Chapter 5

Experiments

5.1. EXPERIMENT-I

5.1.1. Dose Selection of Arjunolic acid to reduce the arsenic mediated toxicity on female reproductive organs:

5.1.1.1. Aims and objectives:

To observe the minimal effective doses of arjunolic acid to define its therapeutic effects against arsenic-induced toxicity in the reproductive organs of female albino rats.

5.1.1.2. Experimental design:

Wistar rats weighted of 130±10 g were preferred for the experiment. During the period of experiment, a normal pellet diet was provided to all animals of all groups. The rats were distributed in five groups containing six rats in each group. The experimental program was as followed:

Group I Vehicle treated control,

Group II Sodium arsenite treated (1.0 mg /100gm body weight),

Group III Sodium arsenite (1.0 mg /100gm body weight) plus arjunolic acid (0.5 mg/100gm body weight) (Sigma-Aldrich, St. Louis, MO),

Group IV Sodium arsenite (1.0 mg /100gm body weight) plus arjunolic acid (1.0 mg/100gm body weight),

Group V Sodium arsenite (1.0 mg /100gm body weight) plus arjunolic acid (1.5 mg/100gm body weight).

Sodium arsenite (1.0 mg /100gm body weight) and arjunolic acid (0.5 mg/100gm body weight, 1.0 mg/100gm body weight, 1.5 mg/100gm body weight) were prepared separately with distilled water. The rats were treated for eight days via oral gavage with arsenic and arjunolic acid as stated above. To execute the same volume of physical stress among the animals, the Group I

(control group) was supplied with the same amount of water as a vehicle through gavage. The rats of Group II were fed with only sodium arsenite at the dose of 1.0 mg /100gm body weight and the rats of Group III were treated with same doses of sodium arsenite and arjunolic acid at the doses of 0.5 mg/100gm body weight. Group IV rats were ingested with same doses of sodium arsenite and arjunolic acid at the doses of 1.0 mg/100gm body weight and the rats of Group V were treated with the same doses of sodium arsenite plus arjunolic acid at the doses of 1.5 mg/100gm body weight. The body weights of all rats were documented. Rats were anesthetized by intramuscular injection of ketamine HCl at the dose of 24 mg/kg body weight to collect blood and female sex organs and finally euthanized by the intramuscular injection of an overdose of ketamine following the standard protocol of CPCSEA. Into a separate sterile bag, the samples were kept in the insulated container having a temperature of -20^0C temperature.

5.1.1.3. Results:

5.1.1.3.1. General Observation:

Animals were provided with a standard rat chow at the composition of crude protein: 23.0%, crude fat: 3.0%, crude fiber: 7.0%, calcium: 1.0-2.5%, phosphorus: 0.9%, sodium: 0.5-1.0%, moisture: 12.0% during the experiment. The intake of water was insignificantly increased in arsenic ingested group than vehicle-treated control group. The water intake in arjunolic acid group at the dose of 1.0 mg and 1.5 mg was higher insignificantly than the dose of 0.5 mg in arjunolic acid group. In terms of the percentage of body mass, the ovarian-uterine somatic index was considered. A marked reduction of the ovarian and uterine wet weight was noticed (Table 1.1.1) in arsenic intoxicated group but the restoration of these organs' weight was achieved following the treatment with arjunolic acid towards normalcy (Table 1.1.1). All the doses of arjunolic acid established a significant restoration of ovarian and uterine somatic indices but

arjunolic acid at the doses of 1.0 mg and 1.5 mg per 100 gm body weight were more effective than 0.5 mg per 100 gm body weight against As^{3+} mediated toxicity (Table 1.1.1).

5.1.1.3.2. Observation of Lipid Peroxidation End Products:

A higher level of MDA and CD was observed significantly in arsenic intoxicated rats than that of the control group (Table 1.1.2). This elevated level of lipid peroxidation end products was significantly abolished in these sex organs by the treatment of arjunolic acid in arsenic intoxicated rats (Table 1.1.2). Arjunolic acid at the dose of 0.5 mg showed no significant change whereas 1.0 mg and 1.5 mg per 100 gm body weight showed the similar type of significant effect (Table 1.1.2).

5.1.1.3.3. Effect of Arjunolic acid on Antioxidants Enzymes:

In comparison with the control group the activity of SOD, catalase, and peroxidase was diminished significantly in arsenic ingested group (Fig. 1.1.1). Arjunolic acid significantly elevated the activity of SOD, catalase, and peroxidase in contrast to sodium arsenite treated groups (Fig. 1.1.1). In uterine SOD and catalase arjunolic acid showed no significant change at the dose of 0.5 mg but showed the similar type of significant effect at the dose of 1.0 mg and 1.5 mg per 100 gm body weight. In case of uterine peroxidase, the dose of arjunolic acid with 0.5 mg did not establish any significant change whereas 1.0 mg and 1.5 mg per 100 gm body weight dose were significantly differed from each other. Arjunolic acid at the dose of 1.5 mg was more significant than 1.0 mg per 100 gm body weight (Fig. 1.1.1).

5.1.1.3.4. Observation of Ovarian Steroidogenesis:

In arsenic-treated rats the activities of ovarian Δ^5, 3β-HSD, 17β-HSD, and LH, FSH, and estradiol were suppressed significantly in contrast to the control group (Fig. 1.1.2). For maintaining the activities of steroidogenic key enzymes and hormones, arjunolic acid played an

important role to keep these activities towards normalcy (Fig. 1.1.2). For the maintaining of ovarian steroidogenic key enzymes and serum estradiol level, arjunolic acid at the dose of 0.5 mg exhibited no significant change whereas 1.0 mg and 1.5 mg per 100 gm body weight exhibited the similar type of significant change (Fig. 1.1.2). There was no significant variation between the doses of arjunolic acid in the case of serum LH and FSH level (Fig. 1.1.2).

Table-1.1.1

	Body Weight (gm)		Organo-somatic indices (gm)		Water intake (ml/100gm body weight)
	Final	Initial	Ovary in pair	Uterus	
Control	128.2±1.62	122.8±3.09	0.067±0.002	0.177±0.016	10.30± 0.93
As^{3+}	136.4±3.00	125.8±2.98	0.040±0.004***	0.126±0.021***	13.49± 1.06
As^{3+}+AA(0.5mg/100gm Body weight)	132.6±2.93	124.6±1.68	0.047±0.002†	0.155±0.006††	12.18 ± 1.55
As^{3+}+AA(1.0mg/100gm Body weight)	134.2±2.68	126.6±1.75	0.057±0.04††	0.166±0.011†††	12.24±1.27
As^{3+}+AA(1.5mg/100gm Body weight)	136.2±1.27	125.0±2.15	0.062±0.004††	0.172±0.003†††	12.41±1.55

Table 1.1.1: The data are mean ± SE, n=6, ANOVA followed by the Post Hoc Dunnett test were used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic treated groups. †p < 0.05, ††p < 0.01, ***/†††p < 0.001.

Table-1.1.2

	Control	As^{3+}	As^{3+}+AA (0.5mg/100gm)	As^{3+}+AA (1.0mg/100gm)	As^{3+}+AA (1.5mg/100gm)
MDA (nmole/gm)	33.62±3.10	50.58±4.04**	46.60±2.60	34.48±1.50†	33.3±2.16†
CD (nmole/gm)	15.29±2.51	24.78±2.44*	17.48±4.13	11.94±0.98†	14.08±1.60†

Table 1.1.2: The data represent mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p < 0.05, **p<0.01.

Figure-1.1.1

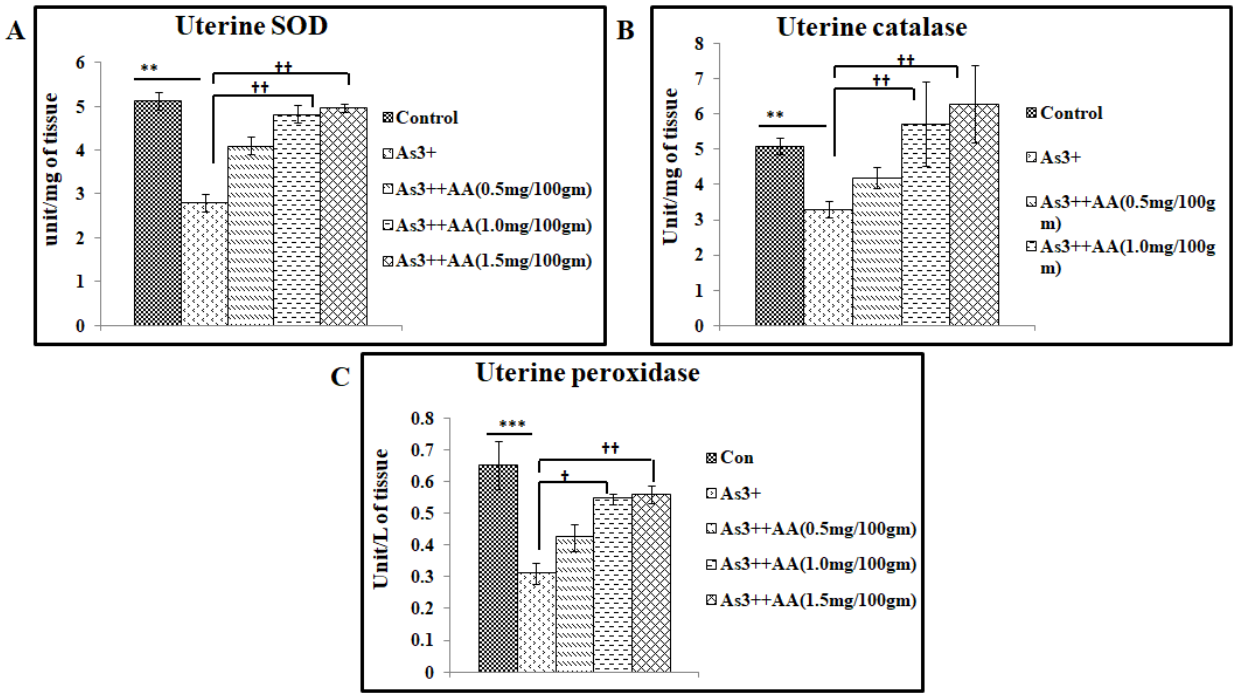


Fig. 1.1.1: The data are represented as mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p < 0.05, **/††p < 0.01, ***p < 0.001.

Figure-1.1.2

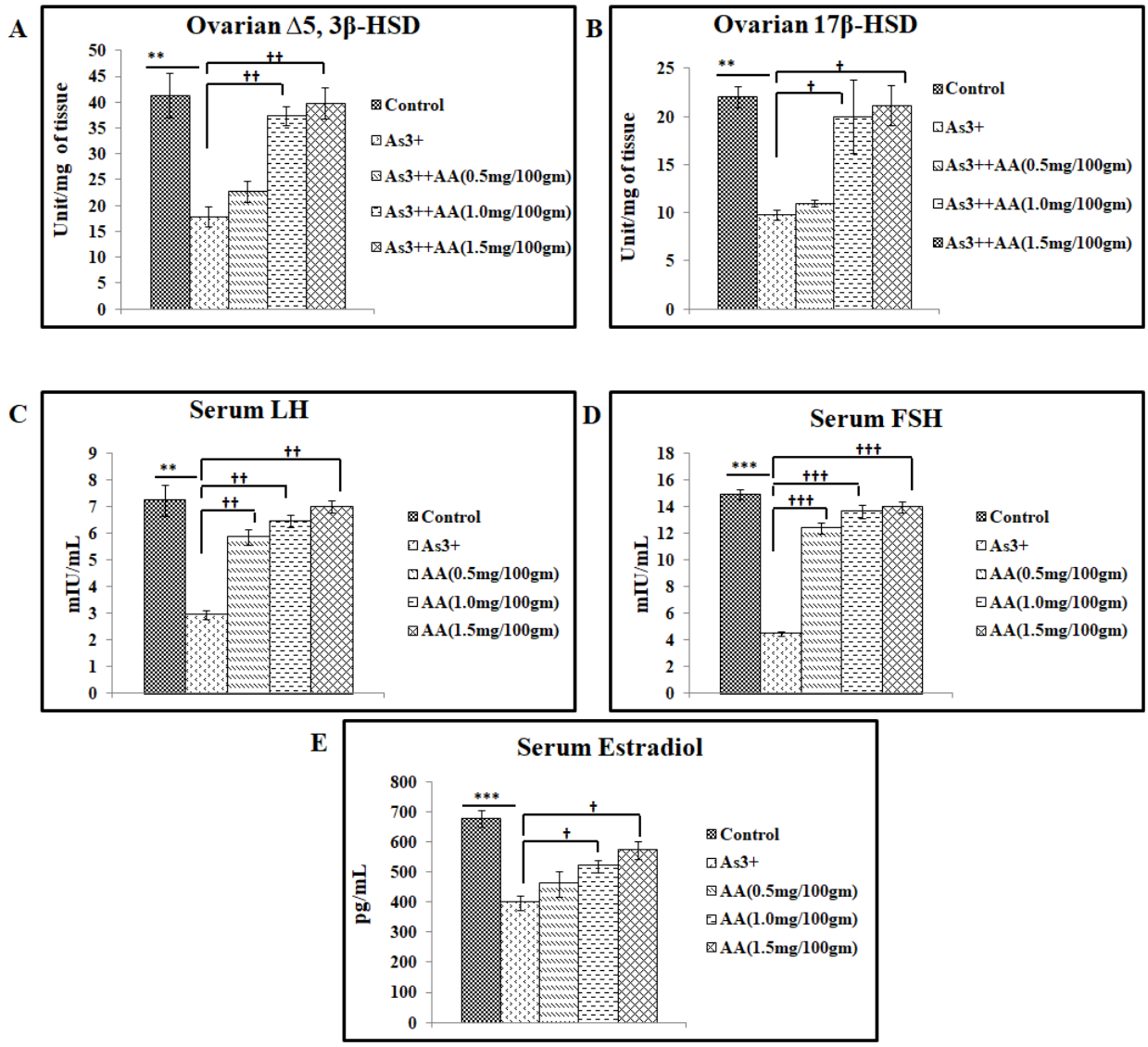


Fig. 1.1.2: The data represent mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †p < 0.05, **/††p < 0.01, ***/†††p < 0.001.

5.1.2. Dose Selection of Vitamin B_{12} to reduce the arsenic mediated toxicity on female reproductive organs:

5.1.2.1. Aims and objectives:

To reveal the effective dose of vitamin B_{12} for the reduction of the arsenic-induced hazards in the reproductive organs of female Wistar rats.

5.1.2.2. Experimental design:

Six rats in each group were distributed in the following manner. Normal rat chow in pellet form was given to the rats.

Group I Vehicle treated control,

Group II Sodium arsenite treated (1.0 mg /100gm body weight),

Group III Sodium arsenite (1.0 mg /100gm body weight) plus vitamin B_{12} (0.07 µg/100gm body weight) (SRL),

Group IV Sodium arsenite (1.0 mg /100gm body weight) plus vitamin B_{12} (0.09 µg/100gm body weight),

Group V Sodium arsenite (1.0 mg /100gm body weight) plus vitamin B_{12} (0.1 µg/100gm body weight).

Sodium arsenite at the dose of 1.0 mg /100gm body weight and vitamin B_{12} at the doses of 0.07 µg/100gm body weight, 0.09 µg/100gm bodyweight, and 0.1 µg/100gm bodyweight were prepared separately with distilled water. The same volume of water was supplied through gavage among the Group I (control group) rats to show the same amount of physical stress. The treatment was continued for eight days via oral gavage. The rats of the Group II were ingested with sodium arsenite at the dose of 1.0 mg /100gm body weight and the rats of the Group of III, IV and V were co-administrated with the same dose of sodium arsenite and vitamin B_{12} at the

doses of 0.07 µg/100gm body weight, 0.09 µg/100gm bodyweight, and 0.1 µg/100gm bodyweight. On day nine the body weights of all rats were recorded. An insulated container having a temperature of -20^0C was used to preserve the samples in a separate sterile bag following anesthesia by intramuscular injection of ketamine HCl at the dose of 24 mg/kg body weight and euthanasia by the intramuscular injection of an overdose of ketamine following the ethical guidelines of CPCSEA.

5.1.2.3. Result:

5.1.2.3.1. General Observation:

There was an insignificant higher water intake in the arsenic intoxicated group than the vehicle-treated control group. Vitamin B_{12} (0.09 µg/100gm body weight and 0.1 µg/100gm body weight) groups exhibited insignificant higher water intake than vitamin B_{12} (0.07 µg/100 gm body weights) group. In terms of percentage of body mass uterine-somatic index was expressed. A noteworthy significant wet weight loss of reproductive organs was observed (Table 1.2.1) in arsenic-treated groups but the treatment with vitamin B_{12} re-established the weight of ovaries and uterus towards control value conspicuously. All the doses of vitamin B_{12} were significant in the restoration of ovarian and uterine somatic indices but vitamin B_{12} at the doses of 0.09 µg and 0.1 µg per 100 gm body weight were more significant than 0.07 µg per 100 gm body weight against As^{3+} mediated toxicity (Table 1.2.1).

