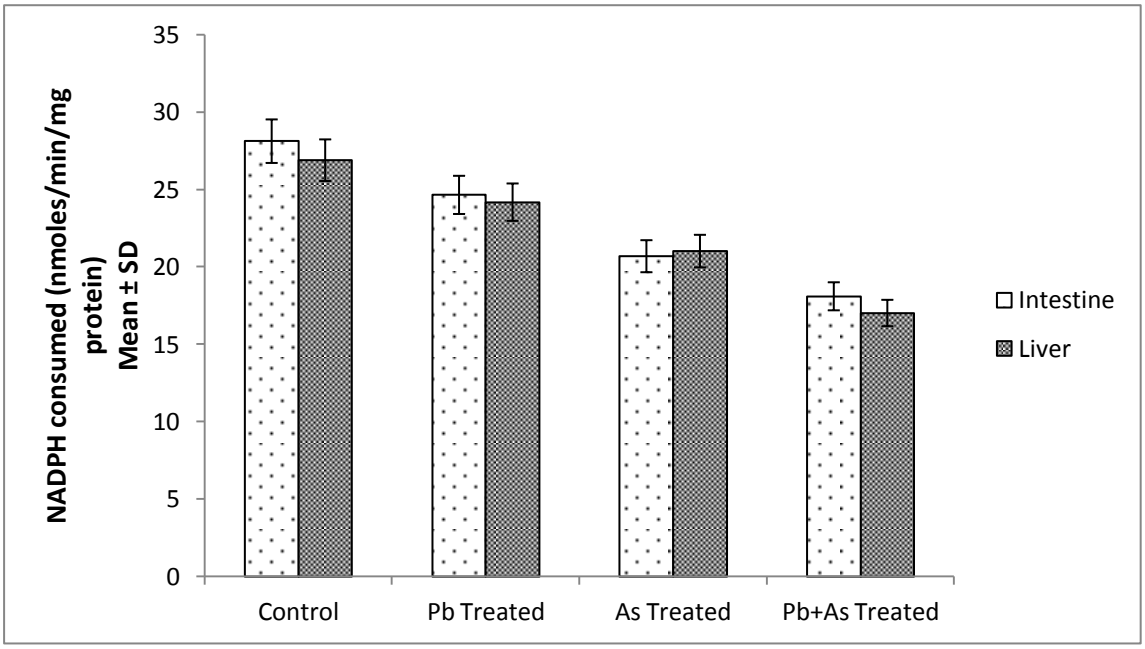


Fig 6: Glutathione S-transferase (GST) activity in macrophages of fish treated simultaneously with leadand arsenic.

Pb, As (intestine) P< 0.01; Pb, As (liver) P< 0.05.

vii) Effect of multi- metal exposure on glutathione peroxidase (GPx) activity of fish macrophages

There was a significance decrease in glutathione peroxidase activity from 5.8±0.33 nmoles/min/mg protein (intestine), 6.6±0.27 nmoles/min/mg protein (liver) in control group to 3.8±0.31 nmoles/min/mg protein (intestine),4±0.29 nmoles/min/mg protein (liver) in multi-metal treated group (Fig: 7).

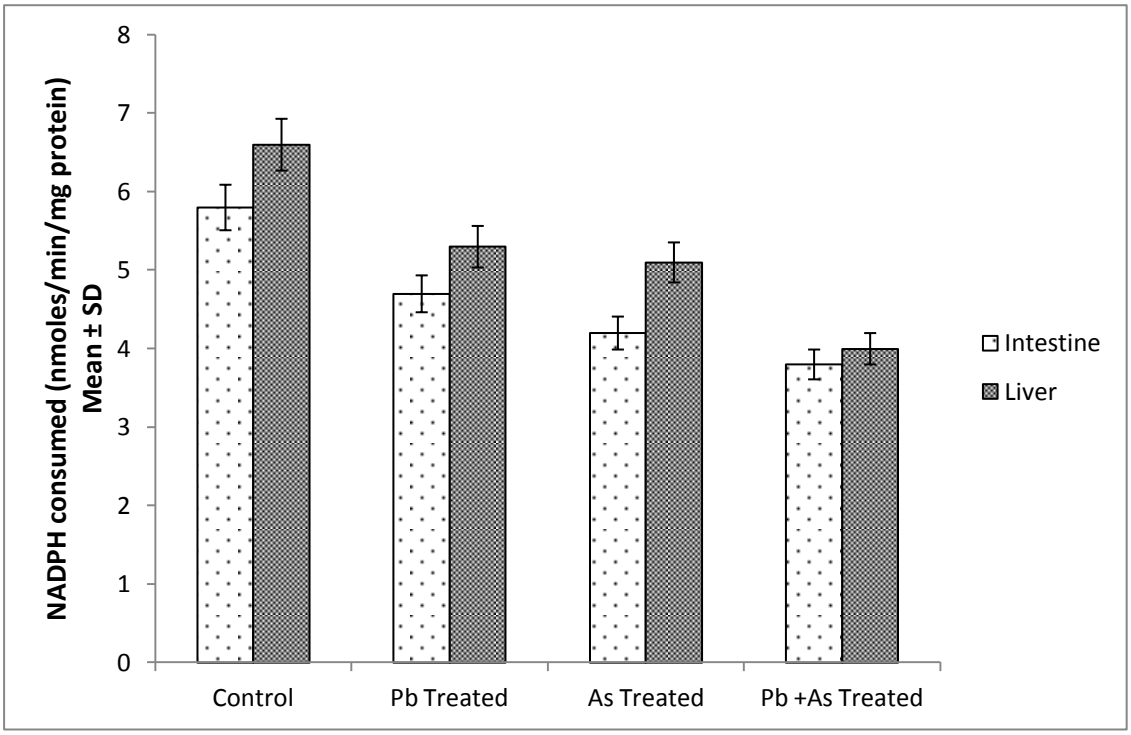


Fig 7: Glutathione peroxidase (GPx) activity in macrophages of fish treated simultaneously with lead and arsenic.

Pb, As (intestine) $P< 0.01$; Pb, As (liver) $P< 0.025$.

viii) Effect of multi- metal exposure on glutathione reductase (GR) activity of fish macrophages

There was a significance decrease in glutathione reductase activity from 3.96±0.21 nmoles/min/mg protein (intestine), 4.51±0.42 nmoles/min/mg protein (liver) in control group to 2.99±0.55 nmoles/min/mg protein (intestine), 2.81±0.52 nmoles/min/mg protein (liver) in multi-metal treated group (Fig: 8).

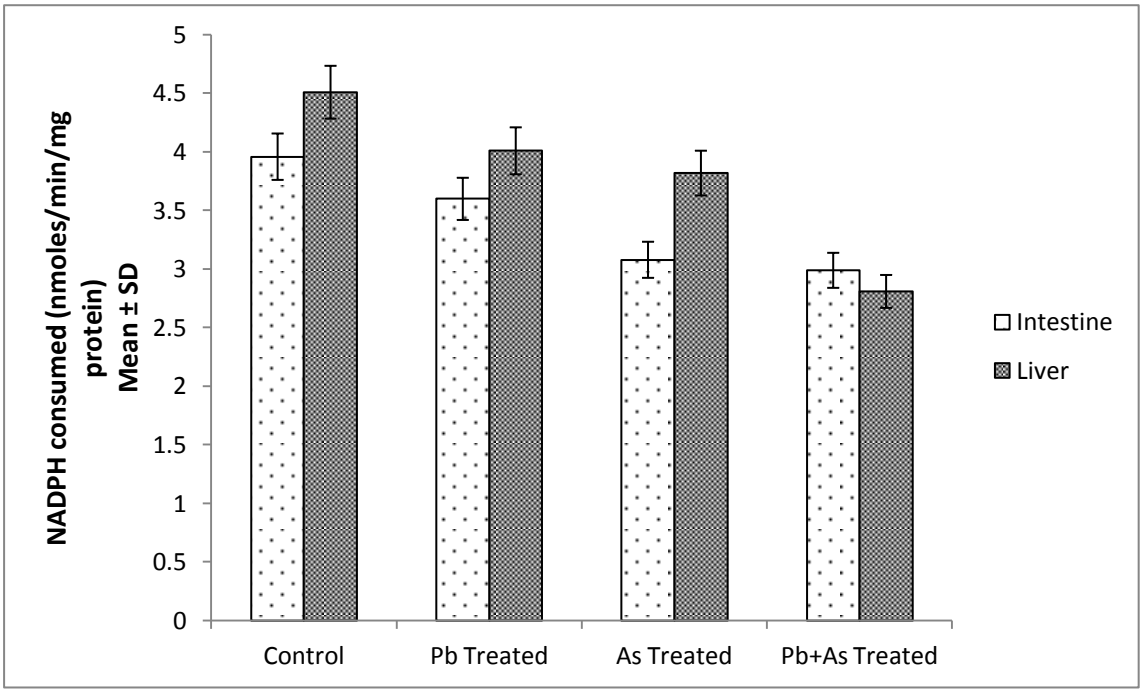


Fig 8: Glutathione reductase (GR) activity in macrophages of fish treated simultaneously with lead and arsenic.

Pb, As (intestine) $P< 0.025$; Pb, As (liver) $P< 0.05$.

ix) Effect of multi- metal exposure on reduced glutathione (GSH) activity of fish macrophages

There was a significance decrease in reduced glutathione activity from 0.823±0.62 nmoles/g tissue (intestine), 0.766±0.59 nmoles/g tissue (liver) in control group to 0.37±0.27 nmoles/g tissue (intestine), 0.382±0.29 nmoles/g tissue (liver) in multi-metal treated group (Fig:9).

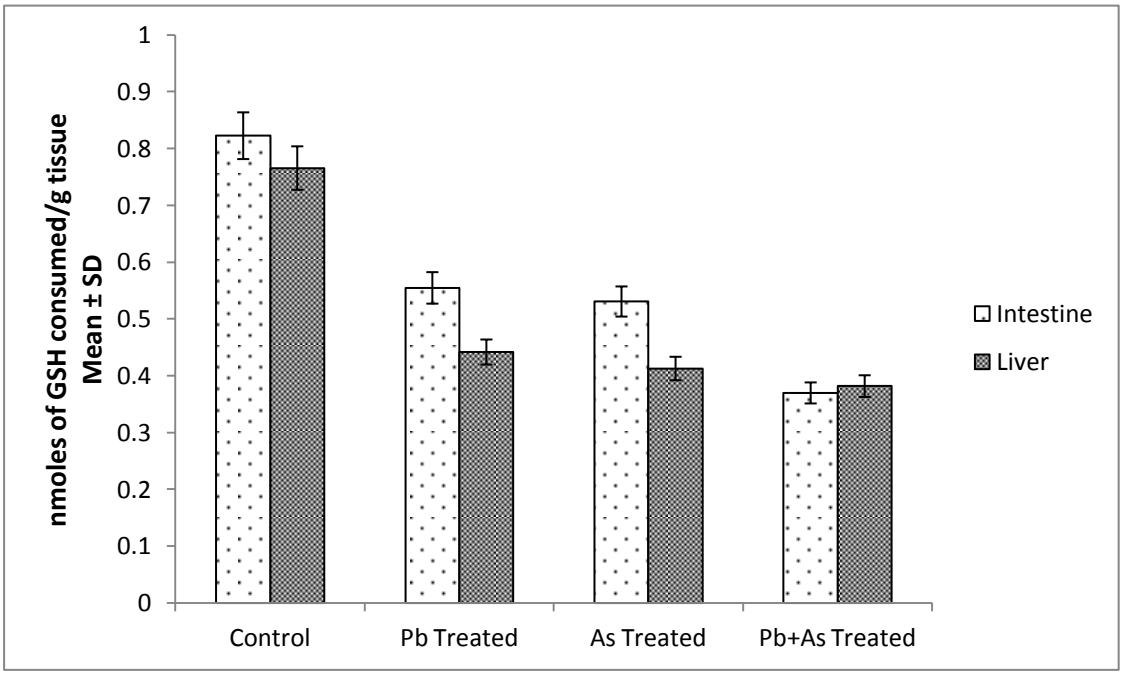


Fig 9: Reduced glutathione (GSH) consumed in macrophages of fish treated simultaneously with leadand arsenic.

Pb, As (intestine) $P< 0.05$; Pb, As (liver) $P< 0.01$.

4.8. STUDY OF EFFECT OF LEAD ACETATE AND SODIUM ARSENITE (*IN VIVO*) SIMULTANEOUSLY ON INNATE IMMUNE RESPONSES IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

i) Effect of multi- metal exposure on phagocytic activity of fish macrophages

There was a significant decrease in phagocytic activity from 21666.66±1.78 % (intestine), 21822.51±1.83 % (liver) in control group to 9714.77±1.55 % (intestine), 9855.22±1.32 % (liver) in multi-metal treated group (Fig: 1).

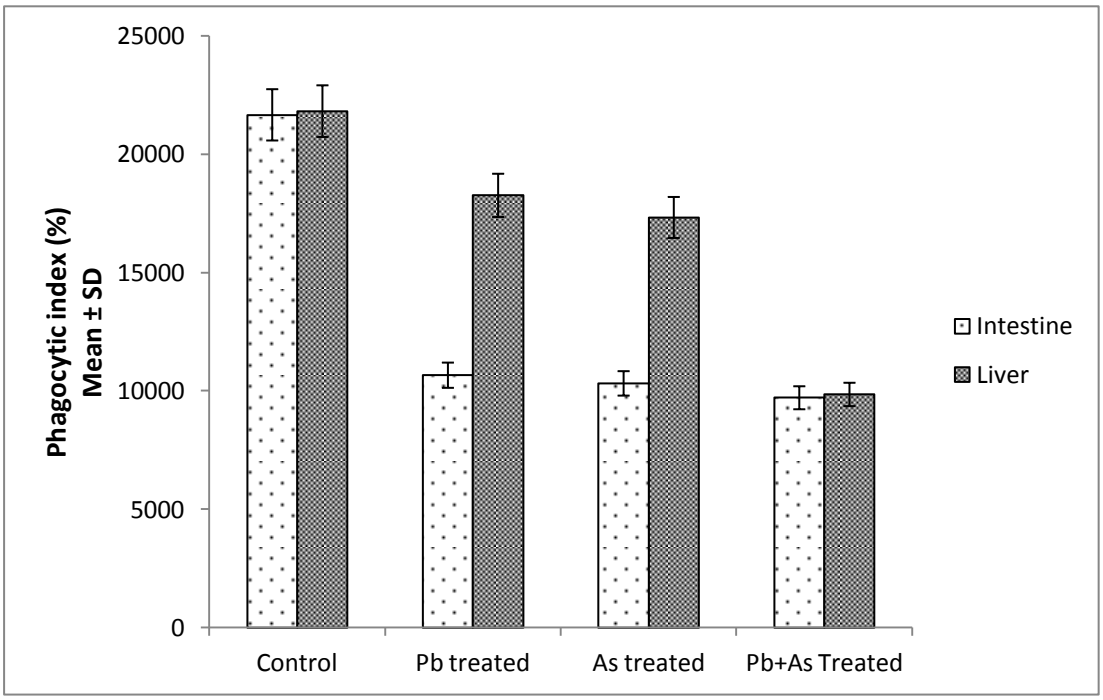


Fig 1: Phagocytic activity in macrophages of fish treated simultaneously with lead and arsenic.

As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

ii) Effect of multi- metal exposure on intracellular killing activity of fish macrophages

There was a significant increase in bacterial viability in multi-metal treated group at 60 min as compared to the control group (Fig: 2).

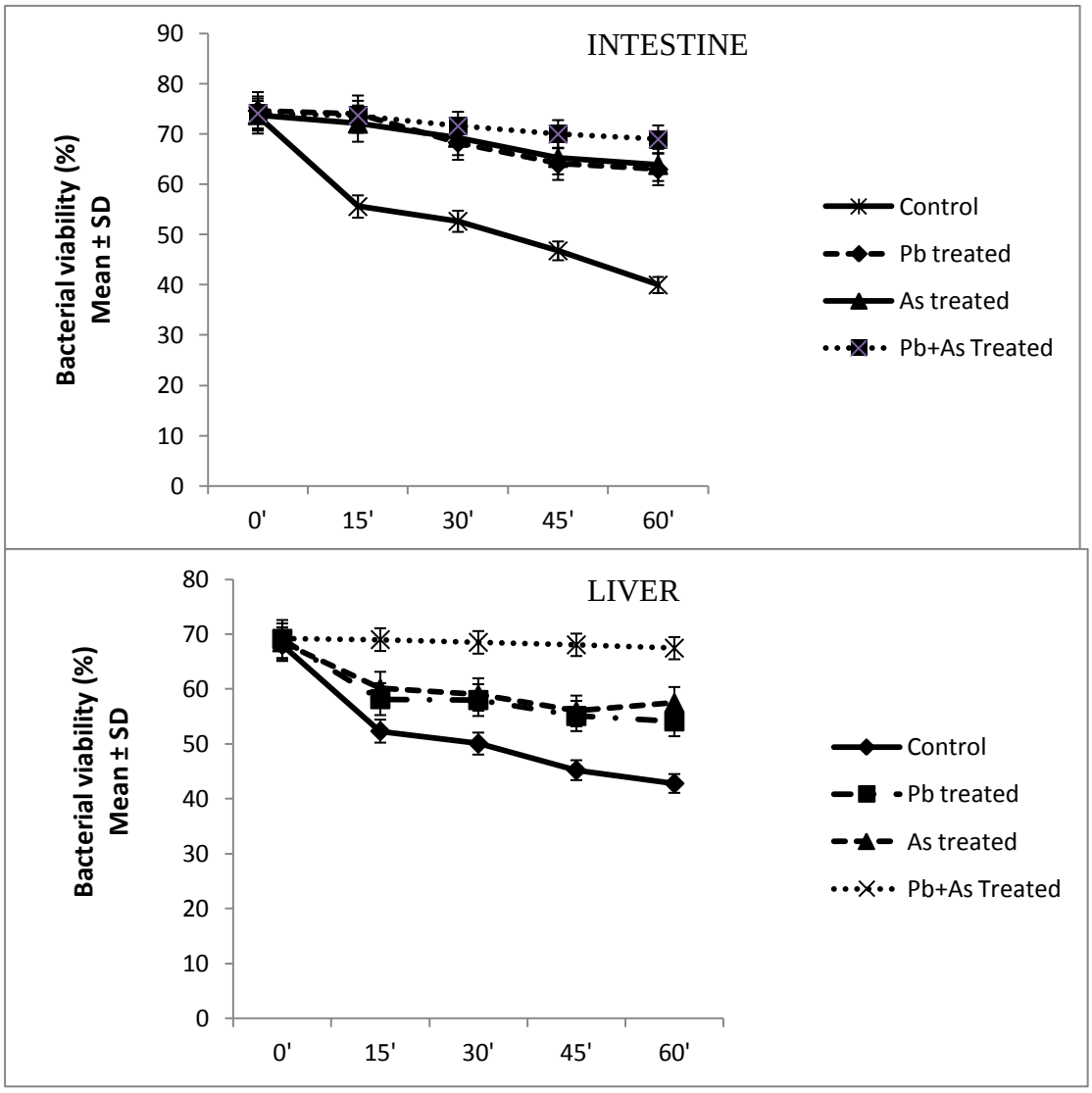


Fig 2: Intracellular killing activity in macrophages of fish treated simultaneously with lead and arsenic.

Intestine- Pb ($P<0.05$); As ($P<0.005$) Liver- Pb ($P<0.01$); As ($P<0.025$)

iii) Effect of multi- metal exposure on chemotactic migration of fish macrophages

There was a significant decrease in chemotactic migration in multi-metal treated group at 60 min as compared to the control (Fig: 3).