5.1.2.3.2. Observation of Lipid Peroxidation End Products:

In comparison to the vehicle-treated control group, the level of uterine MDA and CD was significantly raised in arsenic intoxicated groups (Table 1.2.2). In the treatment with B_{12} the uterine MDA and CD level was diminished significantly in arsenic ingested rats (Table 1.2.2). At

the dose of 0.07 µg, vitamin B_{12} exhibited no significant change but the doses of 0.09 µg and 0.1 µg per 100 gm bodyweight exhibited the similar type of significant effect (Table 1.2.2).

5.1.2.3.3. Effect of Vitamin B_{12} on Antioxidants Status:

In arsenic-treated rats the enzymatic activity of uterine SOD, catalase, and peroxidase was significantly reduced than that of vehicle-treated control group (Fig. 1.2.1). A significant renewal of the activity of SOD, catalase, and peroxidase was prominent following the treatment with B_{12} (Fig. 1.2.1). There was no significant change of vitamin B_{12} at the dose of 0.07 µg in uterine SOD and catalase whereas the doses of 0.09 µg and 0.1 µg per 100 gm body weight were significantly differ from each other (Fig. 1.2.1). The 0.1 µg dose of vitamin B_{12} was more significant than 0.07 and 0.09 µg per 100 gm body weight doses in uterine peroxidase (Fig. 1.2.1).

5.1.2.3.4. Observation of Ovarian Steroidogenesis:

The activities of ovarian Δ^5, 3β-HSD, 17β-HSD and LH, FSH, estradiol were significantly inhibited by the treatment with sodium arsenite in comparison to the control group (Fig. 1.2.2). These activities were noticeably restored by the treatment with vitamin B_{12} towards normalcy (Fig. 1.2.2). In ovarian Δ^5, 3β-HSD vitamin B_{12} at the dose of 0.07 µg was insignificant but the doses of 0.09 µg and 0.1 µg were significantly differ from each other (Fig. 1.2.2). There were no significant differences between the doses of vitamin B_{12} (0.09 µg and 0.1 µg per 100 gm body weight) but the dose of 0.1µg was significant from the other doses of vitamin B_{12} in ovarian 17β-HSD (Fig. 1.2.2). In the rejuvenation of serum LH vitamin B_{12} at the doses of 0.09 µg and 0.1 µg per 100 gm body weight were more significant than the dose of 0.07 µg (Fig. 1.2.2). There was no significant difference between the doses of vitamin B_{12} in the restoration of serum FSH (Fig. 1.2.2). Serum estradiol level showed a similar type of significance of vitamin B_{12} at the dose of

0.09 µg and 0.1 µg per 100 gm body weight whereas the dose of 0.07 µg per 100 gm body weight was insignificant (Fig. 1.2.2).

Table-1.2.1

	Body Weight (gm)		Organo-somatic indices (gm)		Water intake (ml/100gm body weight)
	Final	Initial	Ovary in pair	Uterus	
Control	129.8±1.39	125.6±2.80	0.069±0.004	0.180±0.016	9.71 ± 0.67
As^{3+}	136.6±2.39	128.6±2.25	0.039±0.003***	0.153±0.006**	12.13± 1.35
As^{3+}+B_{12} (0.07µg/100gm Body weight)	132.0±4.11	129.8±2.38	0.048±0.002†	0.163±0.004†	10.86±1.42
As^{3+}+ B_{12} (0.09µg/100gm Body weight)	133.8±3.07	127.0±1.87	0.061±0.004††	0.172±0.004††	11.96 ± 1.26
As^{3+}+ B_{12} (0.1µg /100gm Body weight)	135.6±2.71	127.8±3.02	0.064±0.004††	0.174±0.003††	12.02±1.61

Table 1.2.1: The data are mean ± SE, n=6, ANOVA followed by the Post Hoc Dunnett test were used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †$p < 0.05$, **/††$p < 0.01$, ***$p < 0.001$.

Table-1.2.2

	Control	As^{3+}	As^{3+}+B_{12} (0.07 µg/100gm)	As^{3+}+ B_{12} (0.09 µg/100gm)	As^{3+}+ B_{12} (0.1 µg/100gm)
MDA (nmole/gm)	26.40±3.24	46.46±3.66**	42.28±3.75	29.06±3.21††	24.44±3.34††
CD (nmole/gm)	13.35±2.94	26.85±2.93*	21.37±3.91	15.44±3.82†	14.60±2.69†

Table 1.2.2: The data represent mean ±SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. ***/**†p < 0.05, **/††p < 0.01.

Figure-1.2.1

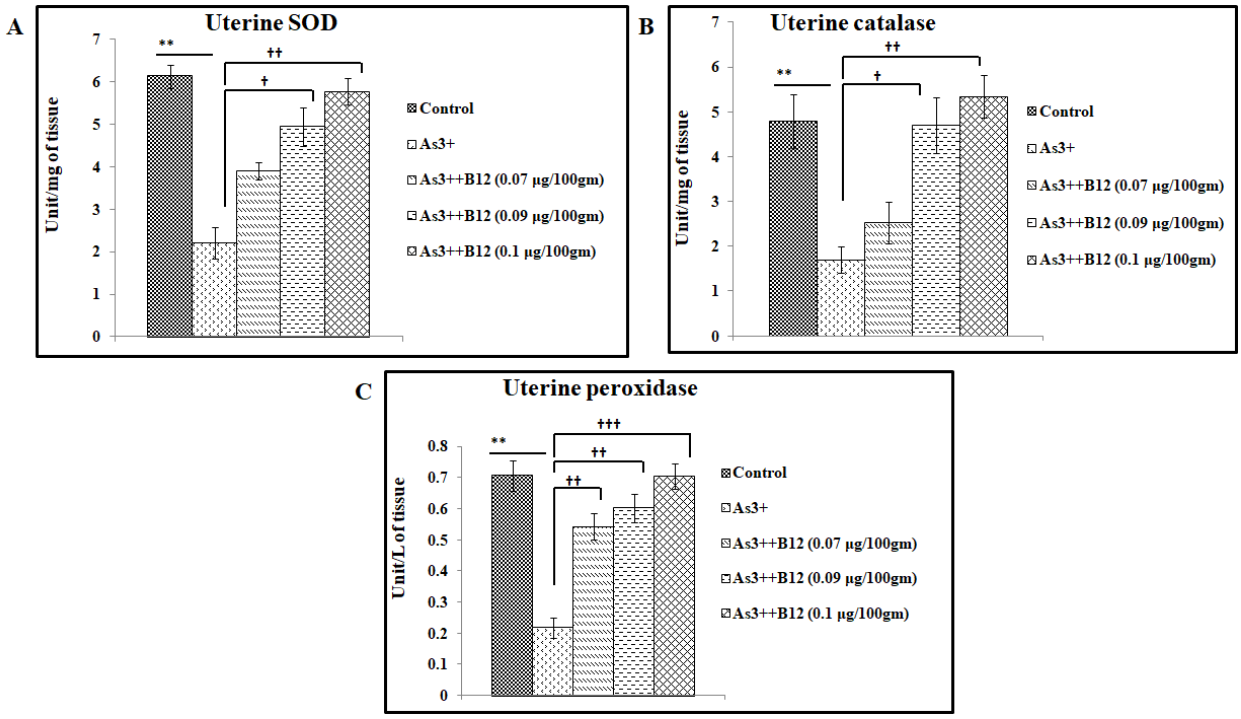


Fig. 1.2.1: The data are represented as mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †p < 0.05, **/††p < 0.01, ***/†††p < 0.001.

Figure-1.2.2

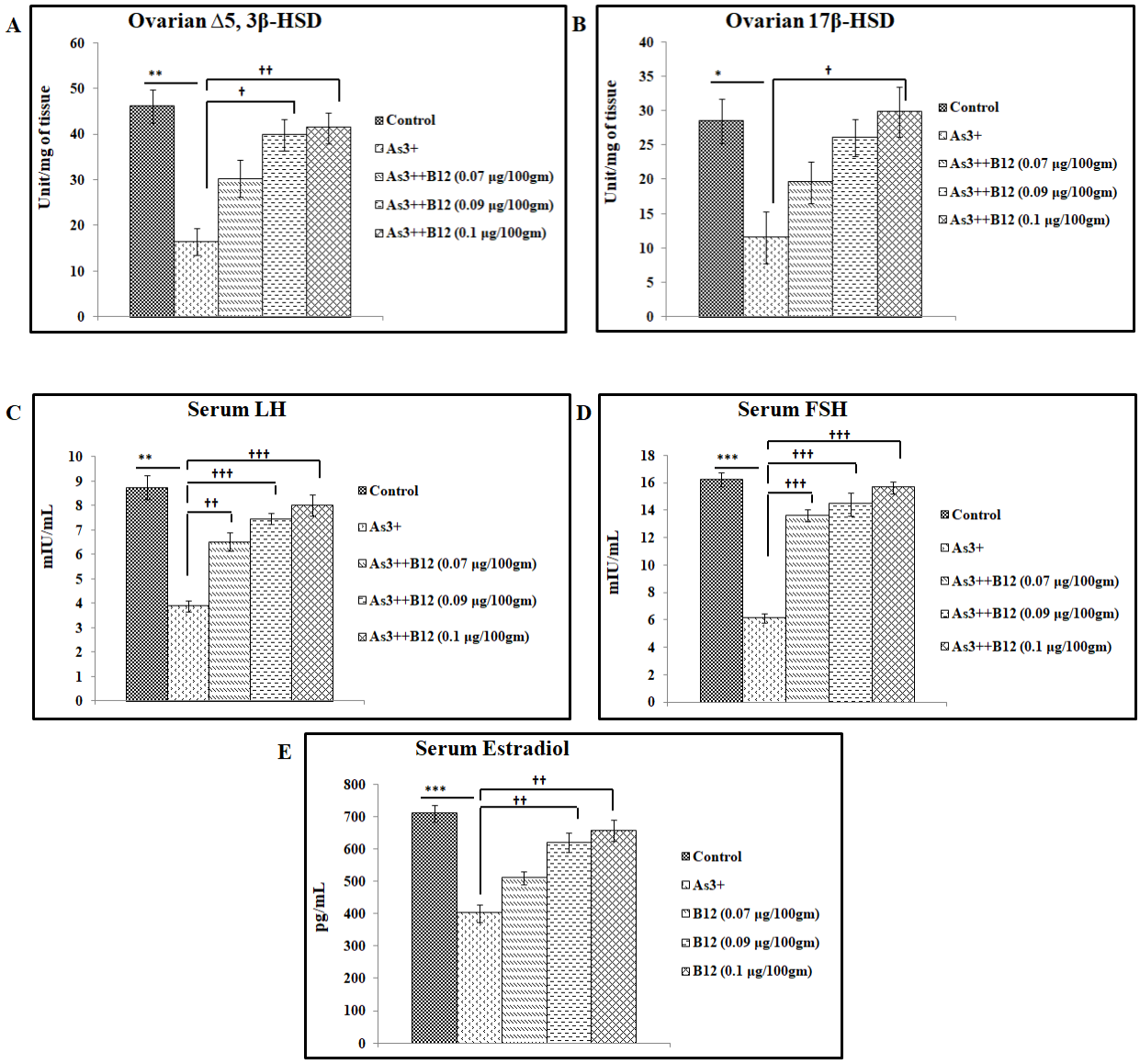


Fig. 1.2.2: The data represent mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. ***/†**p < 0.05, **/††p < 0.01, ***/†††p < 0.001.

5.1.3. DISCUSSION:

Having different biological actions arjunolic acid and vitamin B_{12} show their antioxidant property. Several researchers reported that the uterine tissues are protected through the treatment with vitamin B_{12} from arsenic toxicity in female Wistar rats (Deb et al., 2018). In this experiment, we planned to establish whether arjunolic acid and vitamin B_{12} prevent arsenic-induced repro toxicity in dose-dependent manner. Here, we used three different doses of arjunolic acid i.e. 0.5 mg, 1.0 mg, and 1.5 mg and for vitamin B_{12} we used 0.07 μg, 0.09 μg, and 0.1 μg of doses per 100 gm body weight.

Different cellular macromolecules of uterine tissue are degraded due to the formation of free radicals and oxidative stress (Hultberg et al., 2001). In arsenicated group, the low level of uterine superoxide dismutase and catalase was the consequence of higher level of uterine MDA and CD (Table 1.1.2 and 1.2.2). The tri-valent form of arsenic when associates with H_2O_2 harvests reactive oxygen species (ROS) and generates several lipid peroxides as the terminal products (Wang et al., 2006). Arsenic induces the elevation of uterine MDA, CD, and suppression of the activities of uterine SOD, catatalase, and peroxidase. The dose-dependent study shows that arsenic toxicity distorted the uterine tissue by the production of excess amount of MDA and CD (Table 1.1.2 and 1.2.2) and reduction of intracellular enzymes SOD, catalase, and peroxidase (Fig. 1.1.1 and 1.2.1). And this result is in agreement with the similar finding of Dash et al (Dash et al., 2018). At the time of inflammatory responses during the acute stressful condition, the alteration of redox of cysteines was associated with the inactivation of SOD (Ghosh et al., 2013). Initiation of oxidative stress and generation of superoxide anions ($O_2^{\cdot-}$) in response to arsenic diminishes the mRNA expression of the SOD gene (Rana et al., 2012). H_2O_2 is produced by the transformation of superoxide anion radicals. Arsenic increased the level of free radicals and is

related to the reduced level of this enzyme. In arsenic exposed rats the catalase activity was decreased (Fig. 1.1.1 and 1.2.1). In response to the diminished catalase activity, H_2O_2 detoxification is diminished in uterine tissue. The activity of peroxidase was also reduced in arsenic ingested groups (Fig. 1.1.1 and 1.2.1). This indicates that the accumulation of H_2O_2 in the uterus is associated with programmed cell death (Christine et al., 2010). In this experiment, the treatment with arjunolic acid and vitamin B_{12} with different doses maintains the uterine antioxidant enzymatic activities (Fig. 1.1.1 and 1.2.1) and lipid peroxide end products (Table 1.1.2 and 1.2.2) in arsenic fed rats during its possible own intrinsic antioxidant properties. Other investigators also showed that arjunolic acid (Sinha et al., 2008b) and vitamin B_{12} (Bhattacharjee et al., 2013) protect the activities of enzymatic antioxidants and prevent the level of lipid peroxide end products in arsenic ingested rats. Producing chelate complex with arsenic or removing excess ROS arjunolic acid could protect the antioxidant enzymatic activities (Sinha et al., 2008b). Vitamin B_{12} might play as an antioxidative agent in the way of diminishing the excess arsenic accumulation (Bhattacharjee et al., 2013).

In ovarian steroidogenesis Δ^5, 3β HSD and 17β-HSD are the main enzymes. The plasma levels of estradiol are decreased due to the inhibition of the ovarian steroidogenic enzyme activities (Hinshelwood et al., 1994). The activities of Δ^5, 3β HSD and 17β-HSD are regulated by LH and FSH (Odell et al., 1963). The low level of plasma gonadotrophin could decrease the activities of ovarian Δ^5, 3β-HSD, and 17β-HSD (Ghersevich et al., 1994). In arsenicated group, the levels of LH, FSH, and estradiol were decreased (Fig. 1.1.2 and 1.2.2). Decreased levels of plasma LH and FSH are related to the hypersecretion of ACTH and glucocorticoids (Ghosh et al., 1999). The sensitivity of gonadotroph cells to gonadotrophin-releasing hormone (GnRH) is reduced by the elevated level of plasma glucocorticoids (Chattopadhyay and Ghosh, 2010). Gonadotrophins

may be inhibited by the high level of ACTH (Ogle, 1977). Due to diminished levels of LH, FSH, and estradiol the uterine and ovarian weights were reduced in arsenicated rats. Uterine weights are regulated by estradiol (Edman, 1983), whereas the weight of the ovary is regulated by gonadotrophins (Kulin and Reiter, 1973). In arsenic-treated group, the reduction of the uterine somatic index and uterine malformation are the outcomes of down-regulation of plasma estradiol signaling (Edman, 1983). Earlier it was demonstrated that arsenic could sluggish the ovarian steroidogenic activities via a reduced level of gonadotrophins (Chattopadhyay et al., 1999). The present investigation also revealed the same (Fig. 1.1.2 and 1.2.2).

The previous study showed that a lower dose of arsenic (0.4 ppm) accumulated in uterine tissues (Chattopadhyay and Ghosh, 2010). In different body parts, the lower dose of arsenic treatment for 28 days lead to the generation of the toxic condition in organs (Chattopadhyay et al., 2012; Acharyya et al., 2015). In this experiment, we selected a comparatively higher dose of arsenic (1.0 mg/100 gm body weight) for a comparatively short duration of 2 or 8 days that showed imperative oxidative stress in organs (Manna et al., 2007; Sinha et al., 2008b; Bhattacharya and Haldar, 2013). The higher dose of arsenic is connected with inhibition of antioxidant status and cell injuries (Shi et al., 2004).