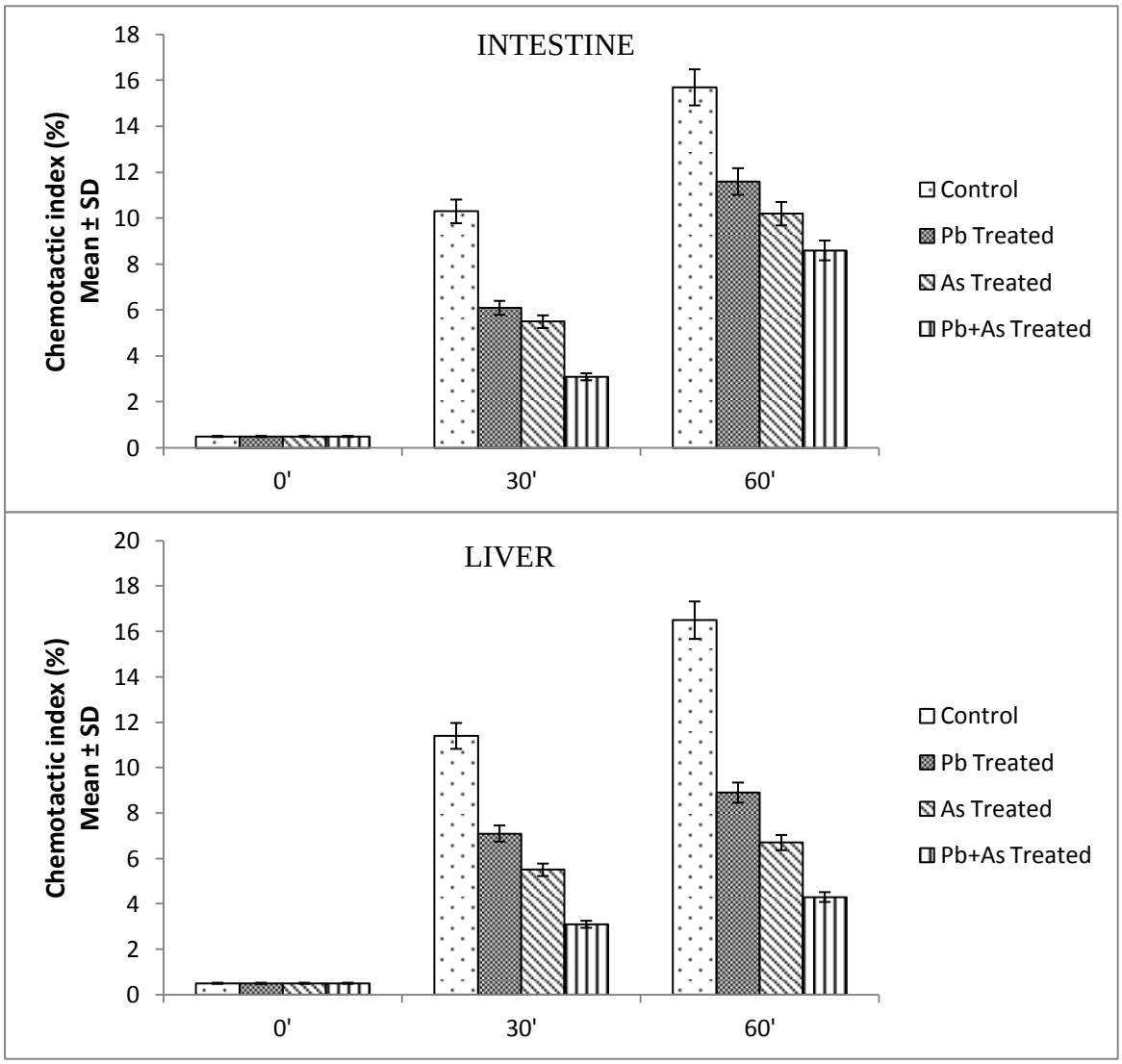


Fig 3: Chemotactic index in macrophages of fish treated simultaneously with lead and arsenic.

Intestine- Pb ($P<0.04$); As ($P<0.025$) Liver- Pb ($P<0.02$); As ($P<0.001$).

iv) Effect of multi- metal exposure on cell adhesion of fish macrophages

There was a significant decrease in cell adhesion in multi-metal treated group at 60 min as compared to the control group (Fig: 4).

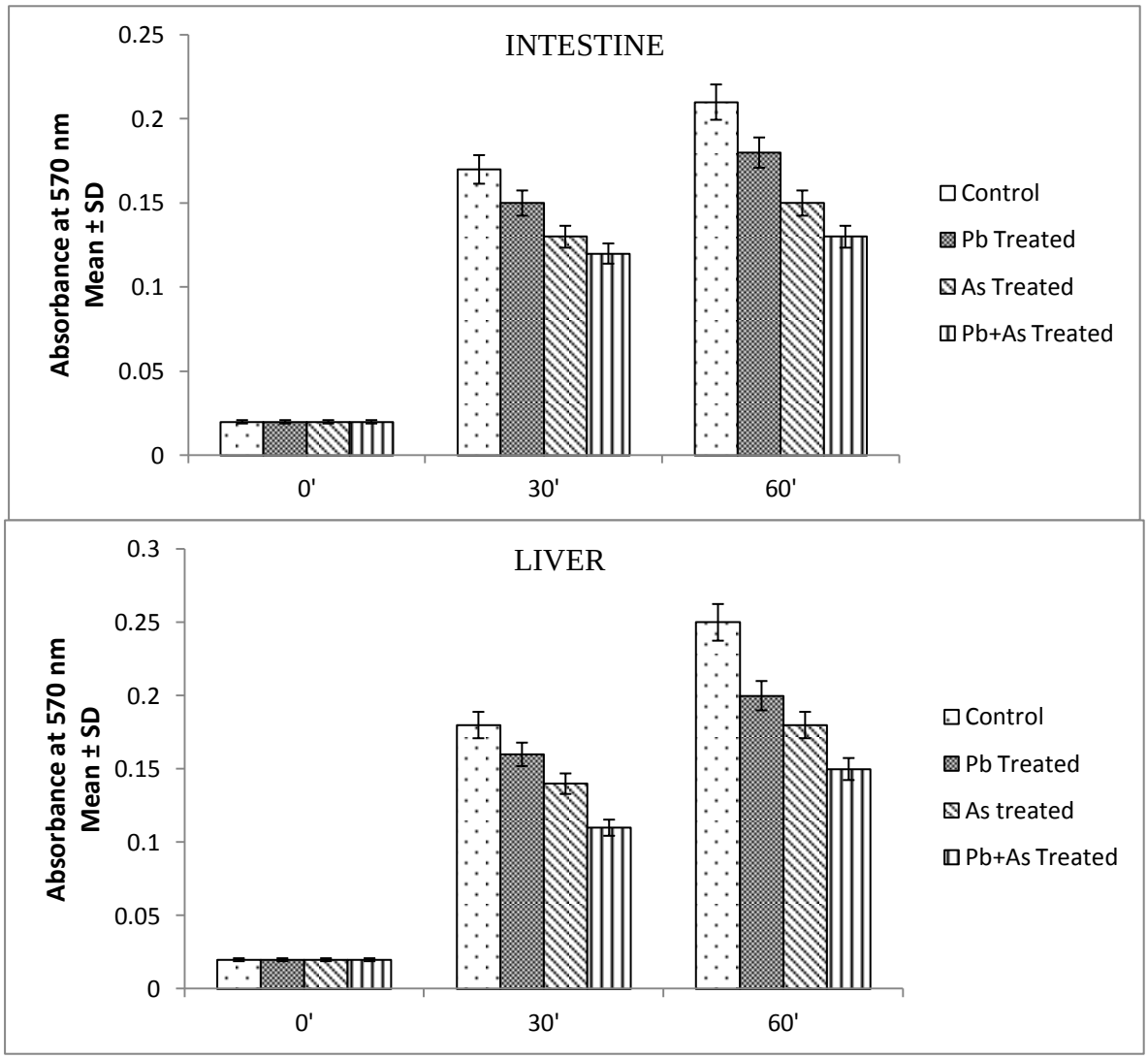


Fig 4: *In vitro* cell adhesion in macrophages of fish treated simultaneously with lead and arsenic. Intestine- Pb ($P<0.025$); As ($P<0.004$) Liver- Pb ($P<0.01$); As ($P<0.025$)

v) Effect of multi- metal exposure on nitric oxide (NO) release of fish macrophages

There was a significant decrease in nitric oxide release from 50.02±0.31 (intestine), 48.61±0.33 (liver) in control group to 30±0.028 (intestine), 32±0.26 (liver) in multi-metal treated group

(Fig: 5).

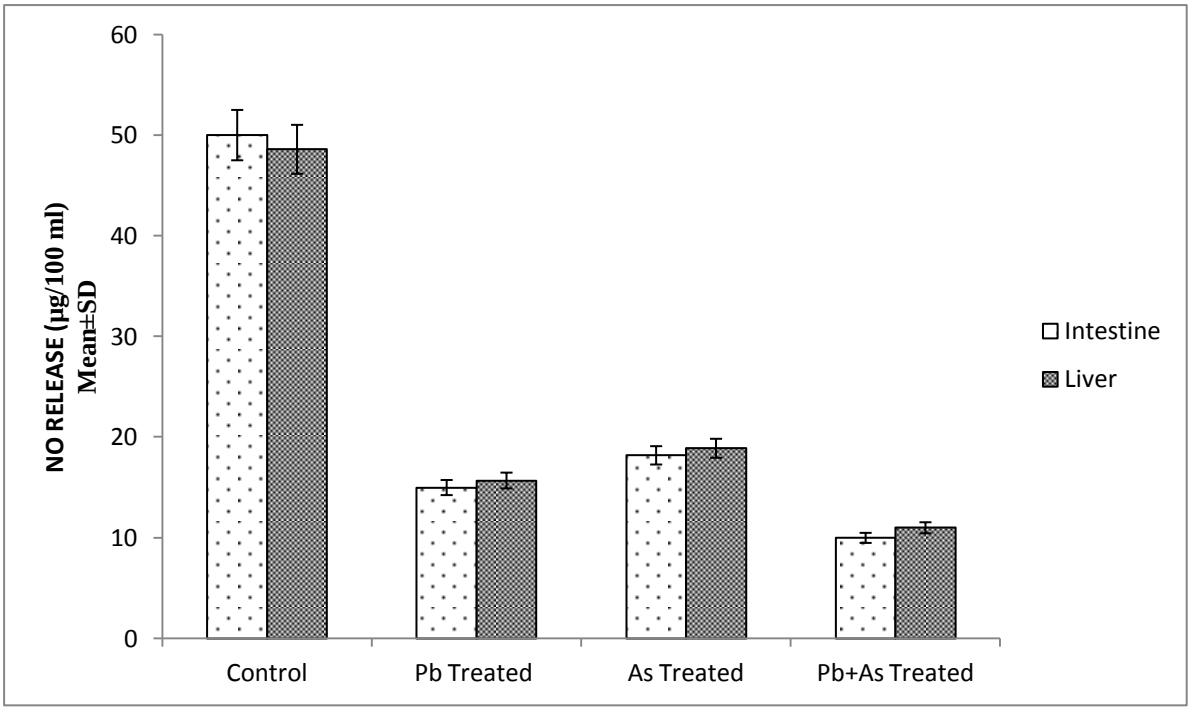


Fig 5: Nitric oxide release in macrophages of fish treated simultaneously with lead and arsenic.