Diminishing the ovarian and uterine stress excretion of arsenic from the organs is imperative. Involvement of the methyl group in the biomethylation process of inorganic arsenic leads to the conversion of the trivalent form of arsenic to the pentavalent form of arsenic (Cullen et al., 1984). Using one-carbon (1C) metabolism S-adenosylmethionine (SAM) and methyltransferase enzyme plays a crucial role in the detoxification of arsenic from the body through the modulation of the methylation process via methionine cycle. During the maintenance of endogenous methionine level methionine cycle utilizes vitamin B_{12} and folic acid where vitamin B_{12} acts as a

co-factor to synthase the methionine enzyme and catalyzes the endogenous methionine synthesis from the S-adenosylhomocysteine (Sahin et al., 2003). Spiegelstein et al explored that vitamin B_{12} may promote the methylation process for the elimination of arsenic through urine from the reproductive organs (Spiegelstein et al., 2003). In this experiment, vitamin B_{12} could effectively reduce the arsenic-induced uterine oxidative stress through maintaining the enzymatic level, lipid peroxide end products, and ovarian steroidogenesis level.

The free radicals which were produced in arsenic-treated group were ameliorated by the treatment with arjunolic acid. In the way of protection of ovarian steroidogenesis, arjunolic acid might renovate the uterine weights (Maity et al., 2018). Manna et al., 2008b explored that the activity of the peroxidase was restored by arjunolic acid in arsenic-treated rats (Manna et al., 2008b) and a possible interplay between arjunolic acid and peroxidase might be inevitable in the present investigation. The above results indicated that arjunolic acid decreases the level of lipid peroxide end products and increases the activity of antioxidants. Arsenic can bind with the two vicinal equatorial –OH groups of arjunolic acid. To develop a five-membered chelate complex, the OH groups of arjunolic acid may be associated with As^{III} (with a lone pair of electrons of oxygen). The chelate complex may be responsible for eliminating free toxins and diminishing oxidative damages. One carboxylic hydrogen atom of the chelate complex is accountable for free radical scavenging (Sinha et al., 2008b). To maintain the standard regulation of various cell signaling pathways, arjunolic acid has been documented for a probable chelating action with arsenic (Manna et al., 2007).

Oxidative stresses are reduced by the glutathione sparing effect of B_{12} via the involvement of the signaling molecules that encouraging methionine synthase activity (Veber et al., 2008). Vitamin B_{12} is also implicated for its direct reaction with reactive oxygen and nitrogen species (Veber et

al., 2008). Vitamin B_{12} can protect the uterine tissue by reducing the level of lipid peroxide end products (Bhattacharjee et al., 2013) and elevates the above antioxidant enzymes' activities (Bhattacharjee and Pal, 2014b).

Considering the results of the present experiment we conclude that arjunolic acid at the dose of 1.0 mg/100gm body weight is more effective than the other doses (0.5 mg/ 100gm body weight and 1.5mg/100gm body weight). This experiment also explored that vitamin B_{12} at the dose of 0.09μg/100gm body weight is more effective than other doses (0.07 μg/ 100gm body weight and 0.1 μg/100gm body weight) against arsenic.

5.2. EXPERIMENT-II

5.2.1. Preventive effect of Arjunolic acid and vitamin B_{12} against arsenic-induced female reproductive disorders in Wistar rats:

5.2.1.1. Aims and objectives:

This experiment is done to find out the preventive role of arjunolic acid and vitamin B_{12} alone or jointly against arsenic-induced toxicity on the female reproductive system of Wistar rats.

5.2.1.2. Animal Selection and Treatment:

Wistar rats of 130±10 g body weight were distributed in seven groups with six in each. The followings are the experimental schedule.

Group I Vehicle treated control,

Group II Sodium arsenite treated (1.0 mg /100gm body weight),

Group III Arjunolic acid group (1.0 mg/100gm body weight) (Sigma-Aldrich, St. Louis, MO),

Group IV Sodium arsenite (1.0 mg/100gm body weight) plus arjunolic acid (1.0 mg/100gm body weight) treated group,

Group V Vitamin B_{12} group (0.09µg/100gm body weight),

Group VI Sodium arsenite (1.0 mg/100gm body weight) plus vitamin B_{12} (0.09µg/100gm body weight) treated group,

Group-VII: Sodium arsenite (1.0 mg/100gm body weight) plus arjunolic acid (1.0 mg/100gm body weight), plus vitamin B_{12} (0.09µg/100gm body weight) treated group.

Oral gavage was introduced to the rats for sixteen days. Sodium arsenite (1.0 mg /100gm body weight), arjunolic acid (1.0 mg /100gm body weight), and vitamin B_{12} (0.09 µg/100gm body weight) were prepared separately with distilled water. The same volume of water was supplied orally among the Group I (vehicle treated control group) for sixteen days. For initial eight days,

the rats of Group III (arjunolic acid group), IV (sodium arsenite plus arjunolic acid group), V (vitamin B_{12} group), VI (sodium arsenite plus vitamin B_{12} group) and VII (sodium arsenite plus arjunolic acid plus vitamin B_{12} group) were treated with arjunolic acid and B_{12} alone and in combined manner. The remaining group designated for arsenic treatment was ingested only with water orally for the initial eight days. From day nine onwards, the rats of the group II (sodium arsenite group), IV (sodium arsenite plus arjunolic acid group), VI (sodium arsenite plus vitamin B_{12} group) and VII (sodium arsenite plus arjunolic acid plus vitamin B_{12} group) were treated with sodium arsenite for eight days, and the rest of the groups were supplied with vehicle (water) through oral gavage. The estrous cycle pattern of all rats was observed. The bodyweight of all rats was recorded on day seventeen and then anesthetized by intramuscular injection of ketamine HCl at the dose of 24 mg/kg body weight to collect blood and female sex organs. To an insulated ice container having a temperature of -20^0C temperature, the samples were kept in a separate sterile bag. Finally, following the ethical guidelines of CPCSEA the rats were euthanized by the intramuscular injection of an overdose of ketamine.

5.2.1.3. Results:

5.2.1.3.1. General Observations:

During the experiment, a standard rat chow was given to the animals as mentioned in experiment I. The water intake was higher in arsenic-treated group than the vehicle-treated control group. In arjunolic acid placebo group, the water intake was insignificantly higher than that of arsenic plus arjunolic acid group. The water intake was also insignificantly higher in the B_{12} placebo group than that of arsenic plus B_{12} group. In terms of the percentage of body mass uterine-somatic index was expressed (Table 2.1). Significant weight loss of female repro-organs was observed in arsenic ingested group when compared with control (Table 2.1). Pretreatment with arjunolic acid

and B_{12} alone or in combination noticeably restored these reproductive organs' weight deterioration (Table 2.1).

5.2.1.3.2. Vaginal smear study:

During the treatment schedule, the pattern of the estrous cycle was observed regularly. Synchronization of the estrous cycle was noted after 2-3 days of pretreatment with arjunolic acid, vitamin B_{12} at the initial eight days. During eight days of treatment with arsenic; the vaginal smear showed a prominent prolonged diestrous stage after 2-3 days in contrast to the vehicle-treated control group, but arjunolic acid, B_{12} pretreated group, and pretreatment of arjunolic acid plus B_{12} combination exhibited a normal estrous cycle pattern (Fig. 2.1).

5.2.1.3.3. Observation of Lipid Peroxidation End Products:

The level of uterine MDA and CD was increased in arsenic group than that of vehicle-treated control group (Table 2.2). Nevertheless, pretreatment of arjunolic acid and B_{12} alone or in combination have significantly shown the ability to correct the variation of these lipid peroxidation end products in these sex organs of arsenic intoxicated rats (Table 2.2).

5.2.1.3.4. Effect of Arjunolic acid and vitamin B_{12} on Antioxidants Status:

In comparison with vehicle-treated control group the activity of these above enzymes were reduced in arsenic-treated rats through as evident from the colorimetric study (Fig. 2.2) and electrozymographic imaging as shown in Fig. 2.3. The arjunolic acid and vitamin B_{12} alone or conjointly in pretreatment modes have shown their ability to restore the activity of SOD, catalase, and glutathione peroxidase.

5.2.1.3.5. Serum LDH status:

The necrotic nature of the tissue appeared as electrozymographic impression showed a comparatively higher expression of serum LDH in arsenicated animals in contrast to control

animals (Fig. 2.3). Higher expression of LDH band was abolished in was in arsenic ingested group when they were pretreated with arjunolic acid and B_{12} alone or in combination (Fig. 2.3) and thereby prevents the cytotoxic nature of arsenic.

5.2.1.3.6. Status of measured vitamins and homocysteine:

The level of serum homocysteine was elevated and the circulating level of serum B_{12} and folic acid was reduced in arsenic intoxicated rats in comparison to the vehicle-treated control group (Fig. 2.4). Pretreatment of arjunolic acid and B_{12} alone or in combination significantly reversed the effects of arsenic on the level of vitamin B_{12}, folic acid, and homocysteine (Fig. 2.4).

5.2.1.3.7. Observation of Ovarian Steroidogenesis:

In contrast with the vehicle-treated control group the activities of the ovarian Δ^5, 3β-HSD and 17β-HSD were hampered remarkably in As^{3+} treated group (Fig. 2.5). The activities of these steroidogenic key enzymes were restored when pretreated with arjunolic acid and vitamin B_{12} alone or both towards the vehicle-treated control.

5.2.1.3.8. Ovarian and Uterine histopathology:

Inducing follicular atresia and deterioration of follicles arsenic could also reduce the number of graafian follicles when compared with the vehicle-treated control group (Fig. 2.6A). Ovaries of arsenic intoxicated group showed diminished numbers of primary classes of preantral and antral follicles. Pretreatment with arjunolic acid and B_{12} prevented the above effects of arsenic on ovarian histomorphology by showing normal numbers of regressive follicles and matured follicles (Fig. 2.6A).

In arsenic-treated rats the secretory glands of the uterus were diminished and degenerations of uterine layers such as perimetrium, myometrium, and endometrium were prominent by the appearance of thinning of these layers. These changes of uterine histomorphology by arsenic

were successfully prevented by arjunolic acid and B_{12} alone or in combination in pretreated mode (Fig. 2.6B).

Table-2.1

	Body weight(g)		Organo-somatic indices(g)		Water intake (ml/100gm body weight)
	Initial	Final	Ovary in pair	Uterus	
Control	125.83±2.44	131.71±4.32	0.063±0.007	0.146±0.014	6.38±0.30
As^{3+}	130.16±2.77	139.00±1.73	0.049±0.004*	0.114±0.013**	7.40±0.38
AA	127.83±2.88	136.83±1.79	0.089±0.005	0.182±0.019	6.41±0.11
As^{3+}+AA	124.85±4.58	133.71±2.80	0.074±0.01†	0.149±0.023††	5.58±0.29
B_{12}	128.57±4.81	135.71±3.26	0.082±0.009	0.203±0.022	5.83±0.93
As^{3+}+B_{12}	127.85±2.69	133.14±3.00	0.069±0.011	0.195±0.029†††	5.53±0.35
As^{3+}+AA +B_{12}	130.00±3.07	137.66±2.73	0.052±0.004	0.126±0.03†	4.98±0.67

Table 2.1: The data are mean ± SE, n=6, ANOVA followed by the Post Hoc Dunnett test were used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†$p < 0.05$, **/††$p < 0.01$, †††$p < 0.001$.

Figure-2.1

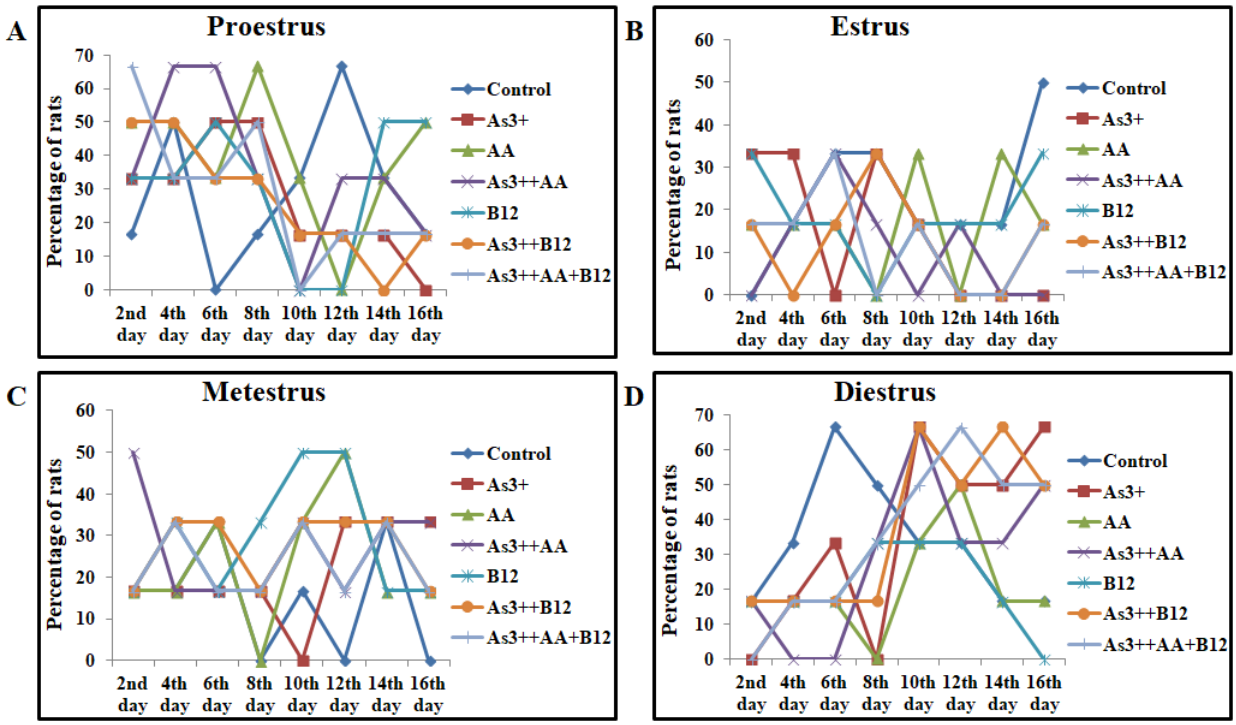


Fig. 2.1: Pattern of the estrous cycle. The regular pattern of the estrous cycle was maintained by the treatment with arjunolic acid and B_{12} alone or in combination in preventive treatment and the regular pattern was abolished to the irregular pattern in arsenic-treated rats.

Table-2.2

	Control	As^{3+}	AA	As^{3+}+AA	B_{12}	As^{3+}+B_{12}	As^{3+}+AA+B_{12}
MDA (nmole/gm)	29.69±0.25	53.97±1.17***	32.00±0.76	41.43±1.86†	32.13±2.50	34.88±2.89†	27.32±2.56††
CD (nmole/gm)	8.14±0.30	28.05±0.69***	13.00±0.29	14.43±0.33†††	13.83±0.49	15.37±0.24†††	13.99±0.248†††

Table 2.2: The data show mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †$p < 0.05$, ††$p < 0.01$, ***/†††$p < 0.001$.