As (liver) P<0.02; Pb (liver) P< 0.01; Pb, As (intestine) P< 0.05;

vi) Effect of multi- metal exposure on myeloperoxidase (MPO) release of fish macrophages

There was a significant decrease in myeloperoxidase release from 43.51±0.44 (intestine), 46.01±0.36 (liver) in control group to 19.03±0.29 (intestine), 19.98±0.36 (liver) in multi-metal treated group (Fig: 6).

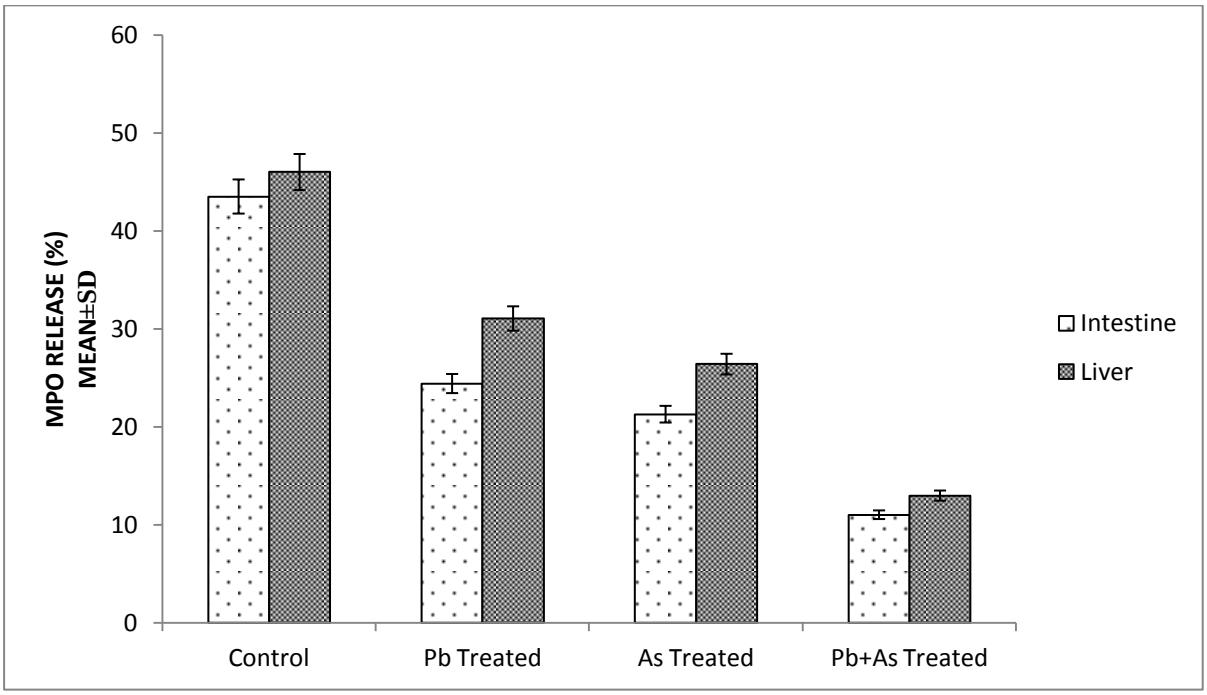


Fig 6: Myeloperoxidase release in macrophages of fish treated simultaneously with leadand arsenic.

Pb, As (intestine) P<0.02; Pb(liver) P< 0.05; As (liver) P< 0.04.

vii) Effect of multi- metal exposure on TNF-αrelease of fish macrophages

There was a significant decrease in TNF-α release in multi-metal treated group both from macrophage lysate and plasma as compared to the control group (Fig: 7).

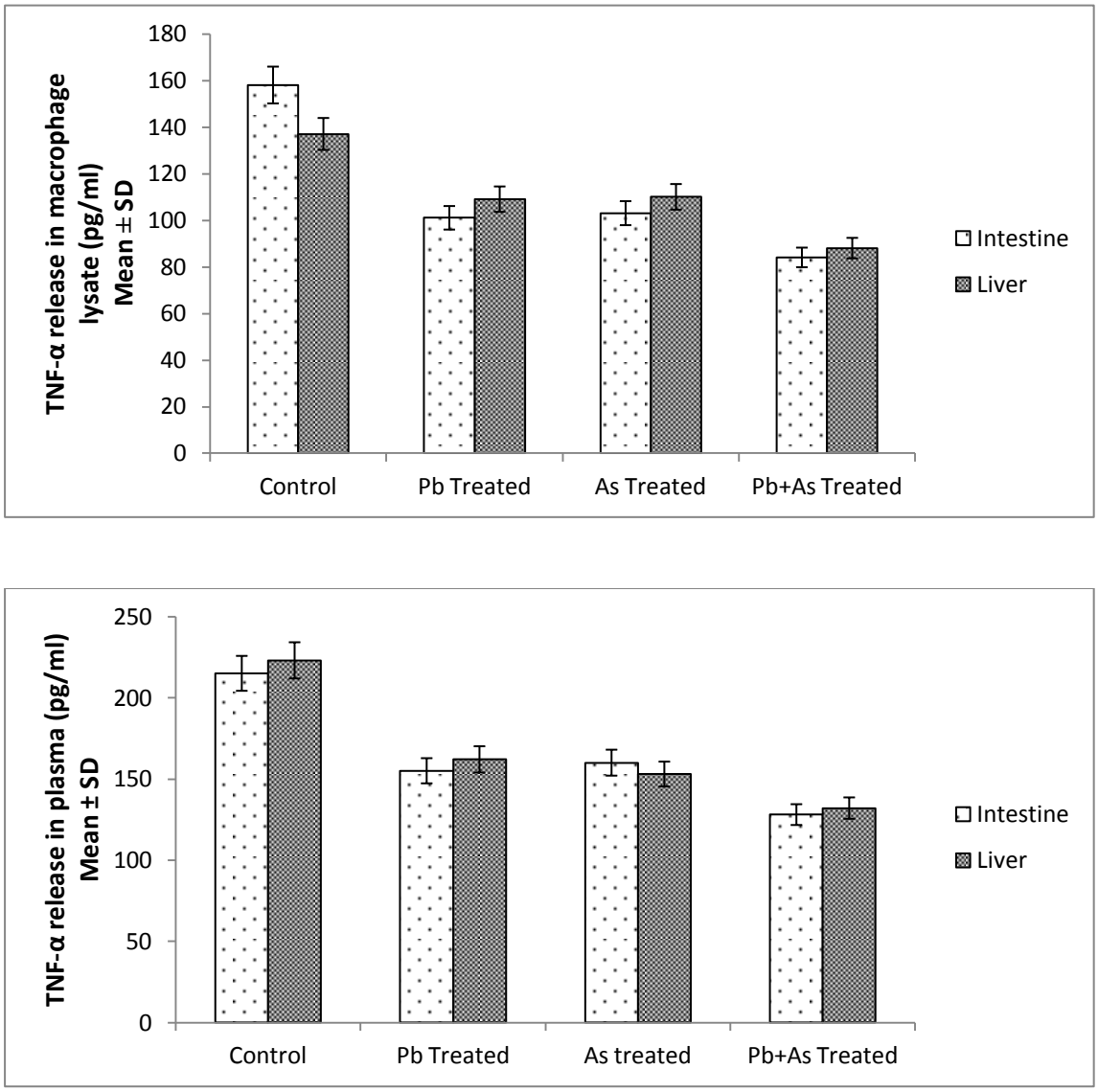


Fig 7: (a) TNF-α released from cell lysate of multi- metal treated group (b) TNF-α released from plasma of multi- metal treated group.

As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

viii) Effect of multi- metal exposure on IL- 1b release of fish macrophages

There was a significant decrease in IL-1b release from 0.074±0.29 (intestine), 0.078±0.31 (liver) in control group to 0.03±0.35 (intestine), 0.032±0.32 (liver) in multi-metal treated group (Fig: 8).

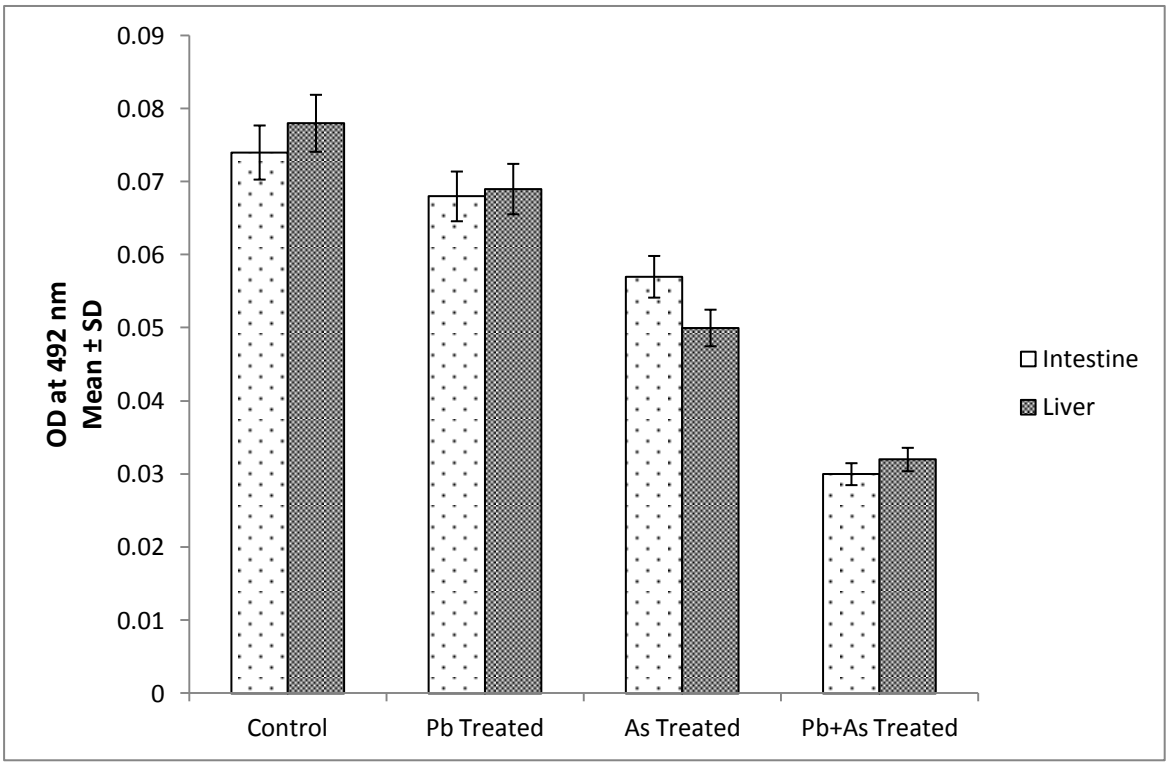


Fig 8: IL-1b released from plasma of multi- metal treated group.

As (liver) P<0.02; Pb, As (intestine) P< 0.05; Pb (liver) P< 0.01.

Chapter 4: Isobologram study on the interactivity effects between lead and arsenic exposure (*in vivo*) in macrophages from fish *Channa punctatus*

4.9. DETERMINATION OF SYNERGY BY AN ISOBOLOGRAM APPROACH BETWEEN LEAD ACETATE AND SODIUM ARSENITE ON

(i) innate immune function- nitric oxide (NO)

Multimetal exposure			
Dose levels	Arsenic (mg/L)	Lead (mg/L)	Mean NO release (mg/100ml)
a	0.059	2.35	11.97 ± 0.638
b	0.118	4.71	11.51 ± 1.849
c	0.236	9.42	10.17 ± 0.382
d	0.472	18.84	8.32 ± 1.102
Single metal exposure			
Dose levels	Arsenic (mg/L)	Mean NO release (mg/100ml)	
a	0.059	18.13 ± 1.484	
b	0.118	16.33 ± 0.16	
c	0.236	15.76 ± 0.788	
d	0.472	14.88 ± 0.366	
Dose levels	Lead (mg/L)	Mean NO release (mg/100ml)	
a	2.35	17.27 ± 0.245	
b	4.71	15.99 ± 0.106	
c	9.42	14.61 ± 0.212	
d	18.84	13.06 ± 0.457	
Control	Mean NO release (mg/100ml)		
	50.02 ± 0.797		

Table 4.1: Effect of repeated single and combined exposure (in vivo) to lead and arsenic on nitric oxide (NO) release from fish intestinal macrophages

The experimental observations from the view point of the biological experiment with fish are actually experimental data from the statistical point of view. In order to analyze the experimental results the popular technique of analysis of variance (ANOVA) has been used to determine whether percentage release of nitric oxide (NO) varies due to exposure types of metals, or dose levels or both incorporating combination of metals. The experiment of metal exposure (lead and arsenic) on fish to study the release of NO is designed such that a two-way ANOVA with four levels having four observations per level is feasible. However such a standard design obviously excludes the control group. It is to be noted that the control group has only one level and four observations. The results have been tested on the basis of the standard two- way classification with four observations per cell excluding the control group in the concluding section. However, to begin with the standard model is modified to make it compatible with the experimental results.

A brief overview on the technique of ANOVA:

The total variation present in a set of observable quantities may under certain circumstances be partitioned into a number of components associated with the nature of classification of data. The systematic procedure of achieving this is called the analysis of variance (ANOVA). A linear model is assumed because it is fundamental to the set up of ANOVA. Further, all errors are assumed to be independent random variables with zero expectation and constant variance (homoscedastic). In order to estimate the parameters of the linear model, the principle of least squares have been applied, which in effect implies minimizing the sum of squares of errors with respect to the parameters of linear models.

Treatment of the experimental data:

The experimental design consists of two factors arsenic (factor A) and lead (factor B). For the present analysis the combination of arsenic and lead in the experiment is not considered as a separate factor. The two factors arsenic (A) and lead (B) are used at four levels each- the levels being doses.

Table 1 (Materials and Methods, Experimental Design - 3) illustrates that there are four separate sections or blocks. The first three can be easily represented as two-way classification with four observations per cell. But the fourth block i.e., 'control' has only one level with four observations.

Block 1 is assumed to be that of bimetal exposure, block 2 and 3 to be those of arsenic (A) and lead (B) (single metal exposure) and block 4, the control. The following notations denote percentage NO observations. (The respective blocks denoted in superscript).

y^1_{iik} = k^{th} observation exposed to the i^{th} level (dose) of A along with the i^{th} level of B in block 1.

y^2_{ik} = k^{th} observation exposed to the i^{th} level of A only in block 2.

y^3_{ik} = k^{th} observation exposed to the i^{th} level of B only in block 3.

y^4_k = k^{th} observation.

It is to be noted that the level of arsenic is denoted by i, while that of lead is denoted by j.

But for this experiment i and j mean the same thing because, both i = 1, 2, …, 4 and j = 1, 2, …, 4, and in block 1, there **are no cross-overs**. That is, the first level of A is combined with the first level of B, the second level of A is combined with the second level of B, and so on.

Thus i = j and y_{iik}^{1} , y_{ik}^{2} and y_{ik}^{3} .

Further let,

α_i = the effect due to i^{th} level of A.

β_i = the effect due to the i^{th} level of B.

γ_i = the effect due to the combination of the i^{th} level of A and the i^{th} level of B, which will be called the interaction between A and B.

Ideally, γ_{ii} should have been written but here it hardly matters with no cross-combinations (–).

The linear model(s) are now ready to be presented as represented by the set of the following four equations.

$$y_{iik}^{1} = \mu_1 + \alpha_i + \beta_i + \gamma_i + e_k^{(1)} \quad \text{(i)}$$

$$y_{ik}^{2} = \mu_2 + \alpha_i + e_k^{(2)} \quad \text{(ii)}$$

$$y_{ik}^{3} = \mu_3 + \beta_i + e_k^{(3)} \quad \text{(iii)}$$

$$y_{k}^{4} = \mu_4 + e_k^{(4)} \quad \text{(iv)}$$

} The linear model (1)

Here i = 1, 2, …, 4; k = 1, 2, …, 4. Since, no square or cubic terms occur on the RHS of each equation, they are all linear models. It is assumed that μ is the fixed effect due to blocks, e.g., μ_1 is fixed effect due to the first block, similarly μ_2, μ_3 and μ_4. In ANOVA it is assumed that,

$$y_{iik}^{1} = \mu^{(1)} = e_k \quad \text{(*)}$$

where $\mu^{(1)}$ is the error.

The true value $\mu^{(1)}$ is that part which is due to assignable causes, and the portion which remains is the error, e_k, which is due to various chance causes. The true value $\mu^{(1)}$ is again assumed to be a linear function of, fixed effect due to the block (μ_1), effect due to i^{th} level of A(α_i), effect due to i^{th} level of B (β_i) and the combined effect due to i^{th} level of A together with that B (γ_i).

Thus, equation (*) becomes,

$$y_{iik}^{(1)} = \mu_1 + \alpha_i + \beta_i + \gamma_i + e_k^{(1)} \qquad \text{(i)}$$

μ_1, μ_2, …, μ_4, α_i, β_i, γ_i are all unknown constants called parameters of the model. It can be written that μ_ℓ ($\ell = 1, \ldots, 4$) as the effect due to the ℓ^{th} block.

It is to be remembered that, α_i is an effect due to the i^{th} level of A, common to all observations belonging to this class of A.

Similarly we have β_i and γ_i. **Moreover, α_i is selected in such a manner that it is the deviation of the general effect present in all observations from the mean of the i^{th} level of A**. This is applicable to β_i and γ_i as well.

In other words, α_i, β_i, and γ_i are framed such that, $\sum_{i=1}^{4} \alpha_i = \sum_{i=1}^{4} \beta_i = \sum_{i=1}^{4} \gamma_i = 0$.

This can be taken as an additional assumption. To estimate the parameters the principle of least squares is applied. This is done as follows:

First, it is defined, $e_k^{(1)} = (y_{iik}^1 - \mu_i - \alpha_i - \beta_i - \gamma_i)$

Second, the sums of squares of errors are

$$S_1 = \sum_{i=1}^{4} \sum_{k=1}^{4} (y_{iik}^{(1)} - \mu_1 - \alpha_i - \beta_i - \gamma_i)^2 \equiv \sum_{i=1}^{4} \sum_{k=1}^{4} e_k^{(1)^2}$$

$$S_2 = \sum_{i=1}^{4} \sum_{k=1}^{4} (y_{ik}^{(2)} - \mu_2 - \alpha_i)^2 \equiv \sum_{i}^{4} \sum_{k}^{4} e_k^{(2)^2}$$

$$S_3 = \sum_{i=1}^{4} \sum_{k=1}^{4} (y_{ik}^{(3)} - \mu_3 - \beta_i)^2 \equiv \sum_{i}^{4} \sum_{k}^{4} e_k^{(3)^2}$$

and $S_4 = \sum_{k=1}^{4} (y_k^{(4)} - \mu_4)^2 = \sum_{k=1}^{4} e_k^{(4)^2}; \quad (i = 1,...,4)$

Third, S_1, S_2, …, S_4 is minimised with respect to the parameters. This is done by setting the first order partial derivatives of all the sum of squares of errors w.r.t. the parameters to zero. Then the estimated values of the parameters are solved from the normal equations.

$$\frac{\partial S_1}{\partial \mu_1} = \frac{\partial S_1}{\partial \alpha_i} = \frac{\partial S_1}{\partial \beta_i} = \frac{\partial S_1}{\partial \gamma_i} = 0 \qquad i = 1, \ldots, 4 \qquad (1)$$

$$\frac{\partial S_2}{\partial \mu_2} = \frac{\partial S_2}{\partial \alpha_i} = 0 \qquad (2)$$

$$\frac{\partial S_3}{\partial \mu_3} = \frac{\partial S_3}{\partial \beta_i} = 0 \qquad (3)$$

and $\frac{\partial S_4}{\partial \mu_4} = 0 \qquad (4)$

There are 16 parameters in all and 16 normal equations from conditions (1), (2), (3) and (4), so that they can be solved to obtain the estimates of the parameters.

To simplify matters the different notations for the different means are written. The means that are being dealt with are

$y_{ii0}^{(1)} = \frac{1}{4} \sum_{k=1}^{4} y_{iik}^{(1)}$, is the mean of the ith levels of A and B in block 1.

$y_{i0}^{(2)} = \frac{1}{4} \sum_{k=1}^{4} y_{ik}^{(2)}$ is the mean of the ith level of A in block 2.

$y_{i0}^{(3)} = \frac{1}{4}\sum_{k=1}^{4} y_{ik}^{(3)}$ is the mean of the i[th] level of B in block 3.

$y_0^{(4)} = \frac{1}{4}\sum_{k=1}^{4} y_k^{(4)}$ is the mean of the control group – block 4.

Finally, the grand means for each block are written as under.

$y_0^{(1)} = \frac{1}{4}\sum_{i=1}^{4} y_{ii0}^{(1)}$ is the grand mean of block 1.

$y_0^{(2)} = \frac{1}{4}\sum_{i=1}^{4} y_{i0}^{(2)}$ is the grand mean of the second block, i.e., block 2.

$y_0^{(3)} = \frac{1}{4}\sum_{i=1}^{4} y_{i0}^{(3)}$ is the grand mean of block 3.