Figure-2.2

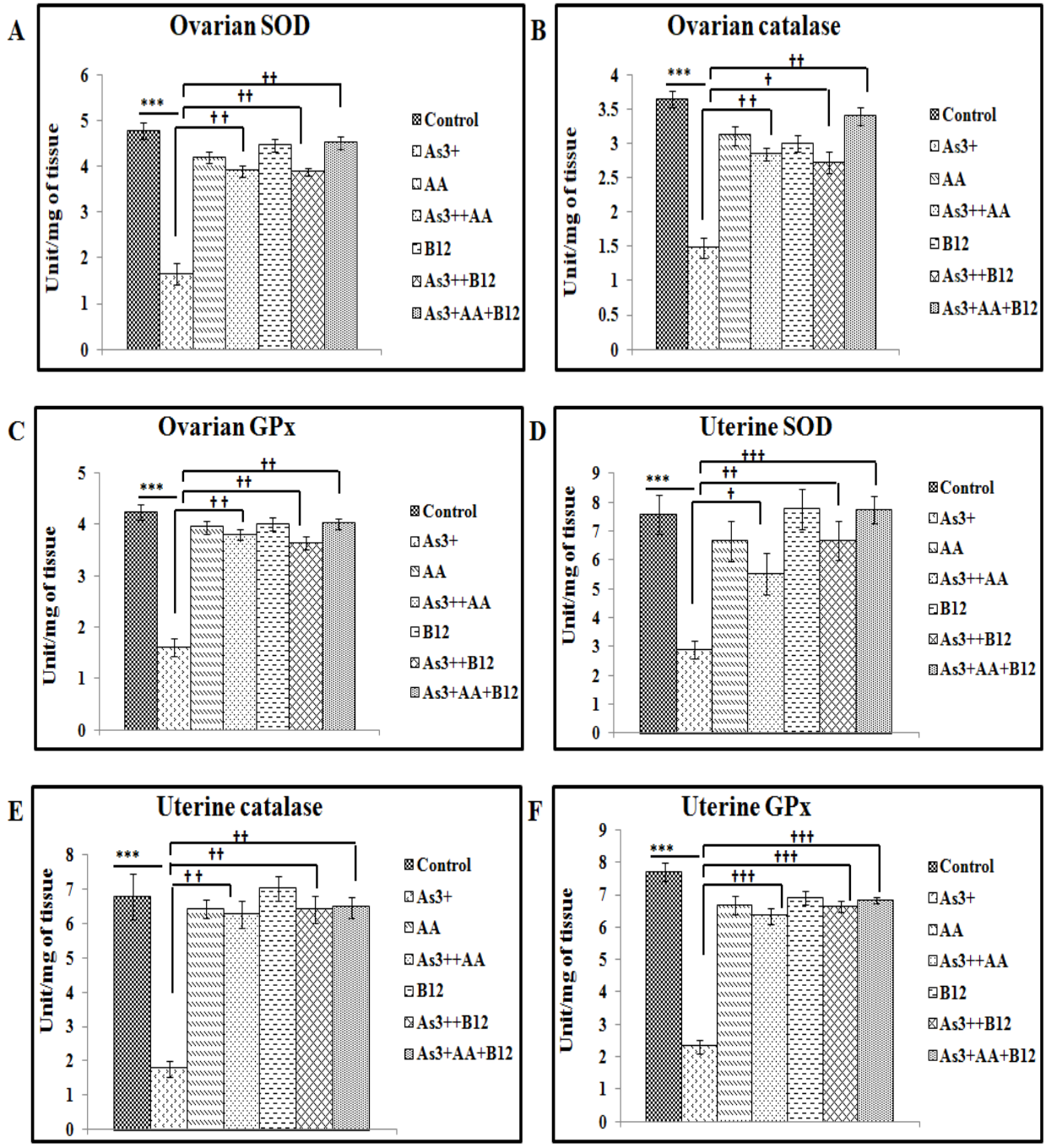


Fig. 2.2: The data show mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test was used. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †$p < 0.05$, ††$p < 0.01$, ***/†††$p < 0.001$.

Figure-2.3

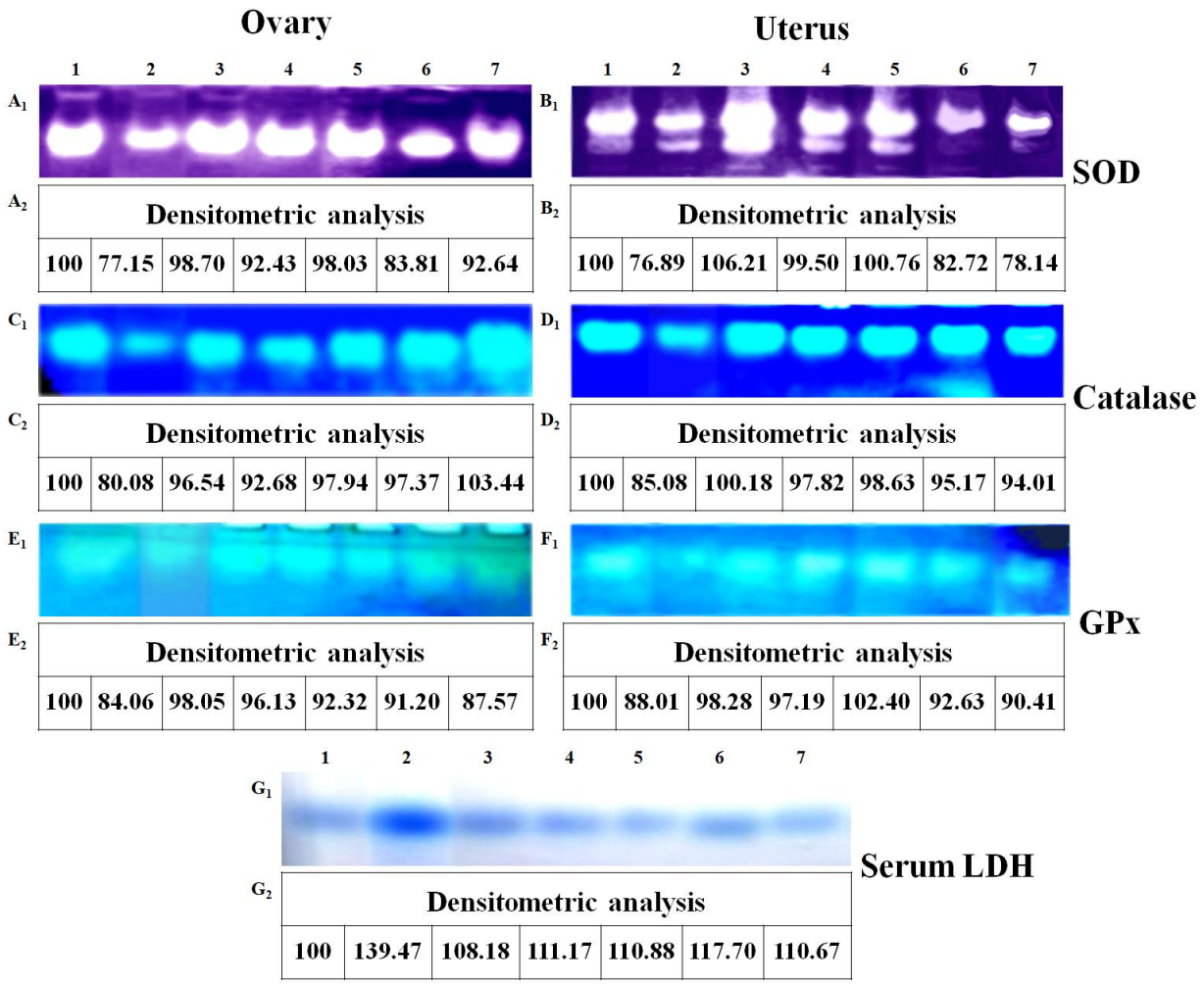


Fig. 2.3: (A, B, C, D, E, F, and G). The same protein was electrophoresed in each lane of 12% and 8% native gel and shown the ovarian, uterine SOD, catalase, and GPx activity. Serum LDH activity was executed to the extent of cellular damage. Lane distribution lane 1: control; lane 2: As^{3+}; lane 3: arjunolic acid; lane 4: As^{3+}+ arjunolic acid; lane 5: B_{12}; lane 6: As^{3+}+ B_{12}; lane 7: As^{3+}+ arjunolic acid+ B_{12}. By the densitometric analysis, the electrozymographic bands were shown numerically.

Figure-2.4

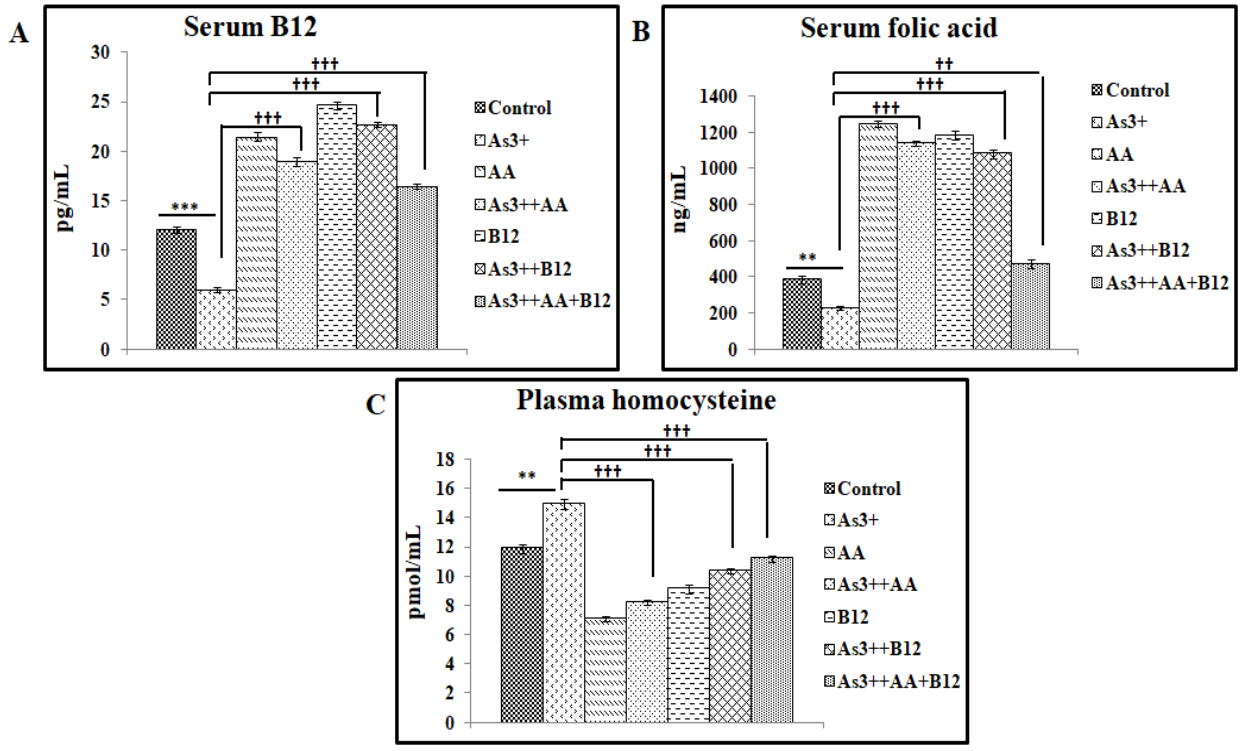


Fig. 2.4: In the As^{III} ingested group the level of homocysteine was elevated and the level of these B vitamins was diminished. The data show mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. **/††$p < 0.01$, ***/†††$p < 0.001$.

Figure-2.5

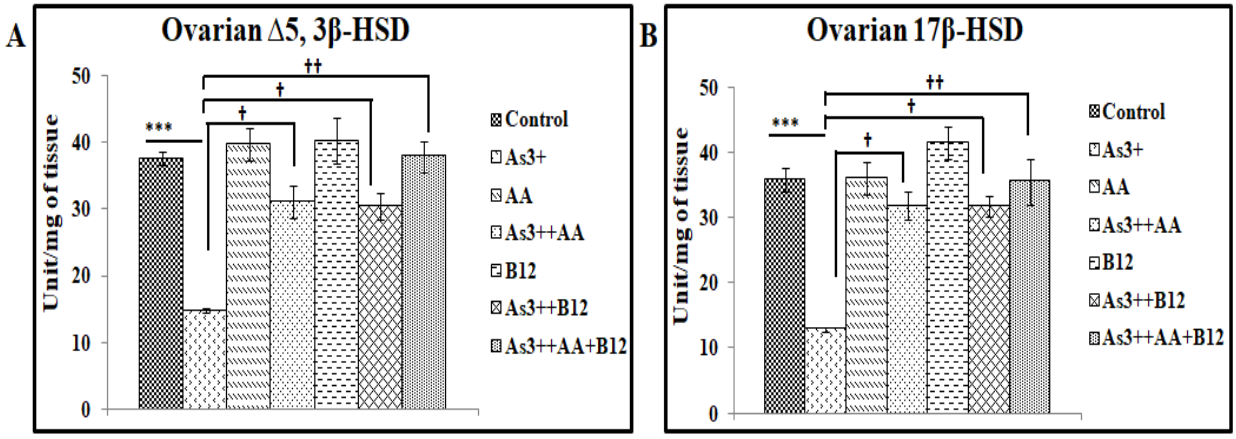
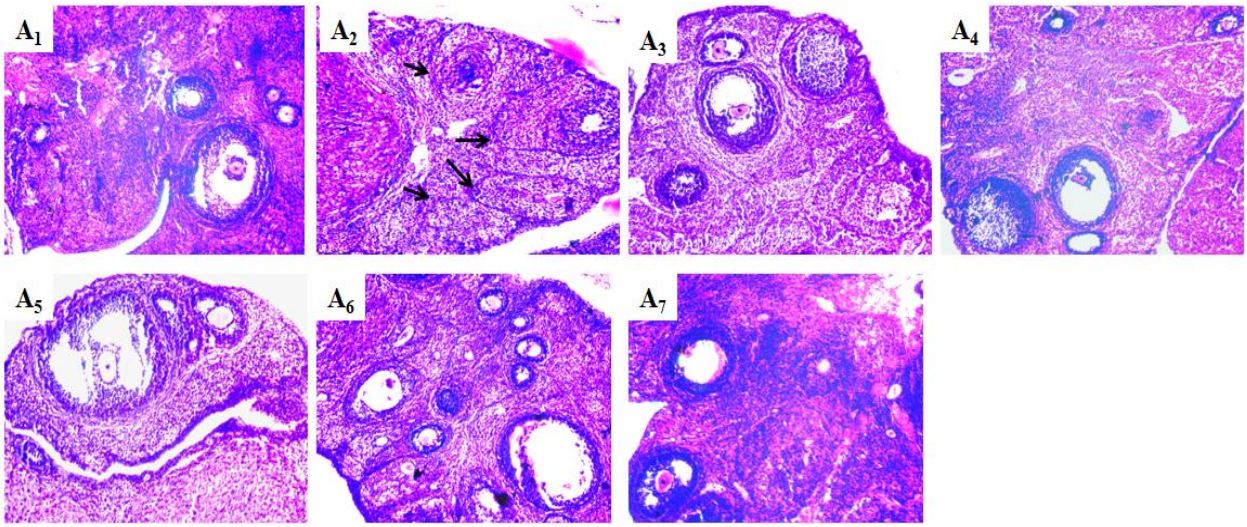


Fig. 2.5: The level of ovarian steroidogenic enzymes was reduced in As^{III} intoxicated group. Arjunolic acid and B_{12} restore the level of these enzymes. The data show mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †$p < 0.05$, ††$p < 0.01$, ***$p < 0.001$.

Figure-2.6



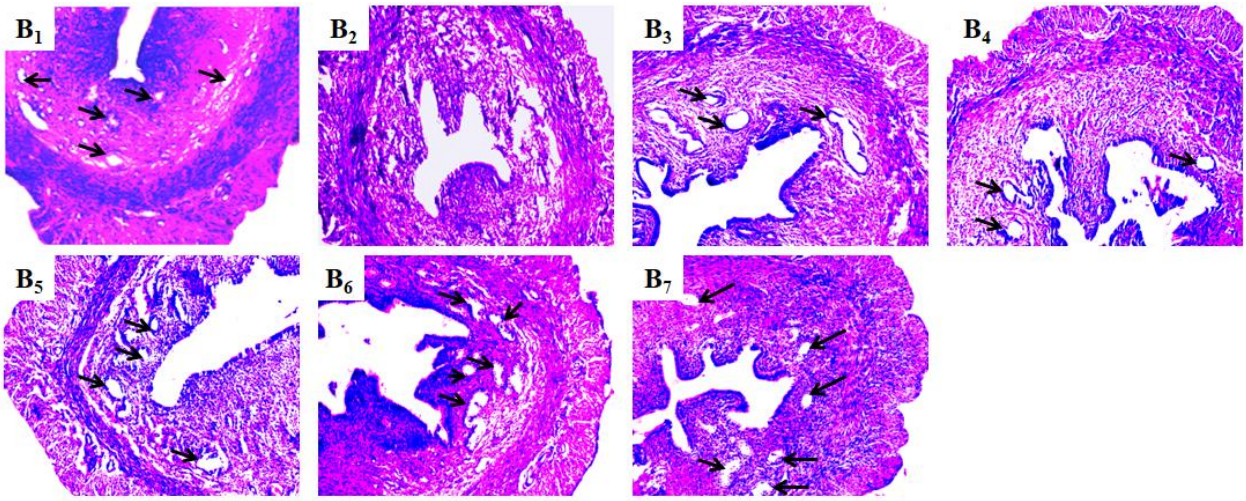


Fig. 2.6: (A and B) In histoarchitechture, the tissue of ovary and uterus were sectioned at 5 µM thickness. After staining with eosin and hematoxylin (Harris) the section was watched under microscope. (A) In ovarian histoarchitechture in arsenic ingested rats the follicular atresia was seen and alone or jointly by the treatment with arjunolic acid and B_{12} the follicles were reappeared. Panel distribution; A_1: control, A_2: As^{3+}, A_3: arjunolic acid, A_4: As^{3+}+ arjunolic acid, A_5: B_{12}, A_6: As^{3+}+ B_{12}, A_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate atresia.

(B) The secretory glands were lost in arsenic-treated group and the preventive effect was seen in arjunolic acid, B_{12}, and the combination group. Panel distribution; B_1: control, B_2: As^{3+}, B_3: arjunolic acid, B_4: As^{3+}+ arjunolic acid, B_5: B_{12}, B_6: As^{3+}+ B_{12}, B_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate secretory glands.