Solving the normal equations (16 in all) for the parameters (16 in all) the least squares estimates of the parameters are derived as (derivation not shown) :

$\hat{\mu}_1 = y_0^{(1)}$, $\hat{\mu}_2 = y_0^{(2)}$, $\hat{\mu}_3 = y_0^{(3)}$ and $\hat{\mu}_4 = y_0^{(4)}$

$\hat{\alpha}_i = y_{i0}^{(2)} - y_0^{(2)}$, $\hat{\beta}_i = y_{i0}^{(3)} - y_0^{(3)}$ and

$\hat{\gamma}_i = \left(y_{ii0}^{(1)} - y_0^{(1)}\right) - \left(y_{i0}^{(2)} - y_0^{(2)}\right) - \left(y_{i0}^{(3)} - y_0^{(3)}\right)$

or, $\hat{\gamma}_i = y_{ii0}^{(1)} - y_{i0}^{(2)} - y_{i0}^{(3)} + y_0^{(2)} + y_0^{(3)} - y_0^{(1)}$

for all i = 1, …, 4.

Three separate null hypotheses shall have to be tested. They are

$H_{01} : \alpha_i = 0 \ \forall_i$; $H_{02} : \beta_i = 0 \ \forall i$ and $H_{03} : \gamma_i = 0 \ \forall i$

The sum of squares due to error, or error sum of squares, ESS is obtained by,

ESS = ESS due to block 1 + ESS due to block 2 + ESS due to block 3 + ESS due to block 4.

or, ESS = $\sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{iik}^{(1)}-\hat{\mu}_1-\hat{\alpha}_i-\hat{\beta}_i-\hat{\gamma}_i\right)^2+$

$$+\sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(2)}-\hat{\mu}_2-\hat{\alpha}_i\right)^2+\sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(3)}-\hat{\mu}_3-\hat{\beta}_i\right)^2+\sum_{k=1}^{4}\left(y_{k}^{(4)}-\hat{\mu}_4\right)^2$$

Now the degrees of freedom of ESS have to be specified. Degree of freedom in any statistical problem is defined as number of observations minus number of parameters estimated. Thus the degrees of freedom for each block written down as follows:

Block 1: d.f. = 16 – 4 = 12 16 – 13 = 3

Block 2: d.f. = 16 – 4 = 12 16 – 1 = 15

Block 3: d.f. = 16 – 4 = 12 16 – 1 = 15

Block 4: d.f. = 4 – 1 = 3 4 – 1 = 3

Hence d.f. carried by ESS is calculated as

D.F. = 12 + 12 + 12 + 3 = 39

Analogous to ESS, we define total sum of squares as

TSS=$\sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{iik}^{(1)}-y_0^{(1)}\right)^2+\sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(2)}-y_0^{(2)}\right)^2+\sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(3)}-y_0^{(3)}\right)^2+\sum_{k=1}^{4}\left(y_{k}^{(4)}-y_0^{(4)}\right)^2$

with D.F. = (16 – 1) + (16 – 1) + (16 – 1) + (4 – 1) = 15 + 15 + 15 + 3 = 48.

It is to be noted that the grand means of each block are all estimated parameters.

Now if H_{01} is true, then $\alpha_i = 0 \ \forall$ i, so that the original model reduces to,

$y_{iik}^{1} = \mu_1 + \beta_i + \gamma_i + e_k^{(1)}$

$y_{ik}^{2} = \mu_2 + e_k^{(2)}$ The linear model (2)

$y_{ik}^{3} = \mu_3 + \beta_i + e_k^{(3)}$

$y_{k}^{4} = \mu_4 + e_k^{(4)}$

with the usual assumptions, $\sum_i \beta_i = \sum_i \gamma_i = 0$. The least squares estimates of the parameters are as under:

$\hat{\mu}_\ell' = \hat{\mu}_\ell$ for all $\ell = 1, \ldots, 4$. That is, estimates of the fixed effects remain unchanged.

$\hat{\beta}_i' = y_{i0}^{(3)} - y_0^{(3)} = \hat{\beta}_i$ as before.
and $\hat{\gamma}_i' = y_{ii0}^{(1)} - y_0^{(1)} - y_{i0}^{(3)} + y_0^{(3)} \neq \hat{\gamma}_i$

Now, the ESS under the assumption that H_{01} is true is given by,

$$\text{ESS(1)} = \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{iik}^{(1)} - \hat{\mu}_1 - \hat{\beta}_i - \hat{\gamma}_i'\right)^2 + \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(2)} - \hat{\mu}_2\right)^2 + \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(3)} - \hat{\mu}_3 - \hat{\beta}_i\right)^2 + \sum_{k=1}^{4}\left(y_k^{(4)} - \hat{\mu}_4\right)^2$$

The d.f. for ESS(1) is given by

D.F.(1) = 12 + 15 + 12 + 3 = 42

Clearly, [ESS(1) – ESS] = SS_1 (say) is the sum of squares due to A only.

Its degrees of freedom are 42 – 39 = 3.

Analogously, ESS under the assumption that $H_{02} : \beta_i = 0 \ \forall i$, is true, is given by,

$$\text{ESS(2)} = \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{iik}^{(1)} - \hat{\mu}_1 - \hat{\alpha}_i - \hat{\gamma}_i''\right)^2 + \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(2)} - \hat{\mu}_2 - \hat{\alpha}_i\right)^2 + \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(3)} - \hat{\mu}_3\right)^2 + \sum_{k=1}^{4}\left(y_k^{(4)} - \hat{\mu}_4\right)^2$$

This carries, 12 + 12 + 15 + 3 = 42 degrees of freedom clearly,

[ESS(2) – ESS] = SS_2, is the sum of squares due to B alone, which has 42 – 39 = 3 degrees of freedom. This is because d.f.s. are additive. Finally, if H_{03} is true, the linear model becomes,

$$\begin{aligned} y_{iik}^{(1)} &= \mu_1 + \alpha_i + \beta_i + e_k^{(1)} \\ y_{ik}^{(2)} &= \mu_2 + \alpha_i + e_k^{(2)} \\ y_{ik}^{(3)} &= \mu_3 + \beta_i + e_k^{(3)} \\ y_k^{(4)} &= \mu_4 + e_k^{(4)} \end{aligned}$$

Linear model-3

with the usual assumptions $\sum_i \alpha_i = \sum_i \beta_i = 0$. Here the least squares estimates are,

$\hat{\mu}_\ell'' = \hat{\mu}_\ell$ for all $\ell = 1, 2, ..., 4$ as before.

$\hat{\alpha}_i'' = \hat{\alpha}_i$ and $\hat{\beta}_i'' = \hat{\beta}_i$ for all $i = 1, ..., 4$ as before

The sum of squares of errors when H_{03} is true is given by

$$\text{ESS(3)} = \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{iik}^{(1)} - \hat{\mu}_1 - \hat{\alpha}_i - \hat{\beta}_i\right)^2 + \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(2)} - \hat{\mu}_2 - \hat{\alpha}_i\right)^2 + \sum_{i=1}^{4}\sum_{k=1}^{4}\left(y_{ik}^{(3)} - \hat{\mu}_3 - \hat{\beta}_i\right)^2 + \sum_{k=1}^{4}\left(y_k^{(4)} - \hat{\mu}_4\right)^2$$

D.F. = 15 + 12 + 12 + 3 = 42

Thus, sum of squares due to AB is given by,

ESS(3) – ESS = SS_3 carries 42 – 39 = 3 d.f.

Finally, the ANOVA table is constructed below.

Source of Variation	D.F.	Sum of Squares (SS)	Mean square SS/DF	F Statistics
A	3	ESS(1) – ESS = SS_1	$MS_1 = SS_1/3$	$MS_1/MSE \sim F_{3,39}$
B	3	ESS(2) – ESS = SS_2	$MS_2 = SS_2/3$	$MS_2/MSE \sim F_{3,39}$
AB	3	ESS(2) – ESS = SS_3	$MS_3 = SS_3/3$	$MS_3/MSE \sim F_{3,39}$
Error	39	ESS	MSE = ESS/39	
Total	48	TSS		

If $F_\ell > F_{\alpha(3,39)}$, then reject the null hypothesis (whichever), where, ℓ = 1, 2, 3.

α = 0.05, 0.01, etc.

If $F_\ell > F_\alpha$ (3, 39), then null hypothesis is rejected at 100α% level.

Calculations : To simplify the calculations, the following relations have been written down, which help in computing the sum of squares due to errors.

ESS :

$$\sum_i \sum_k \left(y_{iik}^{(1)} - \hat{\mu}_1 - \hat{\alpha}_i - \hat{\beta}_i - \hat{\gamma}_i\right)^2 = \left(\sum_i \sum_k y_{iik}^{(1)^2} - 4.\sum_i y_{ii0}^{(1)^2}\right) \quad (1)$$

$$\sum_i \sum_k \left(y_{ik}^{(2)} - \hat{\mu}_2 - \hat{\alpha}_i\right)^2 = \left(\sum_i \sum_k y_{ik}^{(1)^2} - 4.\sum_i y_{i0}^{(1)^2}\right) \quad (2)$$

$$\sum_i \sum_k \left(y_{ik}^{(3)} - \hat{\mu}_3 - \hat{\beta}_i\right)^2 = \left(\sum_i \sum_k y_{ik}^{(3)^2} - 4.\sum_i y_{i0}^{(3)^2}\right) \quad (3)$$

and $$\sum_{k=1}^{4}\left(y_k^{(4)}-\hat{\mu}_4\right)^2=\sum_k y_k^{(4)^2}-4.y_0^{(4)^2} \qquad (4)$$

ESS_1 :

$$\sum_i\sum_k\left(y_{iik}^{(1)}-\hat{\mu}_1-\hat{\beta}_i-\hat{\gamma}_i'\right)^2=\left(\sum_i\sum_k y_{iik}^{(1)^2}-4.\sum_{i=1}^{4} y_{ii0}^{(1)^2}\right) \qquad (1)$$

$$\sum_i\sum_k\left(y_{ik}^{(1)}-y_0^{(1)}\right)^2=\left(\sum_i\sum_k y_{ik}^{(1)^2}-16.y_0^{(2)^2}\right) \qquad (2)$$

$$\sum_i\sum_k\left(y_{ik}^{(3)}-\hat{\mu}_3-\hat{\beta}_i\right)^2=\left(\sum_i\sum_k y_{ik}^{(3)^2}-4.\sum_i y_{i0}^{(3)^2}\right) \qquad (3)$$

and $$\sum_{k=1}\left(y_k^{(4)}-\hat{\mu}_4\right)^2=\sum_k y_k^{(4)^2}-4.y_0^{(4)^2} \qquad (4)$$