5.2.1.4. DISCUSSION:

Arsenic at the dose of 1.0 mg/100gm bodyweight could damage the ovarian and uterine tissue. Tissue death by apoptosis, necrosis, cancer, and other abnormalities are evident as a consequence of As^{III} intoxication and its organic derivatives (Calatayud et al., 2013). In arsenicated group, the low level of uterine superoxide dismutase and catalase are the outcomes of a high level of uterine MDA and CD (Table 2.2). The tri-valent form of arsenic when associates with H_2O_2 harvests reactive oxygen species (ROS) and generates several lipid peroxides and conjugated diene as the end products (Wang et al., 2006).

In the electrophoretic study, the intensity of enzymatic bands in arsenic-treated group was decreased compared to vehicle-treated control group in ovarian and uterine tissue (Fig. 2.3). The redox modification of cysteines was connected with the inactivation of SOD, which was revealed at the time of inflammatory responses throughout acute asthmatic condition (Ghosh et al., 2013). $Arsenic^{III}$ also decreases the mRNA expression of the SOD gene, which increases oxidative stress and decreases the nullification of superoxide anion radicals ($O2^{\cdot-}$) (Rana et al., 2012). Then H_2O_2 is produced from the conversion of the superoxide anion. During electrophoretic study, we showed that catalase activity was reduced. The detoxification of H_2O_2 from ovarian and uterine tissue was retarded due to decreased activity of catalase. In arsenic-treated group faint band of GPx (Fig. 2.3E and 2.3F) was seen due to accumulation of H_2O_2 in ovarian and uterine tissue during the programmed cell death (Do et al., 2003). The decreased level of these enzymes was linked with the increased level of free radicals during arsenic metabolism.

Serum lactate dehydrogenase (LDH) is a necrotic marker. The serum LDH level was higher in necrotic cells than normal cells (Zhang et al., 2015). In the present study, the serum LDH level was increased in arsenic exposed group, which was shown as a prominent band of

electrozymogram (Fig. 2.3G). During elevation of the serum LDH, the apoptotic tissue lesions were found in uterine histological tissue sections. Stimulation of collagen secretion might be correlated with an increased level of serum LDH as this plays an important role in fibrotic changes in the uterus (Iglesias et al., 1988). Cell transformation and apoptosis were also found in arsenic-exposed group due to the high level of ROS generation (Zhang et al., 2015).

During ovarian Steroidogenesis, Δ^5, 3β-HSD and 17β-HSD are the major enzymes. Data showed that the activities of these ovarian steroidogenic enzymes were diminished in arsenicated rats (Fig. 2.5) and finally decreased the estradiol signaling (Hinshelwood et al., 1994). Circulating levels of LH and FSH are directly proportional towards the regulation of Δ^5, 3β HSD and 17β HSD activities (Odell et al., 1963; Ghersevich et al., 1994). Here, the low level of plasma gonadotrophin decreased along with the suppressed activities of ovarian 3β-HSD and 17β-HSD in response to arsenic.

Our previous study showed that a lower dose of arsenic (0.4 ppm) accumulated in uterine tissues (Chattopadhyay and Ghosh, 2010). But the lower dose of arsenic given for 28 days enumerated the toxic effects of arsenic in different body parts (Chattopadhyay et al., 2012; Acharyya et al., 2015). In our experiment, we used a comparatively higher dose of arsenic (1.0 mg/100 gm body weight) with the lower dose of arjunolic acid (1.0 mg/100 gm body weight) (Hemalatha et al., 2010; Vasanthi and Parameswari, 2012) and slightly higher dose of B_{12} (0.09μg/100 gm body weight) (Mukherjee et al., 2006; Acharyya et al., 2015) to search out the preventive effects of these bio-molecules on female reproductive organs. The regular pattern of the estrous cycle was abolished towards the irregular pattern in arsenic-treated rats (Parshad et al., 1990). Following 3 to 4 days of the pretreatment with arjunolic acid and B_{12} alone or in combination established a

synchronized estrous cycle pattern by replacing consistent metestrous or diestrous stage in arsenic-treated rats (Fig. 2.1).

The results of this experiment showed that the levels of vitamin B_{12} and folic acid were reduced and homocysteine was elevated in arsenic treated group (Fig. 2.4). There was an inverse relationship between the B_{12}-folate and serum LDH level (Hoffbrand et al., 1966; Keskin and Keskin, 2015). So, these two vitamins are important to prevent the necrosis of reproductive organs. The decreased level of vitamin B_{12} and folic acid was associated with the reduced level of arsenic detoxification through the delay in biliary arsenic excretion (Kile and Ronnenberg, 2008; Hall et al., 2009). The biliary emission of arsenic might be promoted by B_{12} and folic acid in the methylated form (As^{III}). Polycystic ovarian syndrome (Maleedhu et al., 2014) and ovarian carcinoma (Corona et al., 1997) occur due to the assembly of homocysteine in the ovary. In polycystic ovarian syndrome, the increased homocysteine level was decreased by B_{12} and folic acid (Kilicdag et al., 2005). In this experiment, restoration of the circulating level of B_{12} and folic acid (Jayarajah, 2005) might be promoted by arjunolic acid and vitamin $B_{12,}$ which in turn suppressed homocysteine (Fig. 2.4). Homocysteine lowering ability of B vitamins is already a proven fact (Jeremy et al., 2007).

The irregularities of ovarian functions may be explained in two ways. The numbers of atretic follicles are elevated and the numbers of healthy follicles are reduced due to the low levels of plasma gonadotrophins and estradiol (Gore-Langton and Daniel, 1990) in arsenicated rats. Arsenic generates oxidative stress in the ovary (Sun, 1990) which is substantiated by the diminished level of SOD, catalase, GPx, and the enhanced level of MDA and CD. Due to the consumption of As^{III} ovarian follicular deterioration was found in arsenicated group (Fig. 2.6A) (Chattopadhyay et al., 2003). Oxidative stress occurs in primordial and preovulatory follicles due

to the damage of antioxidant enzymatic defenses (Tarin, 1996). The number of antral follicles and ovarian granulosa cells was reduced due to the elevated level of homocysteine (Singh et al., 2009). A higher-grade embryo production was also diminished due to the increased level of plasma homocysteine (Ocal et al., 2012).

Estradiol manages the growth and proliferation of uterine layers (Patil et al., 1998). In arsenic intoxicated rats (Fig. 2.6B) cellular injuries occurred in uterine layers which were accompanied with the loss of secretory cells of uterine tissue with the alteration of the endometrial layer. The injury may be associated with the loss of secretory cells of uterine tissue along with the alteration of the endometrial layer. Deterioration of uterine histomorphology (Fig. 2.6B) due to arsenic-induced oxidative stress (Beltran-Garcia et al., 2000) as established from the low level of uterine SOD, catalase, GPx, and high level of uterine MDA and CD (Table 2.2). In arsenicated uterine tube cell degeneration, thinning of the muscular layer and reduced endometrial glands were found. Oxidative stress was produced by the arsenic in uterine tissue by the endometrial ROS generation (Chatterjee and Chatterji, 2010). The high level of ROS production is associated with the low level of estrogen that initiates endometrial cycle disruption in arsenic-treated group (Akram et al., 2010).

The pentavalent form of arsenic is converted into the trivalent form of arsenic during inorganic arsenic metabolism. Methyl group is included with the As^{III} by this inorganic arsenic metabolism in the biomethylation process (Cullen et al., 1984). By modulating the methylation process; arsenic is detoxified from the body via methionine cycle where methyltransferase enzyme and S-adenosylmethionine (SAM) using one-carbon (1C) metabolism have an important role. Vitamin B_{12} and folic acid are utilized by the methionine cycle for maintaining the endogenous methionine level where vitamin B_{12} acts as a co-factor to synthesize the methionine enzyme and

catalyze the endogenous methionine synthesis from the S-adenosylhomocysteine (Sahin et al., 2003).

Arjunolic acid could prohibit the free radicals production in arsenicated group. The regular estrous cycle revival through the treatment with arjunolic acid in arsenic-treated rats indicated the restoration of estradiol signaling (Bennett, 2001). Arjunolic acid could decrease the female repro toxicity through altering the circulating level of vitamin B_{12}, folic acid, and homocysteine in arsenicated rats and could acts on the uterus and ovaries by sustaining the level of Δ5, 3β-HSD and 17β-HSD (Maity et al., 2018). Containing the two vicinal equatorial –OH groups arjunolic acid has the ability to bind with arsenic. Involving with As^{III} (with a lone pair of electrons of oxygen) the OH groups of arjunolic acid may develop a five-member chelate complex. Therefore arjunolic acid prevents oxidative damages. The chelate complex eradicates the free toxins through inhibiting oxidative damages. The free radical scavenging activity of arjunolic acid requires one carboxylic hydrogen atom of the chelate complex. In various cell signaling pathways; the arsenic-induced transformation could be altered by the arjunolic acid via chelation therapy (Manna et al., 2007).

Glutathione sparing effect of B_{12} can decrease the oxidative stress by altering the signaling molecules and stimulating the methionine synthase activity, which has a direct reaction with reactive oxygen and nitrogen species (Veber et al., 2008). Low SOD activity and insufficiency of vitamin B_{12} could accumulate the H_2O_2 in the uterus (Jia and Domenico, 2010). Initiation of Severe oxidative stress and demolition of cellular redox homeostasis is materialized by the deficiency of B_{12} (Bito et al., 2017). Vitamin B_{12} could improve the activity of SOD, catalase, GPx, and reduce the level of lipid peroxide end product in arsenicated rats (Bhattacharjee and Pal, 2014b). Averting the DNA fragmentation and histological alteration vitamin B_{12} could

prevent apoptotic change and cellular damage (Friso and Choi, 2002). In arsenic intoxicated rats B_{12} could improve the ovulation process through altering the development of the ovum (Bennett, 2001).

In conclusion, from the above results arjunolic acid and B_{12} alone showed the enhanced beneficial preventive effects in the restoration of body growth, organs weight, antioxidant status of ovary and uterus against arsenic-induced female reproductive hazards in Wistar rats. These biomolecules can improve the histoarchitechture of ovary and uterus. The deterioration of tissue through apoptosis and necrosis is prevented probably by the elimination of arsenic from organs and lowering of lipid peroxide end products and serum LDH. Via methylation process arsenic may be removed from the ovary and uterus where methionine plays an important role. The above study will be helpful for the development of a preventive treatment strategy in the arsenic affected people. Further study is necessary for this field.

5.3. EXPERIMENT-III

5.3.1. Protective effect of Arjunolic acid and B_{12} on female reproductive organs against arsenic toxicity in Wistar rats:

5.3.1.1. Aims and objectives:

This experiment was executed in female reproductive organs of Wister rats to search out the protective role of arjunolic acid and B_{12} on arsenic-induced hazards alone or in combination.

5.3.1.2. Animal Selection and Treatment:

Distributing six rats in each group the rats (130±10 g body weight) were allocated into seven groups. The experimental program was followed as:

Group I This group was a vehicle-treated control group,

Group II In this group the rats were treated with sodium arsenite (1.0 mg /100gm body weight),

Group III Arjunolic acid (1.0 mg/100gm body weight) (Sigma-Aldrich, St. Louis, MO),

Group IV Sodium arsenite (1.0 mg/100gm body weight) plus arjunolic acid (1.0 mg/100gm body weight) were given to this group,

Group V Vitamin B_{12} (0.09µg/100gm body weight),

Group VI The rats were co-treated with sodium arsenite (1.0 mg/100gm body weight) and vitamin B_{12} (0.09µg/100gm body weight),

Group VII Sodium arsenite (1.0 mg/100gm body weight), arjunolic acid (1.0 mg/100gm body weight), and vitamin B_{12} (0.09µg/100gm body weight) were co-administered in this group of rats in a combined manner.

While treating all groups the rats were treated for eight days through oral gavage in co-administration mode and observed the estrous cycle pattern. After preparing arsenic (1.0 mg /100gm body weight), arjunolic acid (1.0 mg /100gm body weight), and vitamin B_{12} (0.09 µg/100gm body weight) separately with distilled water, the rats of Group II were fed with

sodium arsenite and the arsenicated rats of Group IV, VI, VII were treated with arjunolic acid and vitamin B_{12} in co-administration mode. The rats of Group of III and V were fed only with arjunolic acid and vitamin B_{12}. Throughout the treatment rats' body weights and feeding habits were recorded. Rats were anesthetized by intramuscular injection of ketamine HCl at the dose of 24 mg/kg body weight for collecting blood, uterine horns, and ovaries on day nine. The samples were kept in a separate sterile bag with an insulated ice unit having -20^0C temperature. Finally, rats were euthanized by the intramuscular injection of an overdose of ketamine following the ethical guidelines of CPCSEA.

5.3.1.3. Results:

5.3.1.3.1. General Observations:

Animals were provided with a standard rat chow as mentioned in experiment I. The animals of arsenic fed group had higher water intakes than that of other mentioned experimental groups. In terms of the percentage of body mass uterine-somatic index was calculated. In arsenicated group (Table 3.1) a significant wet weight loss of ovaries and uterus was found. Alone or co-jointly arjunolic acid and B_{12} co-administration meaningfully re-established the weight loss of these reproductive organs towards control value.

5.3.1.3.2. Vaginal smear study:

The pattern of estrous cycles was watched for 8 days frequently. After four days of treatment with arsenic continuous metestrous and diestrous stages were noted in comparison to vehicle-treated control group and after the treatment with arjunolic acid and vitamin B_{12} alone or jointly in arsenic-treated rat's synchronization of estrous cycle was clearly shown in Fig. 3.1.

5.3.1.3.3. Observation of Lipid Peroxidation End Products:

In arsenic intoxicated rats the level of uterine MDA and CD was significantly raised in contrast to the control group (Table 3.2). Nevertheless, arjunolic acid and B_{12} co-treatment alone or in combination had established its ability to correct this alteration of these lipid peroxidation end products in these sex organs of arsenic ingested rats.

5.3.1.3.4. Effect of Arjunolic acid and B_{12} on Antioxidant Status:

Spectrophotometric analysis of ovarian and uterine SOD, catalase, and GPx revealed a significant diminished activity of these enzymes in arsenic exposed rats (Fig. 3.2). Electrozymographic impression also focused on a significant deterioration of the expression of these ovarian and uterine enzymatic proteins [Fig. 3.3 (A-F)]. The reduction of these enzymatic band densities was shown as weak band strength through electrozymographic study in arsenic intoxicated group. In arsenicated group the densities of these enzymatic bands of ovary were decreased to 11%, 20% 22% respectively, and uterus were decreased to 34%, 42%, and 58% respectively from the level of control. The activities of these enzymes were restored by the treatment with arjunolic acid and B_{12} alone or jointly.

5.3.1.3.5. Serum LDH status:

The possibility of necrotic degeneration of the tissue was prominent from the electrozymographic study of serum LDH (Fig. 3.3G) where arsenic exposed group intact and a distinct band was seen with higher intensity (Fig. 3.3G) in contrast to the control group. Co-administration with arjunolic acid and vitamin B_{12} alone or in combination decreased the release of this enzyme as visible by the appearance of a weak band.

5.3.1.3.6. DNA fragmentation and comet assay:

In arsenic intoxicated group, uterine tissue exhibited a greater degree of DNA degradation in comparison to the control group (Fig. 3.4A_1). Through densitometric analysis (Fig. 3.4A_2) it was

clearly found that a greater migration of DNA on an agarose gels in arsenic-treated group along with the less intense band. The damaging of DNA was diminished in arsenicated rats by the treatment with arjunolic acid and vitamin B_{12} alone or jointly.

In arsenic ingested rats from a distinct number of cells the extruded nature of the broken uterine DNA was shown comet formation (Fig. 3.4B). In arsenic-treated group the damage of the cellular DNA was reversed when the rats were co-treated with arjunolic acid and B_{12} alone or in combination and that was documented from the comparative less number of comets found in these groups.

5.3.1.3.7. Observation of Ovarian steroidogenesis:

In sodium arsenite treated rats a remarkable reduction in the activities of ovarian Δ^5, 3β-HSD, 17β-HSD, and LH, FSH, estradiol were detected when compared to the vehicle-treated control group (Fig. 3.5). For maintaining the activities of ovarian steroidogenic key enzymes and hormones alone or jointly arjunolic acid and vitamin B_{12} played an important role towards a normal level.

5.3.1.3.8. Status of measured vitamins and homocysteine:

As^{III} exhibited different effects on the circulating level of vitamin B_{12}, folic acid, vitamin C, and homocysteine (Fig. 3.6). There was a significant increased level of vitamin C and homocysteine and a significant decreased level of vitamin B_{12} and folic acid in arsenicated rats. Co-treatment with arjunolic acid and B_{12} alone or jointly restored the significant level of vitamin B_{12}, folic acid, and homocysteine without showing any significant change of serum vitamin C level (Fig. 3.6).

5.3.1.3.9. Serum SGPT and SGOT level:

SGPT and SGOT were the biomarkers of liver health. In As^{III} ingested group there was a significant elevation of the SGPT and SGOT level (Fig. 3.7). Arjunolic acid and vitamin B_{12} were shown its ability to diminish the levels of these serum enzymes significantly towards the control level.

5.3.1.3.10. Ovarian and Uterine histopathology:

Ovarian histomorphology revealed that the numbers of primary classes of preantral and antral follicles were diminished in response to the treatment of sodium arsenite. A higher follicular regression was prominent by the appearance of there a decreasing number of graafian follicles and elevating numbers of follicular atresia in arsenicated group in comparison to the control group (Fig. 3.8A). Co-treatment with arjunolic acid and or vitamin B_{12} successfully increased the numbers of matured follicles with diminishing numbers of atretic follicles (Fig. 3.8A).

There was a reducing number of uterine secretory glands and the layers of the uterus i.e. perimetrium, myometrium, and endometrium were thinned in arsenic-treated rats. Rats consumed arsenic in association with arjunolic acid and B_{12} alone or jointly (Fig. 3.8B) reestablished the adequate numbers of secretory glands along with the restoration of the breadth of the uterine layers towards normalcy.

Table-3.1

	Body Weight (gm)		Organo-somatic indices (gm)		Water intake (ml/100gm body weight)
	Final	Initial	Ovary in pair	Uterus	
Control	134.0±4.33	121.0±6.06	0.063±0.002	0.160±0.01	10.62 ± 0.6
As^{3+}	137.0±7.94	123.0±8.43	0.041±0.003**	0.118±0.02*	12.9± 0.8
AA	132.4±6.36	130.2±5.88	0.053±0.003	0.145±0.01	11.15±0.8
As^{3+} + AA	131.0±5.89	128.0±7.01	0.050±0.002†	0.140±0.01†	10.92 ± 0.9
B_{12}	130.2±2.02	127.6±1.82	0.052±0.004	0.147±0.011	12.15±0.7
As^{3+}+B_{12}	131.8±1.79	129.4±3.60	0.050±0.002†	0.144±0.013†	11.55±0.9
As^{3+}AA+B_{12}	133.4±2.34	127.2±5.73	0.051±0.002†	0.152±0.011†	11.94±0.3

Table 3.1: In response to sodium arsenite treatment the alteration of body growth, organo-somatic indices, and water intake, and the protection with arjunolic acid and B_{12} alone or in combination were shown. The data show mean ± SE, n=6, ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†$p < 0.05$; **$p < 0.01$.

Figure-3.1

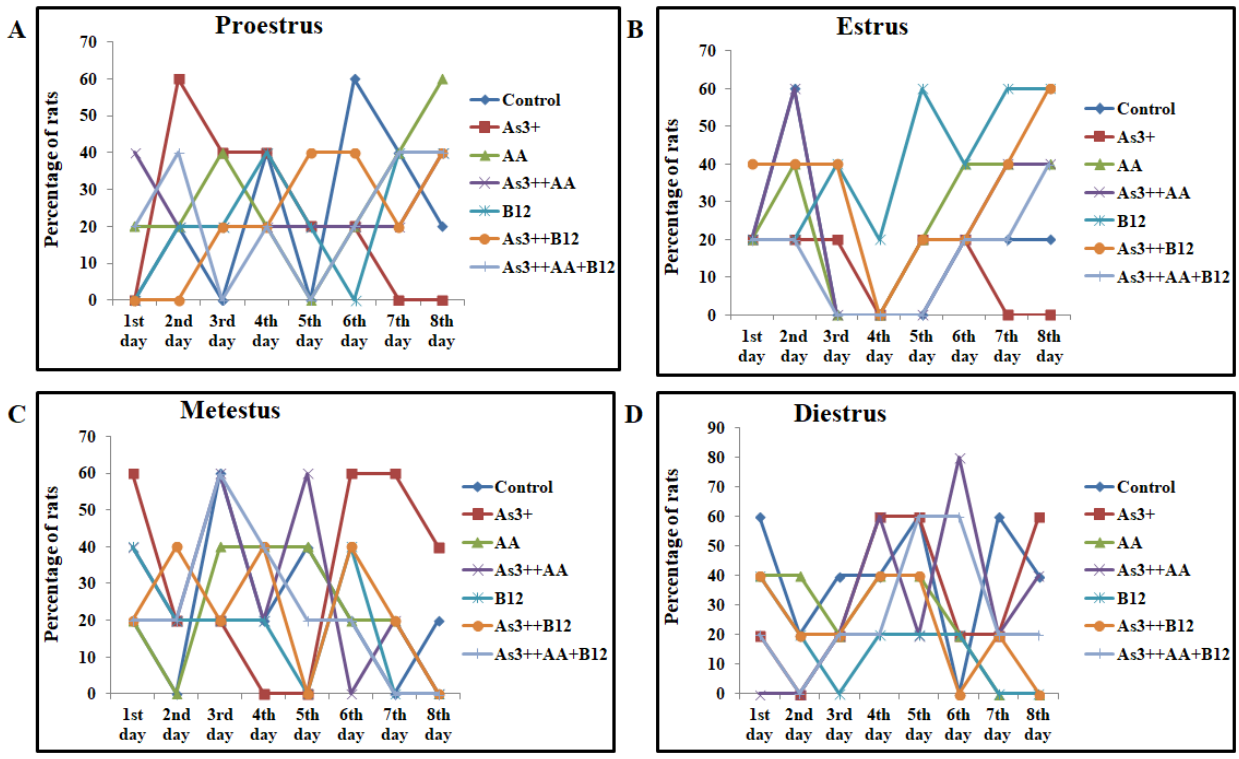


Fig. 3.1: Pattern of the estrous cycle. The irregular pattern was further synchronized to a regular pattern when arjunolic acid and B_{12} alone or jointly supplemented in arsenic-treated rats.

Table-3.2

	Con	As^{3+}	AA	As^{3+}+AA	B_{12}	As^{3+}+B_{12}	As^{3+}+AA+B_{12}
MDA (nmole/gm)	26.22±11.72	32.24±14.42*	30.47±13.62	29.27±13.08	30.80±13.77	29.97±13.4	28.66±12.81
CD (nmole/gm)	17.74±7.93	20.89±8.73*	18.46±8.25	18.17±8.12	18.4±8.22	17.35±7.75	16.86±7.54

Table 3.2: The uterine levels of MDA and CD were protected by the treatment with arjunolic acid and B_{12} alone or in combination. The data represent mean ±SE, n=6. ANOVA followed by the Post Hoc Dunnett test. *$p < 0.05$.

Figure-3.2

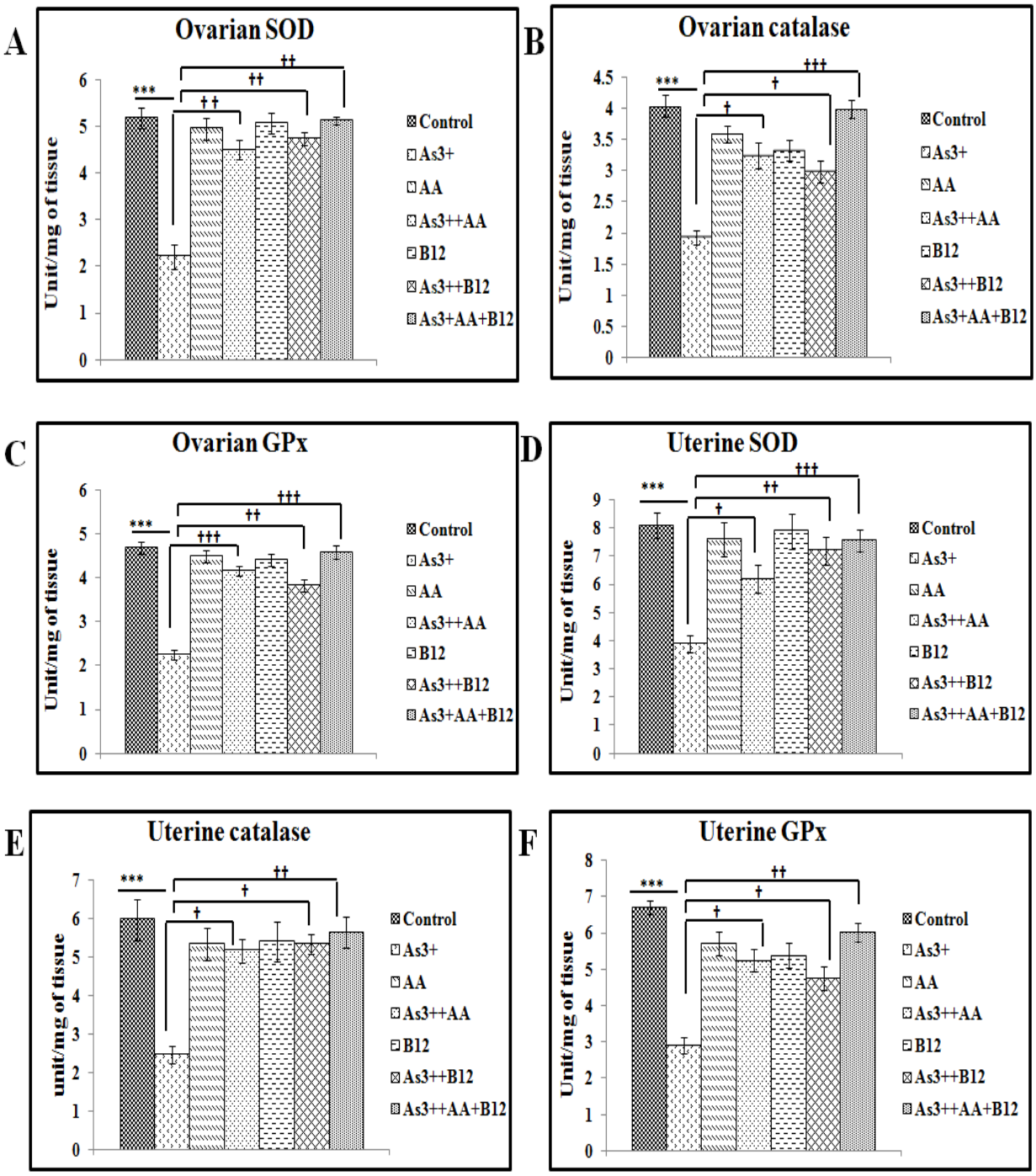


Fig. 3.2: The activity of these enzymes were reduced in arsenic fed rats but restored by the co-treatment with arjunolic acid and vitamin B_{12}. The data show mean ± SE, n=6, ANOVA followed

by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †p<0.05, ††p<0.01, ***/†††p<0.001.

Figure-3.3

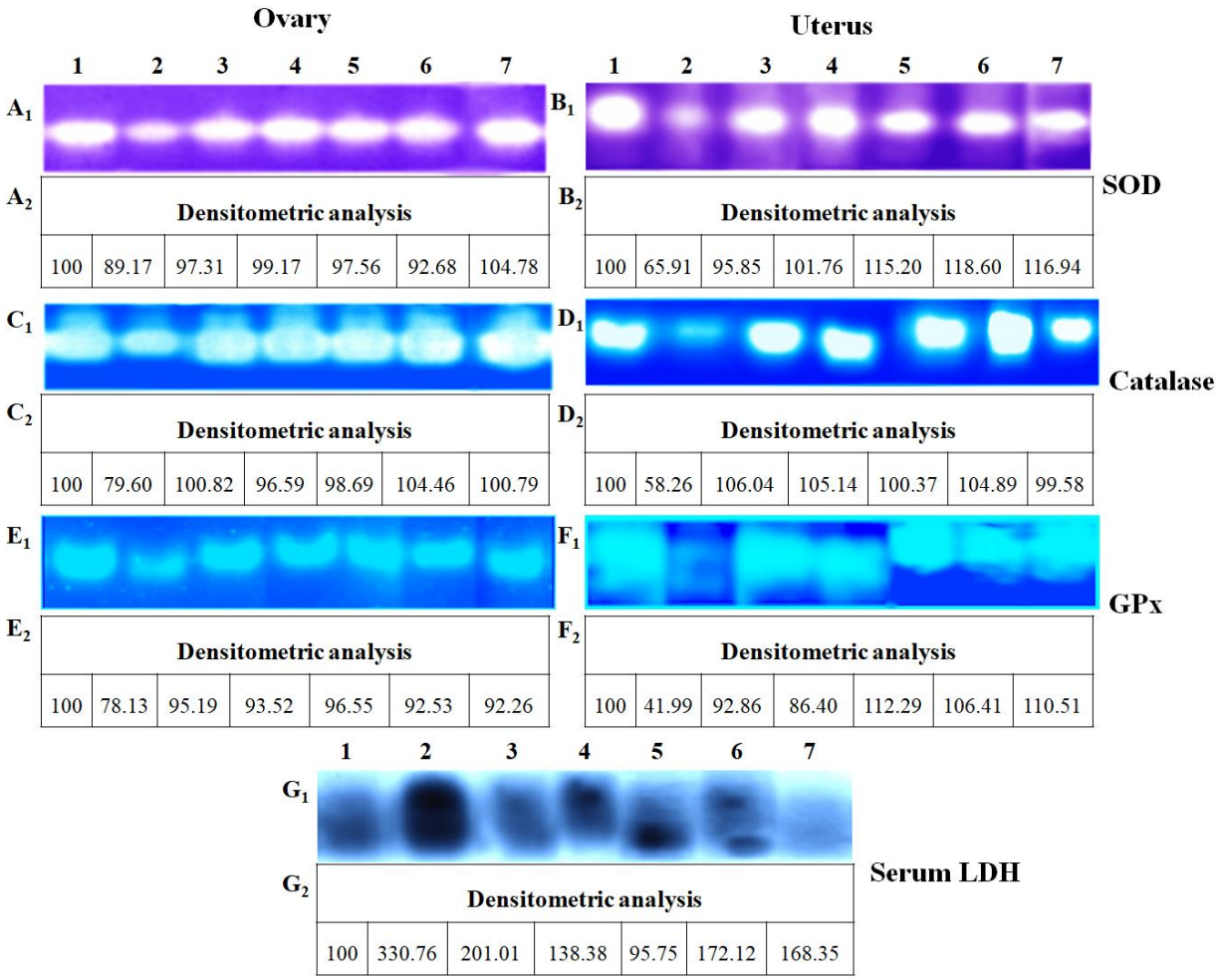


Fig. 3.3: (A-G). On 12% and 8% native gel the activity of ovarian and uterine SOD, catalase, and GPx were shown. The same amount of protein was electrophoresed in each lane. To the extent of cellular damage serum LDH activity was accomplished. Lane distribution; lane 1: control, lane 2: As^{3+}, lane 3: arjunolic acid, lane 4: As^{3+}+ arjunolic acid, lane 5: B_{12}, lane 6: As^{3+}+ B_{12}, lane 7: As^{3+}+ arjunolic acid+ B_{12}. The electrozymographic bands were shown numerically by the densitometric analysis.