ESS_2 :

$$\sum_i\sum_k\left(y_{iik}^{(1)}-\hat{\mu}_1-\hat{\alpha}_i-\hat{\gamma}_i''\right)^2=\left(\sum_i\sum_k y_{iik}^{(1)^2}-4.\sum_{i=1}^{4} y_{ii0}^{(1)^2}\right) \qquad (1)$$

$$\sum_i\sum_k\left(y_{ik}^{(2)}-\hat{\mu}_2-\hat{\alpha}_i\right)^2=\left(\sum_i\sum_k y_{ik}^{(2)^2}-4.\sum_i y_{i0}^{(2)^2}\right) \qquad (2)$$

$$\sum_i\sum_k\left(y_{ik}^{(3)}-y_0^{(3)}\right)^2=\left(\sum_i\sum_k y_{ik}^{(3)^2}-16y_0^{(3)^2}\right) \qquad (3)$$

and $$\sum_{k=1}^{4}\left(y_k^{(4)}-\hat{\mu}_4\right)^2=\left(\sum_k y_k^{(4)^2}-4.y_0^{(4)^2}\right) \qquad (4)$$

ESS_3 :

$$\sum_i\sum_k\left(y_{iik}^{(1)}-\hat{\mu}_1-\hat{\alpha}_i-\hat{\beta}_i\right)^2=\sum_i\sum_k\left(y_{iik}^{(1)}-y_{i0}^{(2)}-y_{i0}^{(3)}+y_0^{(2)}+y_0^{(3)}-y_0^{(1)}\right)^2 \qquad (1)$$

$$\sum_i \sum_k \left(y_{ik}^{(2)} - \hat{\mu}_2 - \hat{\alpha}_i\right)^2 = \left(\sum_i \sum_k y_{ik}^{(2)^2} - 4.\sum_i y_{i0}^{(2)^2}\right) \quad (2)$$

$$\sum_i \sum_k \left(y_{ik}^{(3)} - \hat{\mu}_3 - \hat{\beta}_i\right)^2 = \left(\sum_i \sum_k y_{ik}^{(3)^2} - 4.\sum_i y_{i0}^{(3)^2}\right) \quad (3)$$

and $$\sum_{k=1} \left(y_k^{(4)} - \hat{\mu}_4\right)^2 = \left(\sum_k y_k^{(4)^2} - 4.y_0^{(4)^2}\right) \quad (4)$$

Degree of freedom is defined as,

(No. of quantities involved in a sum) – (no. of equations to which those quantities are subject).

The relevant quantities (sum of squares) are calculated and presented below.

(*) $$\sum_i \sum_k y_{iik}^{(1)^2} = 19,922.283$$

$$4\sum_i^4 y_{ii0}^{(1)^2} = 4(4,978.813) = 19,915.252$$

$$\therefore \quad \left(\sum_i \sum_k y_{iik}^{(1)^2} - 4\sum_i y_{ii0}^{(1)^2}\right) = 7.031 \quad (1)$$

(*) $$\sum_i \sum_k y_{ik}^{(2)^2} = 38,851.909$$

$$4\sum_i y_{i0}^{(2)^2} = 38843.561$$

$$\therefore \quad \left(\sum_i \sum_k y_{iik}^{(2)^2} - 4.\sum_i y_{i0}^{(2)^2}\right) = 8.348 \quad (2)$$

(*) $$\sum_i \sum_k y_{ik}^{(3)^2} = 41,041.250$$

$$4\sum_i y_{i0}^{(3)^2} = 41,036.292$$

$$\therefore \quad \left(\sum_i \sum_k y_{ik}^{(3)^2} - 4.\sum_i y_{i0}^{(3)^2}\right) = 4.958 \qquad (3)$$

(*) $$\sum_i y_{ik}^{(4)^2} = 15,681.128$$

$$4.y_0^{(4)^2} = 15,661.271$$

$$\therefore \quad \left(\sum_i y_k^{(4)^2} - 4y_0^{(4)^2}\right) = 19.857 \qquad (4)$$

Also, $y_0^{(2)} = 49.185; \quad 16y_0^{(2)^2} = 38,706.628$

$$y_0^{(3)} = 50.598; \quad 16y_0^{(3)^2} = 40,962.522$$

and $y_0^{(1)} = 35.005.$

Calculation table for $\sum_i \sum_k \left(y_{iik}^{(1)} - \hat{\mu}_1 - \hat{\alpha}_i - \hat{\beta}_i\right)^2$ in ESS (3)

$\left(y_{iik}^{(1)} - y_{i0}^{(2)} - y_{i0}^{(3)} + y_0^{(2)} + y_0^{(3)} - y_0^{(1)}\right)$

i,k	1	2	3	4
1	- 0.212	- 1.292	- 0.732	- 0.802
2	- 0.292	- 1.982	- 0.602	- 0.462
3	1.048	1.688	0.408	0.918
4	0.688	2.238	- 0.862	0.238

(*) $\sum_i \sum_k \left(y_{iik}^{(1)} - y_{i0}^{(2)} - y_{i0}^{(3)} + y_0^{(2)} + y_0^{(3)} - y_0^{(1)}\right)^2 = 18.721$

Finally, the errors sum of squares are :

ESS = 7.031 + 8.348 + 4.958 + 19.857 = 40.194

ESS (1) = 7.031 + 145.281 + 4.958 + 19.857= 177.127

ESS (2) = 7.031 + 8.348 + 78.728 + 19.857 = 113.964

ESS (3) = 18.721 + 8.348 + 4.958 + 19.857 = 51.884

And

SS_1 = ESS (1) – ESS = 136.933

SS_2 = ESS (2) – ESS = 73.770

SS_3 = ESS (3) – ESS = 11.69

$SS_1/3 = MS_1 = 45.644$

$SS_2/3 = MS_2 = 24.59$ $FSS_3/3 = MS_3 = 3.897$

ESS/39 = 1.031

Testing :

To test H_{01} the relevant test statistic is,

$\frac{MS_1}{MSE} = \frac{45.644}{1.031} = 44.27 > F_{0.01,3.39}$ which is highly significant.

To test H_{02} the relevant test statistic is $\frac{MS_2}{MSE} = 23.85 > F_{0.01,3.39}$ which is also highly significant.

Finally, H_{03} is tested by,

$\frac{MS_3}{MSE} = \frac{3.897}{1.031} = 3.783 > F_{0.05,3.39}$, i.e., significant at 5% level though insignificant at 1% level.

Thus we conclude that the combined effect (multimetal) is significant at 5% level ($P<0.05$).

(1) The impact (in terms of NO release) of arsenic and lead, when applied in tandem in the same doses as applied separately, turns out to be statistically significant at 5% level. This establishes the view that, apart from the individual metal effects, the multimetal exposure is also significant. In other words, the two metals lead and arsenic when applied in tandem are not self destructive or antagonistic in nature. Had it been so, the multimetal exposure would not have been statistically significant. Though the above statistical analysis hints at synergy, it does not directly prove its existence. That is, the above F tests are not conclusive evidences favoring the argument that arsenic and lead have a synergic impact on NO release. In what follows, an Isobologram has been developed which gives conclusive evidence favoring the argument that arsenic and lead have a synergic impact on NO release. It clarifies that the two metals are not self destructive in nature so far as the impact in terms of NO release is concerned.

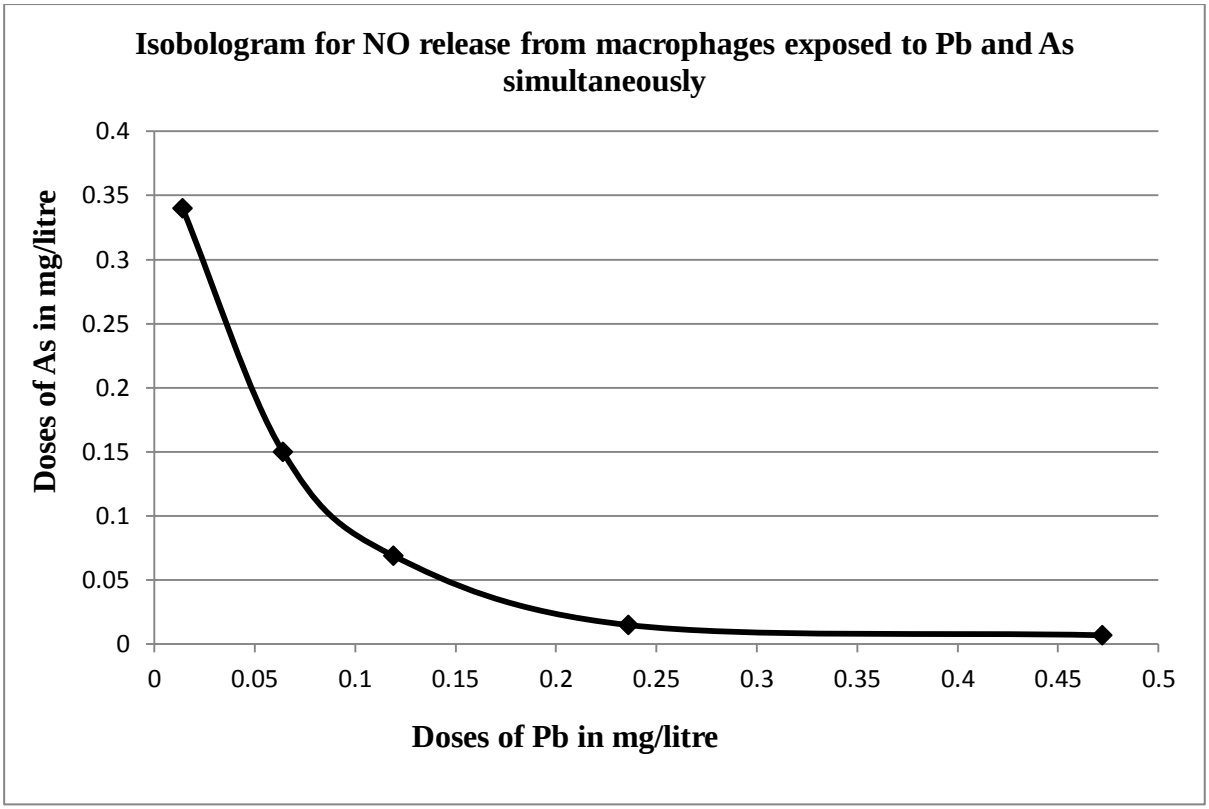


Fig 1: Isobol for NO release from macrophages exposed to lead and arsenic

(2) It is to be noted that the α_i and β_i in Block 1 are different from those in Blocks 2 and 3. This is because each Block gives a separate least squares problem. This is as far as the statistical argument goes. From the biological perspective, it is to be remembered that in this theoretical argument d_A and D_A, and d_B and D_B, respectively, differ from each other. Here

d_A = required dose level of metal A (say As) under multimetal exposure for a particular NO release.