Figure-3.4

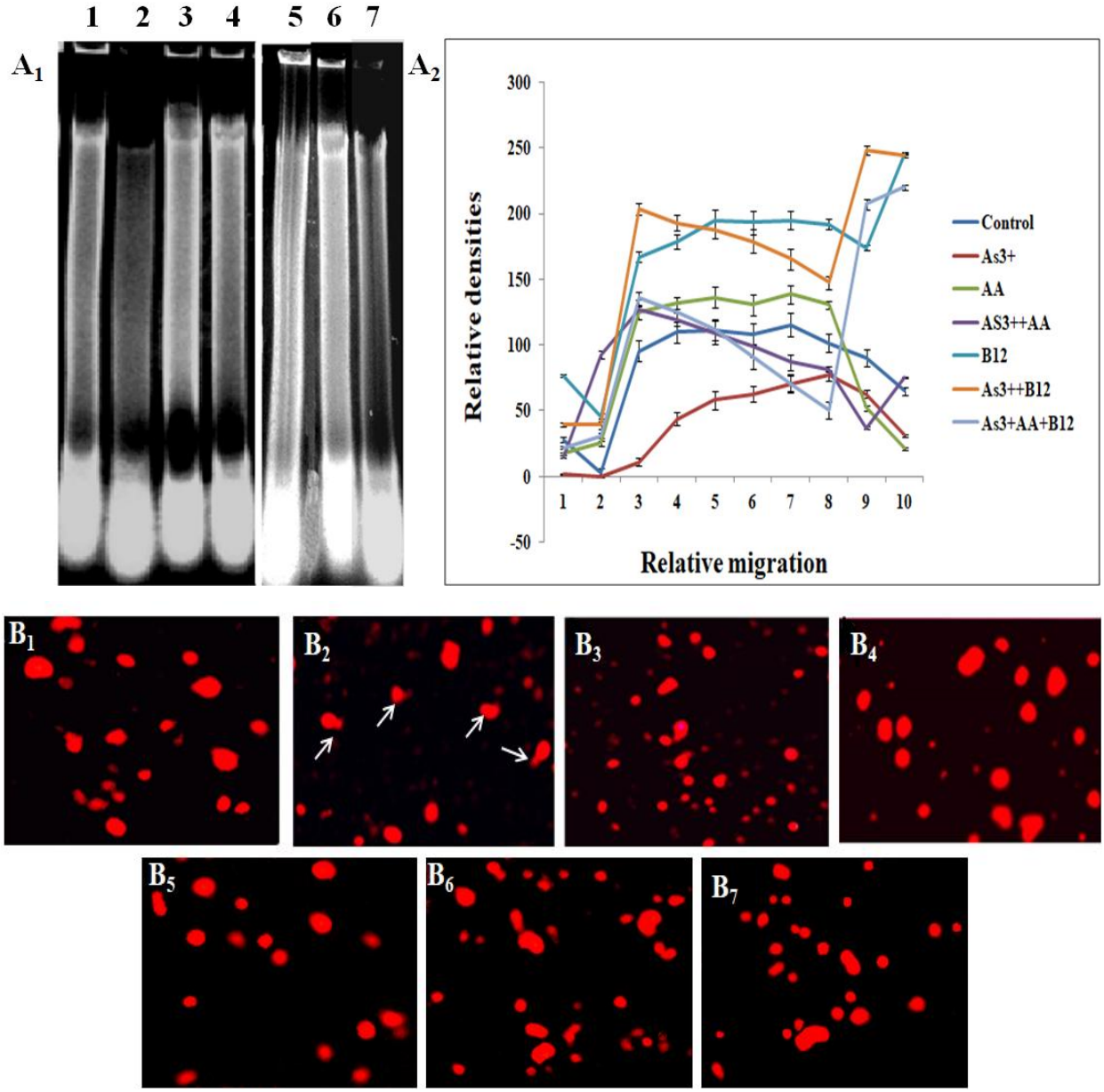


Fig. 3.4: (A_1) After electrophoresis the ethidium bromide stained agarose gel showing the protection of arjunolic acid and B_{12} alone or jointly against arsenic-induced uterine DNA degradation. Lane distribution; lane 1: control, lane 2: As^{3+}, lane 3: arjunolic acid, lane 4: As^{3+}+ arjunolic acid, lane 5: B_{12}, lane 6: As^{3+}+ B_{12}, lane 7: As^{3+}+ arjunolic acid+ B_{12}. (A_2) In

densitometric analysis arsenic-induced uterine DNA degradation was protected by jointly or alone arjunolic acid and B_{12}.

(B) Comet assay shows the protective role of arjunolic acid and B_{12} alone or in combination. Panel distribution; B_1: control, B_2: As^{3+}, B_3: arjunolic acid, B_4: As^{3+}+ arjunolic acid, B_5: B_{12}, B_6: As^{3+}+ B_{12}, B_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate comet.

Figure-3.5

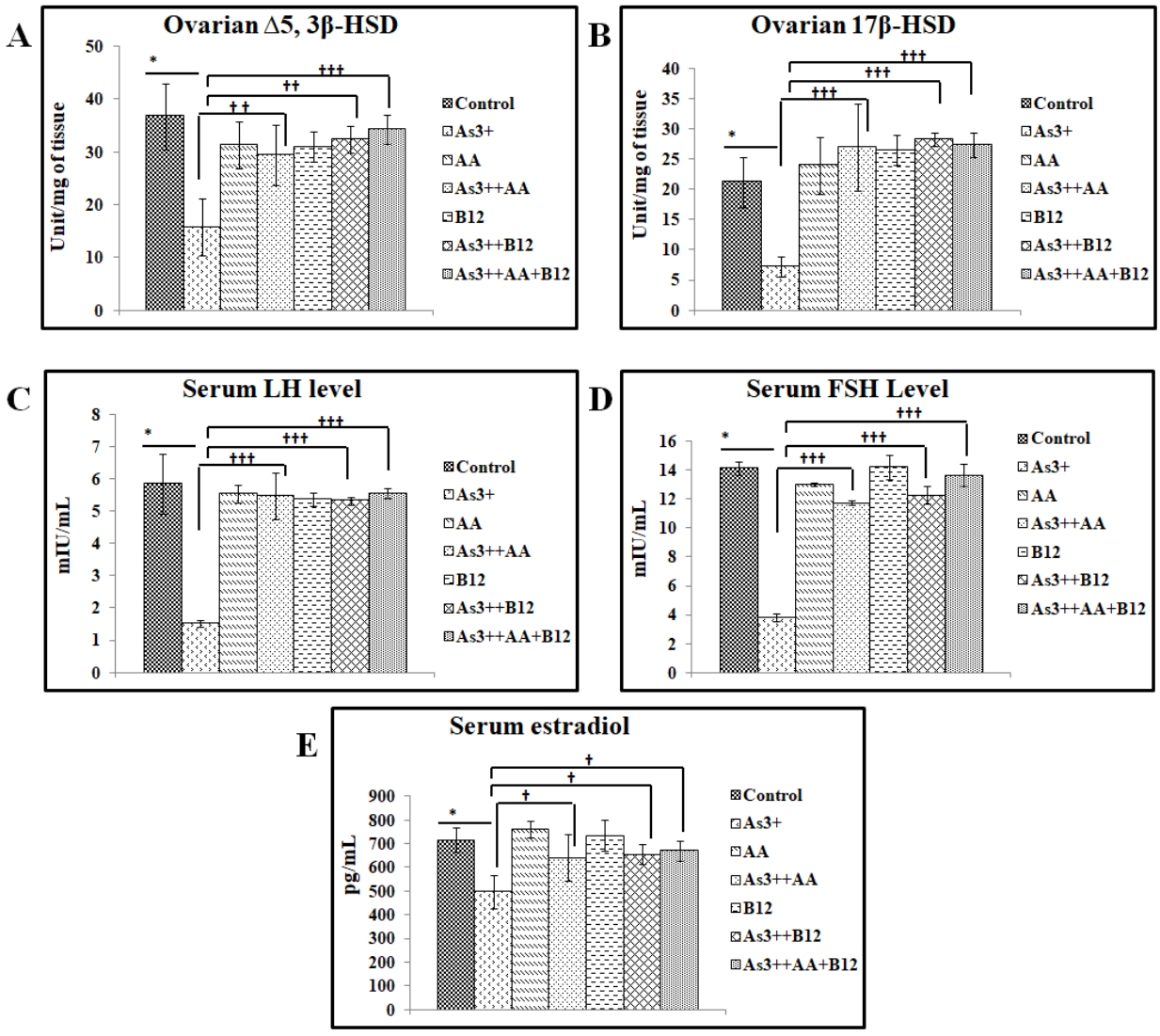


Fig. 3.5: The activity of ovarian steroidogenic enzymes and the level of hormones were restored by the treatment with arjunolic acid and B_{12} alone or jointly. The data showed mean ± SE, n=6,

ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, ††p<0.01, †††p<0.001.

Figure-3.6

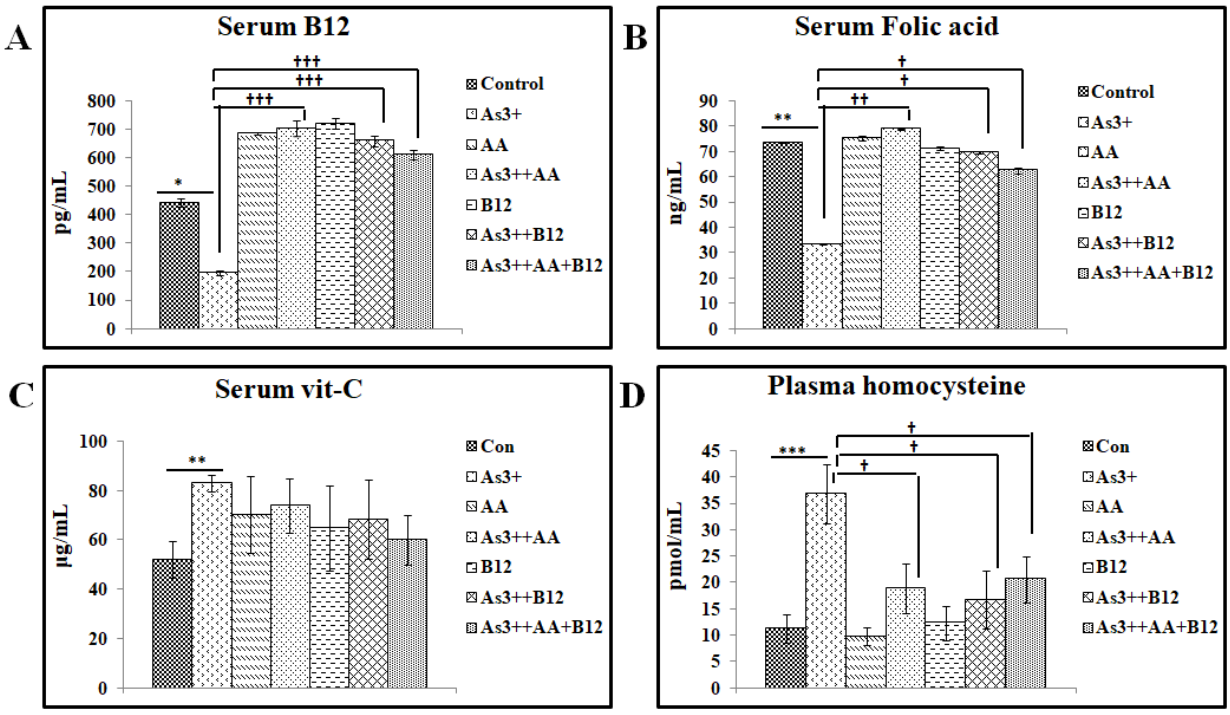


Fig. 3.6: In arsenicated group, the low level of B vitamins was associated with the high levels of vitamin C and homocysteine. Alone or combined treatment of arjunolic acid and B_{12} decreased the levels of homocysteine and increased the level of B vitamins and did not show any significant change of serum vitamin C level. The data show mean ± SE, n=6, ANOVA followed by Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **/††p<0.01, ***/†††p<0.001.

Figure-3.7

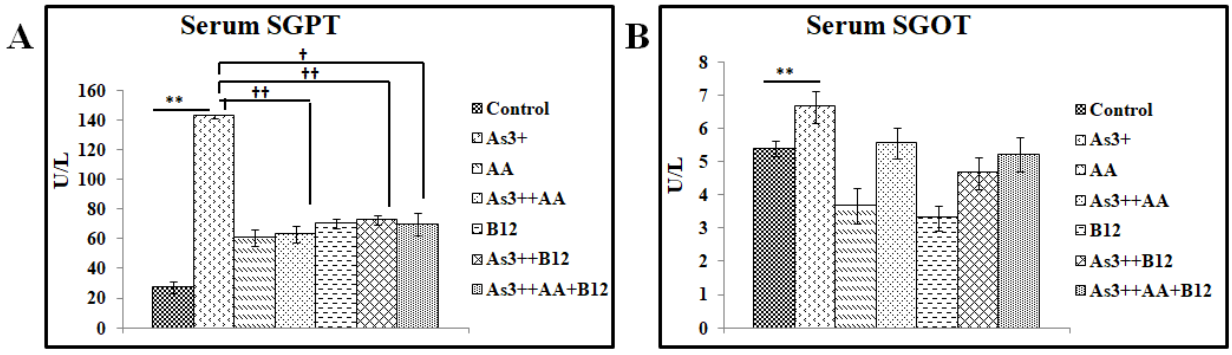


Fig. 3.7: In arsenic-treated group the elevated level of these hepatic markers were diminished by the treatment with arjunolic acid and B_{12} alone or jointly. The data showed mean ± SE, n=6, ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. †p<0.05, **/††p<0.01.

Figure-3.8

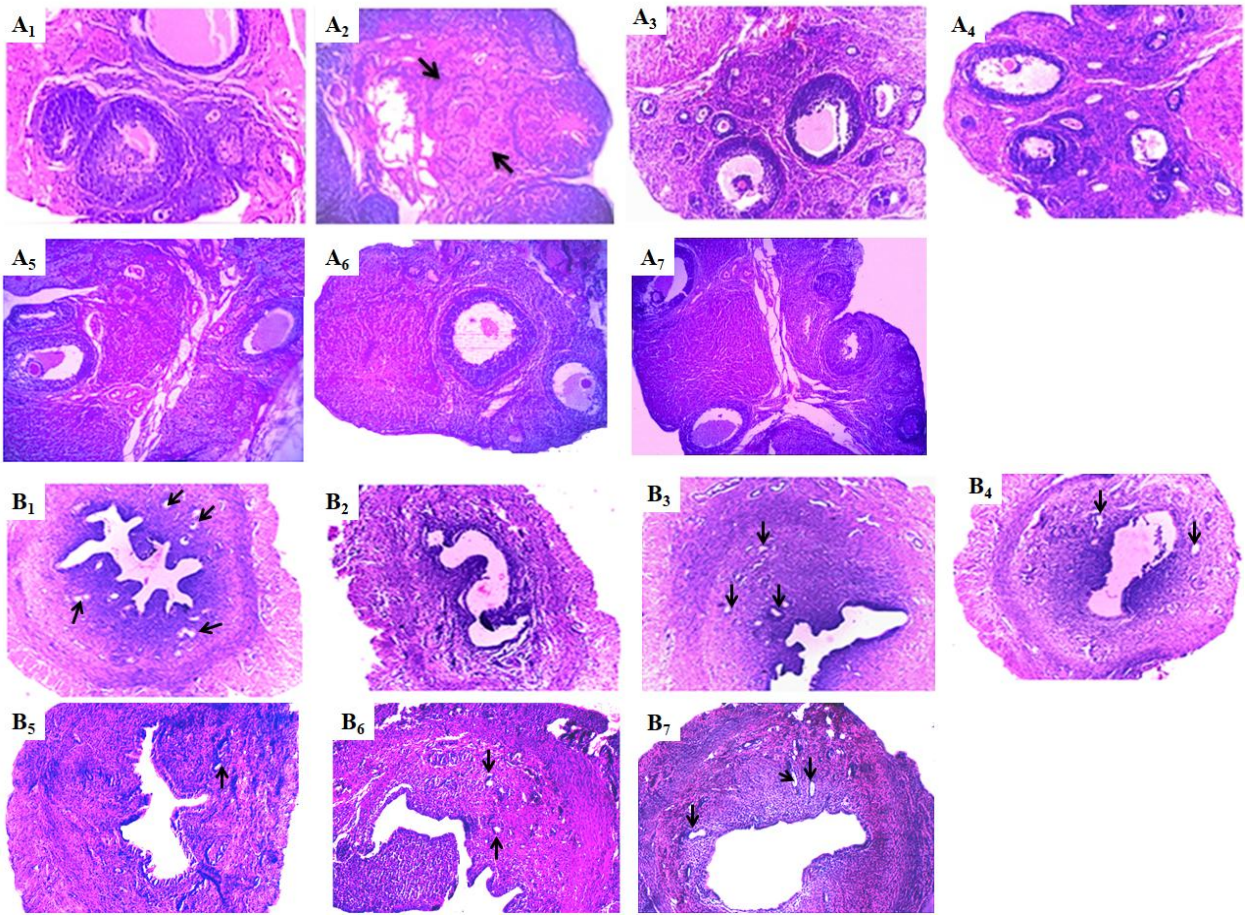


Fig. 3.8: (A and B) Histoarchitechture of ovarian and uterine tissue sectioned at 5 µM thickness and stained with eosin and hematoxylin (Harris). (A) Showing follicular atresia in arsenic ingested rats and the effects were reverted back following the co-administration of arjunolic acid and B_{12}. Panel distribution; A_1: control, A_2: As^{3+}, A_3: arjunolic acid, A_4: As^{3+}+ arjunolic acid, A_5: B_{12}, A_6: As^{3+}+ B_{12}, A_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate follicular atresia.

(B) Uterine secretory glands were lost in arsenicated rats and the recovery of this condition was seen in arjunolic acid, B_{12}, and its combined group. Panel distribution; B_1: control, B_2: As^{3+}, B_3: arjunolic acid, B_4: As^{3+}+ arjunolic acid, B_5: B_{12}, B_6: As^{3+}+ B_{12}, B_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate secretory glands.

5.3.1.4. DISCUSSION:

Due to the reduction of ovarian and uterine SOD, catalase, and GPx the oxidative stress occurs in As^{III} group with the elevation of uterine MDA and CD level (Table 3.2). The trivalent form of arsenic when reacts with H_2O_2 produces reactive oxygen species (ROS) and generates several lipid peroxides and conjugated diene as the end products (Wang et al., 2006).