D_A = required level of metal under single metal exposure for the same release of NO.

Similar definitions apply for d_B and D_B.

In this experiment, α_i's in Block 1, correspond to d_A, while α_i's of Block 2, correspond to D_A. Analogously, β_i's can be related to d_B and D_B.

The Isobologram / Plotting the Isobologram

In order to derive / plot the isobologram from the experimental data, the impact of dose levels of a certain metal on NO release by means of ordinary lease squares regression (2-variable) is first examined.

Normally, the impact of dose levels (single or multimetal) on NO release does not incorporate the control group (values) at any stage. But one feels that the true impact of the dose levels can only be studied if the control-values are viewed as a scale of reference (i.e., measuring rod or yard stick), or an ideal condition. This is so because; they are free from any heavy metal exposure.

To incorporate the control group in the analysis, the impact of a particular dose level of a particular metal is simply measured by the shortfall in NO release from the mean % NO release of the control group.

Moreover, as the isobologram is plotted in a square it is necessary that the dose levels of the 2 metals be transformed to the same scale. This is done by assuming,

$$X = \frac{(\text{dose level of lead in mg/kg bw})}{5} \text{ and}$$

$Y = (\text{dose level of arsenic in mg/kg bw}) \times 4$, so that the dose levels in mg/kg bw simply become 1, 2, 3 and 4 for each metal.

Here

Z_1 = shortfall of NO release from that of the mean value of the control group, for multimetal exposure (i.e., block 1).

The mean NO release for each dose, i.e., the mean NO release for each row in block 1 and so also in blocks 2 and 3 is considered.

Z_2 = shortfall of NO release from that of the mean control value for a particular row mean for block 2, and

Z_3= shortfall of NO release of a particular row mean from mean control value for block 3.

Clearly, Z_1 measures the impact due to multimetal exposure,

Z_2 measures that due to arsenic alone while Z_3 measures the impact due to lead alone.

Model specification for Regression analysis

From the observations it is found that the dose levels of a particular metal and shortfall of NO release are non-linearly related. The two are impact related such that the scatter approximates a log-linear model of the general form $y = ax^b$.

It is otherwise known as power regression (or log-log model). Three separate regressions – one each for block 1, block 2 and block 3 respectivelyare performed.

For Block 1 the model is,

$$z_1 = a_1(X.Y)^{b_1} \quad (1)$$

i.e., $$\ln z_1 = \ln a_1 + b_1 \ln(X.Y) \quad (1a)$$

the following model is fitted for block 2.

$$z_2 = a_2(X.Y)^{b_2} \quad (2)$$

i.e., $$\ln z_2 = \ln a_2 + b_2 \ln X \quad (2a)$$

Analogously for Block 3 the model is fitted

$$z_3 = a_3(Y)^{b_3} \quad (3)$$

i.e., $$\ln z_3 = \ln a_3 + b_3 \ln X \quad (3a)$$

The parameters (*a*'s and *b*'s) of the above 3 equations are estimatedon the basis of the experimental results.

Now, a simplistic assumption is made that the overall effect (in terms of NO release) of the multimetal and single metal exposure is the sum of the single metal (z_2 and z_3) and the multimetal (z_1) effects.

In other words, the effects are assumed to be additive, each effect getting unit weight. Thus, overall effect, *Z* is given by

$$Z = z_1 + z_2 + z_3 \quad (4)$$

The estimated overall effect is obtained as,

$$\hat{Z} = \hat{z}_1 + \hat{z}_2 + \hat{z}_3 \quad (4a)$$

where $\hat{z}_1, \hat{z}_2$ and $\hat{z}_3$ are estimated by fitting models (1a), (2a) and (3a) respectively to the data.

Finally, the overall effect *Z* at a particular level is fixed and the values of *Y* for different levels of *X* are found.

This gives a series of (*X*, *Y*) pairs for the same effect. Plotting *Y* values against different *X*-values, the Isobol for *Z* = 40.

Using the above technique, isobols for various levels of *Z* can be derived. Plotting all these different isobols on the same *X*-*Y* plane gives the isobologram.

Results and Discussion

For block 1 the estimated model is,

$$\hat{z}_1 = 21.17(X.Y)^{0.158}, \quad R^2 = 0.99$$

for Block 2, it is,

$$\hat{z}_2 = 9.14(Y)^{0.447}, \quad R^2 = 0.91$$

while for Block 3 it is,

$$\hat{z}_3 = 8.96(X)^{0.345}, \quad R^2 = 0.97$$

The combined effect (estimated) is,

$$\hat{Z} = 21.17(X.Y)^{0.158} + 9.14(Y)^{0.447} + 8.96(X)^{0.345} \qquad (5)$$

Now assuming $\hat{Z} = 40$, equation (5) becomes,

$$21.17\ (XY)^{0.158} + 9.14\ (Y)^{0.447} + 8.96\ (X)^{0.345} = 40 \qquad (5a)$$

Putting X = 1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 and solving for the corresponding Y values, the following (X, Y) pairs are obtained

On the basis of these (X, Y) scores the isobol for $\hat{Z} = 40$ U is plotted. This is depicted in the diagram.

The nature of the isobol for $\hat{Z} = 40$ provides conclusive evidence favouring the argument that lead and arsenic are synergistically related so far as NO release from cells is concerned. In other words, the said metals have a synergic (or synergistic) impact on cells, in terms of NO release.

(ii) Antioxidant status- Catalase (CAT)

Multimetal exposure			
Dose levels	Arsenic (mg/L)	Lead (mg/L)	Mean CAT activity (U/mg protein)
a	0.059	2.35	2.87 ± 0.036
b	0.118	4.71	2.31 ± 0.049
c	0.236	9.42	2.02 ± 0.038
d	0.472	18.84	1.62 ± 0.02
Single metal exposure			
Dose levels	Arsenic (mg/L)	Mean CAT activity (U/mg protein)	
a	0.059	4.91 ± 0.038	
b	0.118	4.32 ± 0.112	
c	0.236	3.96 ± 0.024	
d	0.472	3.78 ± 0.063	
Dose levels	Lead (mg/L)	Mean CAT activity (U/mg protein)	
a	2.35	5.11 ± 0.237	
b	4.71	4.97 ± 0.125	
c	9.42	4.42 ± 0.24	
d	18.84	3.89 ± 0.357	
Control	Mean CAT activity (U/mg protein)		
	5.78 ± 0.357		

Table 4.2: Effect of repeated single and combined exposure (in vivo) to lead and arsenic on catalase (CAT) activity from fish intestinal macrophages

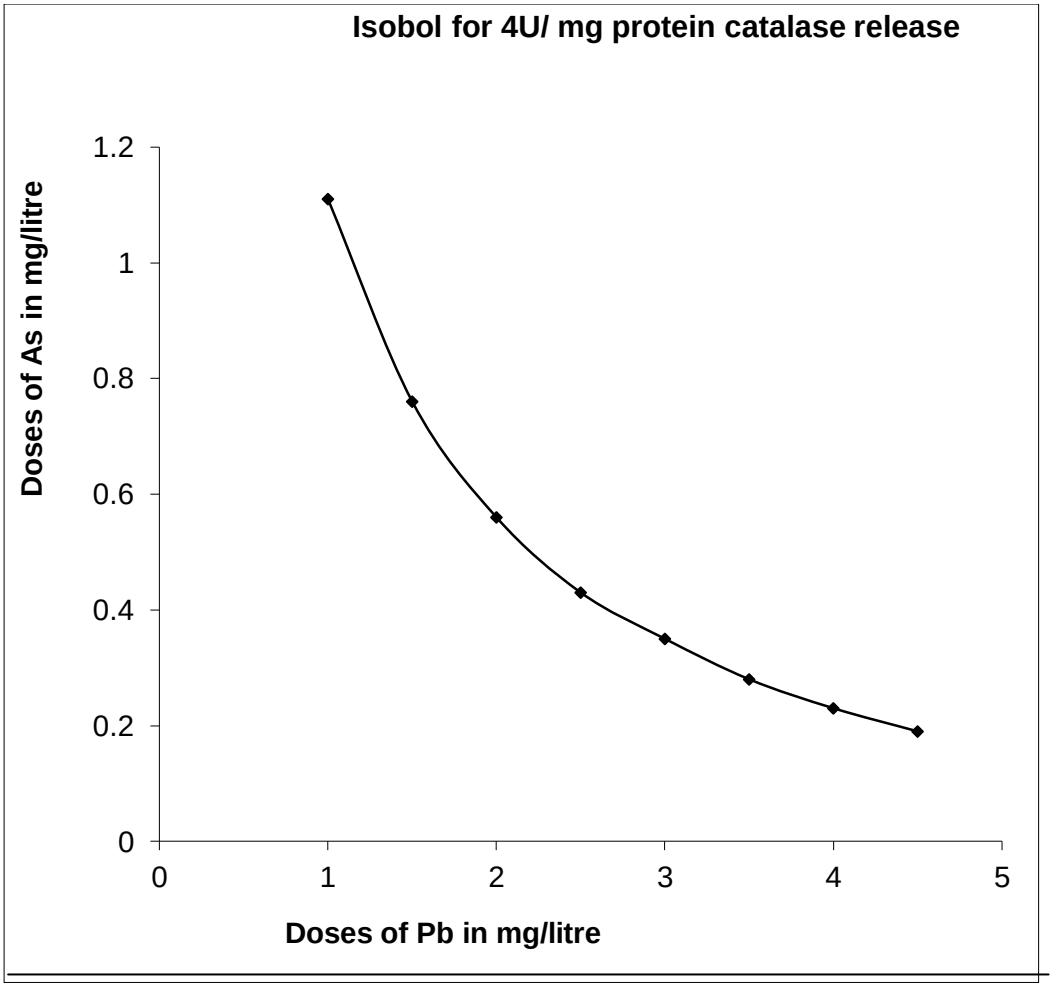


Fig. 2: Isobol for CAT activity from macrophages exposed to lead and arsenic

Similarly, it can be stated that lead and arsenic have a synergic (or synergistic) impact on cells, in terms of CAT activity.