Excess amounts of superoxide anions ($O2^{\cdot-}$) were produced in ovarian and uterine tissue due to the toxicity of arsenic. The weak expression of ovarian and uterine SOD was found in arsenic-ingested group as appeared from the electrozymographic image (Fig. 3.3 A&B) with weak band strength of SOD in comparison to the vehicle-treated control group. The decreased activity of catalase in ovarian and uterine tissue showed the indistinct band density in electrozymogram (Fig. 3.3 C&D) in arsenicated group. Low band density leads to the decreased detoxification of H_2O_2 from ovarian and uterine tissue. In this experiment, the faint band of GPx (Fig. 3.3E and 3.3F) was found in arsenic exposed rats due to the accumulation of H_2O_2 in ovarian and uterine tissue at the time of programmed cell death (Do et al., 2003).

Serum lactate dehydrogenase (LDH) was a biomarker of cancer. The level of serum LDH was higher in the cancerous cell than that of the normal cell (Zhang et al., 2015). In this experiment, the appearance of serum LDH was elevated in arsenic-treated rats that showed in electrozymogram (Fig. 3.3G) as 230% enhanced band density from the vehicle-treated control. The apoptotic tissue injury was found in the histological tissue sections of the uterus during the elevation of the serum LDH. In the changes of uterine fibrous serum, LDH might play an imperative role through stimulating collagen production (Iglesias et al., 1988). Cell transformation and apoptosis were found in arsenic exposed group due to the high level of ROS generation (Zhang et al., 2015).

The decreasing activity of SOD and catalase were connected with DNA damage via ROS and oxidative stress (Wnek et al., 2011) and that was substantiated by DNA smearing (Fig. 3.4A) and single-cell DNA damage (Fig. 3.4B) in arsenic exposed group. DNA damage was started by the formation of ROS and it also suppresses the DNA repair system and repair of oxidative DNA injury (Kligerman et al., 2003). Due to oxidative stress, ROS is generated, and it breaks the protein, lipid, and DNA structure (Liu et al., 2001). During arsenic metabolism S-adenosyl methionine (SAM), methyl donor leads to DNA hypomethylation. The altered SOD activity was due to the methylated form of arsenic that might develop tissue death via apoptosis and necrosis (Jomova et al., 2011). Hypomethylation is the result of oxidative DNA breakage. The inorganic arsenic^{III} and DMA^{III} are more cytotoxic than its less methylated form (MMA^{III}). Inorganic arsenic^{III} and DMA^{III} release more hydroxyl radicals ($HO^{\cdot}$). Hydroxyl radicals damage the DNA extremely under the altered influence of SOD (Zamora et al., 2014). The damage of DNA could further encourage apoptotic and necrotic tissue damage (Vermeulen et al., 2005).

Due to the low level of LH, FSH, and estradiol, the uterine and ovarian weights are diminished in arsenicated rats (Fig. 3.5). Uterine weights are regulated by estradiol (Edman, 1983), whereas the weight of ovary is regulated by gonadotrophins (Kulin and Reiter, 1973). In arsenic-treated group diminution of the uterine somatic index (Table 3.1) and uterine malformation (Fig 3.8B) were the outcome of downregulation of plasma estradiol signaling which was established by the reduced level of estradiol in arsenicated group. Following 3 to 4 days of arsenic treatment, an asynchronized estrous cycle pattern was observed as a consequence of constant metestrous or diestrous stage (Fig. 3.1).

Earlier studies reported a lower dose of arsenic intoxication (0.4 ppm) for a duration of seven estrous cycles accumulated arsenic in uterine tissues (Chattopadhyay and Ghosh, 2010).

Different body parts toxicity was reported at the lower dose of arsenic for 28 days (Chattopadhyay et al., 2012; Acharyya et al., 2015). In this experiment, we used a comparatively higher dose of arsenic (1.0 mg/100 gm body weight) for short period with the lower dose of arjunolic acid (1.0 mg/100 gm body weight) (Hemalatha et al., 2010; Vasanthi and Parameswari, 2012) and slightly higher doses of B_{12} (0.09µg/100 gm body weight) (Mukherjee et al., 2006; Acharyya et al., 2015) to investigate the protective effects of these bio-molecules on female reproductive organs.

In arsenic exposed group, the water intake was higher than that of other groups during the experiment (Table 3.1). Arsenic intoxication was shown to develop gastrointestinal irritation and intense thirst (Goebel et al., 1990) along with a renal failure risk due to shock and dehydration (Giberson et al., 1976).

The liver cells produce SGPT and SGOT. Due to inflammation, the SGPT and SGOT from the liver are leaked into the serum. Thus the levels of these hepatic enzymes are measured in serum. The level of SGPT and SGOT were increased in arsenic fed rats in comparison to the vehicle-treated control group in this experiment (Fig. 3.7). Pathological modification in hepatic cells is one of the important factors behind this change. Hepatocellular necrosis may be the cause of an enhanced level of SGPT and SGOT in serum. So that the permeability of the cell membrane is increased that discharge these enzymes into the blood-stream (Vandenberghe, 1995; Rana et al., 1996). Through the treatment with arjunolic acid and B_{12} alone or in combination decreases the level of these hepatic transaminases (Fig. 3.7).

From our previous studies, we noticed that poisoning of arsenic could imbalance the activities of ovarian steroidogenesis (Chattopadhyay et al., 1999). Our present experiment showed that As^{III} could reduce the activities of these ovarian steroidogenic enzymes (Δ^5, 3β-HSD and 17β-HSD)

(Fig. 3.5). The suppressed activity of ovarian steroidogenic enzymes could further diminish serum estradiol level (Hinshelwood et al., 1994). The gonadotrophin hormones regulate the activities of ovarian steroidogenic enzymes (Odell et al., 1963). Low level of serum LH and FSH suppresses ovarian 3β-HSD and 17β-HSD activity (Ghersevich et al., 1994). A higher concentration of ROS led to oxidative damage to the proteins with the estrogen signaling pathway (Chatterjee and Chatterji, 2010).

Treatment with arjunolic acid and vitamin B_{12} alone or jointly decreased the level of ROS in arsenic-treated rats by restoring the activity of SOD [Fig. 3.2 and 3.3 (A&B)] and catalase [Fig. 3.2 and 3.3 (C&D)] in ovarian and uterine tissue in this experiment. Estrogen regulates the activity of uterine GPx. Maintaining the activity of uterine peroxidase estrogen has an important function, and it improves and propagates the endometrium layer of the uterus (DeSombre and Lyttle, 1979). Due to the endometrial ROS production, the oxidative stress occurs after arsenic ingestion in the uterus (Chatterjee and Chatterji, 2010) which is substantiated here by the low level of uterine SOD, catalase, GPx (Fig. 3.2 and 3.3) and high level of uterine MDA and CD level (Table 3.2). There is a connection between the elevated amount of ROS excretion and lower level of estrogen in arsenicated group that disrupts the endometrial cycle (Akram et al., 2010). Indistinct uterine layers, uterine cellular degenerations were found in arsenic intoxicated rats (Khorasani et al., 2011) where the secretory cells were lost.

In this study follicular distortion was found in arsenic-treated group (Fig. 3.8A). Our earlier study showed that a low amount of arsenic could disintegrate the ovarian follicles (Chattopadhyay et al., 2003). Due to the low level of plasma gonadotrophins and estradiol, the numbers of atretic follicles were increased and the numbers of healthy follicles were diminished (Gore-Langton and Daniel, 1990). Oxidative stress occurs in the ovary through the consumption

of arsenic (Sun, 1990) which was verified by the enhanced level of MDA, CD, (Table 3.2) and the reduced level of SOD, catalase, and GPx (Fig. 3.2 and 3.3). Due to the elevation of homocysteine, the number of antral follicles and ovarian granulosa cells was reduced in response to the low level of estradiol (Singh et al., 2009). A higher-grade embryo production was diminished due to the increased level of plasma homocysteine (Ocal et al., 2012). In this experiment, ovarian and uterine damages in arsenic-treated group were reverted back by the treatment with arjunolic acid and vitamin B_{12} alone or in combination. Arjunolic acid and vitamin B_{12} alone or jointly protected arsenic-induced ovarian and uterine apoptosis, necrosis and distorted ovarian-uterine histo-architecture in arsenicated group may be due to the improvement of uterine DNA from its breakage.

Data showed that the toxicity of arsenic led to an increased level of homocysteine along with a diminished level of vitamin B_{12} and folic acid (Fig. 3.6). An elevated level of serum LDH (Fig. 3.3G) was associated with a low level of B_{12}-folate (Hoffbrand et al., 1966; Keskin and Keskin, 2015). B-vitamins are essentially important for the protection of necrosis in reproductive organs. The accumulation of homocysteine in the ovary might be the cause of polycystic ovarian syndrome (Maleedhu et al., 2014) and ovarian carcinoma (Corona et al., 1997). A surge in serum B_{12}-folate suppresses the generation of homocysteine to combat polycystic ovarian syndrome (Kilicdag et al., 2005). Arjunolic acid and vitamin B_{12} had shown their ability to lower the elevated level of homocysteine via the re-establishment of the circulating level of B_{12} and folic acid in this experiment. Jeremy et al., 2007 also explored that elevation of homocysteine was sluggish by these B-vitamins (Jeremy et al., 2007). Interruption in biliary release of arsenic diminishes arsenic detoxification by suppressing vitamin B_{12} and folic acid (Kile and

Ronnenberg, 2008; Hall et al., 2009) since biliary excretion of arsenic might be endorsed by B_{12}-folate when arsenic is in its methylated form (As^{III}).

In inorganic arsenic metabolism, the pentavalent form of arsenic is transformed into trivalent form. During biomethylation process of inorganic arsenic metabolism, methyl group is integrated with the As^{III} (Cullen et al., 1984). Via methionine cycle, the detoxification of arsenic is proceeded through the modulation of the methylation process using one-carbon (1C) metabolism where S-adenosylmethionine (SAM) and methyltransferase contribute crucial role. Maintaining endogenous methionine level methionine cycle utilizes vitamin B_{12} and folic acid, where B_{12} acts as a co-factor to catalyze the endogenous methionine synthesis from S-adenosylhomocysteine (Sahin et al., 2003). The level of serum vitamin C was enhanced in arsenicated rats, but arjunolic acid and vitamin B_{12} co-treatment alone or jointly did not show any significant change in the level of serum vitamin C (Fig. 3.6). Rats are able to synthesize vitamin C, and it might be possible that due to the arsenic-induced oxidative stress the level of vitamin C was significantly higher in arsenicated rats. Actually, vitamin C in rats could maintain the homeostatic adjustment against oxidative stress primarily towards the limited extend but this vitamin alone cannot prevent the oxidative stress generation for a later period most probably due to the alteration of other antioxidant and oxidative stress markers in due course of arsenic intoxication.

The revival of ovarian and uterine weight in arsenicated rats was the consequence of the restoration of the plasma gonadotrophin levels and ovarian steroidogenesis by arjunolic acid (Chattopadhyay et al., 1999; Chattopadhyay et al., 2003; Chattopadhyay and Ghosh, 2010). Arjunolic acid contributes an important role in maintaining peroxidase activities in arsenic ingested rats (Manna et al., 2008b). The regular estrous cyclicity and peroxidase activity towards control level following the ingestion of arjunolic acid in arsenic-treated rats in this study is

indicating the re-establishment of estradiol signaling (Bennett, 2001). Through altering the circulating level of B_{12}-folate and homocysteine arjunolic acid could counteract female reproductive malfunction through normalization of the morphology of ovarian follicles, uterine layers as well as protecting the activities of LH, FSH, and estradiol. Having two vicinal equatorial –OH groups arjunolic acid could bind with As^{III} and form the chelate complex. Inhibiting the oxidative damages, the chelate complex removes the free toxins (Manna et al., 2007).

B_{12} reduces oxidative stress via glutathione sparing effect and by decreasing reactive oxygen and nitrogen species (Veber et al., 2008). Deficiency of vitamin B_{12} exerts H_2O_2 accumulation in the uterus by reducing SOD activity (Jia and Domenico, 2010). Insufficiency of B_{12} is the cause of destruction of cellular redox homeostasis that leads to severe oxidative stress (Bito et al., 2017). The level of lipid peroxide end products was diminished and reduced activity of SOD, catalase, and GPx were improved by the co-treatment with vitamin B_{12} in arsenic fed rats as corroborated with the study of Bhattacharjee and Pal, 2014b. Vitamin B_{12} could protect the apoptotic change and cellular damage from DNA fragmentation and histological alteration (Friso and Choi, 2002). However, B_{12} helps in the progression of ovulation in arsenic intoxicated rats.

In conclusion, co-treatment with arjunolic acid and B_{12} alone or in combination can protect the genetic constituent and cellular synthetic mechanism. Necrotic tissue deterioration may be protected by vitamin B_{12} and folate in this experiment and it may be feasible from the possibility of arsenic removal from ovarian and uterine tissue. This type of protective action is helpful in the rejuvenation of tissue structural materials and amino acid pool. On the way of the protection of arsenic-induced ovarian-uterine toxicity and carcinogenicity arjunolic acid and B_{12} have an imperative role as active exogenous nutrients. Other hand, arsenic is usually removed from the

body via methylation process where methionine has an important role. Arjunolic acid perhaps traps As^{III} due to its chelating property. It may be possible that arjunolic acid increases the bioavailability of vitamin B_{12} and folate which enhance arsenic detoxification. Hence, arsenic-induced oxidative stress is diminished in ovarian and uterine tissue. The above study will be helpful for the protective treatment strategy in the arsenic affected population. Further study is essential in this field.

5.4. EXPERIMENT-IV

5.4.1. Post-treatment of Arjunolic acid and B_{12} against arsenic-induced female reproductive health hazards:

5.4.1.1. Aims and objectives:

The present experiment was executed to find out the curative efficacy of arjunolic acid and vitamin B_{12} alone or jointly against arsenic-mediated disorders of female reproductive organs of Wistar rats.

5.4.1.2. Animal Selection and Treatment:

The rats were equally distributed in seven groups weighing 130±10 g. The experimental schedule was as followed:

Group-I Vehicle treated control group,

Group-II Sodium arsenite (1.0 mg /100gm body weight) treated group,

Group-III Arjunolic acid (1.0 mg/100gm body weight) (Sigma-Aldrich, St. Louis, MO),

Group-IV Sodium arsenite (1.0 mg/100gm body weight) and arjunolic acid (1.0 mg/100gm body weight),

Group-V Vitamin B_{12} (0.09μg/100gm body weight),

Group-VI Sodium arsenite (1.0 mg/100gm body weight) and vitamin B_{12} (0.09μg/100gm body weight),

Group-VII Sodium arsenite (1.0 mg/100gm body weight), arjunolic acid (1.0 mg/100gm body weight), and vitamin B_{12} (0.09μg/100gm body weight) combination group.

The rats were treated via oral gavage for sixteen days. To introduce the same amount of physical stress the rats of the Group I were supplied with water for sixteen days through gavage orally as a vehicle. For initial eight days the rats of the Group II, Group IV, Group VI and Group VII were

fed with sodium arsenite and the rats of Group III, V were fed with water. From day nine onwards the rats of group III, IV, V, VI and VII were treated with arjunolic acid and B_{12} alone or in combined manner for eight days. From day nine onwards the rats of the Group II were ingested with water orally. The estrous cycle pattern of all rats was observed. On day seventeen the body weights of all rats were recorded and they were anesthetized by intramuscular injection of ketamine HCl at the dose of 24 mg/kg body weight and collect blood and female sex organs. The samples were kept in a separate sterile bag with an insulated ice container having a temperature of -20^0C temperature. Finally, following the ethical guidelines of CPCSEA the rats were euthanized by the intramuscular injection of an overdose of ketamine.

5.4.1.3. Results:

5.4.1.3.1. General Observation:

Standard rat chow was given to the animals as mentioned in experiment I. In arsenic exposed rats water intake was insignificantly higher than that of other groups. In terms of the percentage of body mass uterine-somatic index was expressed. A significant wet weight loss of ovaries and uterus was found in arsenic ingested group (Table 4.1). These reproductive organs' weight loss was returned to the control value by the treatment with arjunolic acid and B_{12} alone or in combination.

5.4.1.3.2. Vaginal smear study:

The estrous cycle was noted regularly for 16 days. Vaginal smear was characterized with the occurrence of prolonged metestrus or diestrus in arsenic-treated rats after 6 days of treatment onwards with arsenic in comparison to control, whereas synchronization of the estrous cycle was prominent after the treatment with arjunolic acid and B_{12} and arjunolic acid and B_{12} group in arsenic intoxicated rats (Fig. 4.1).

5.4.1.3.3. Observation of Lipid Peroxidation End Products:

In arsenicated group, the level of uterine MDA and CD was elevated than the control value (Table 4.2). However, arjunolic acid and vitamin B_{12} alone or in combination had the ability to right this alteration of these lipid peroxidation end products in this sex organ of arsenic exposed rats.

5.4.1.3.4. Effect of Arjunolic acid and Vitamin B_{12} on Antioxidants Status:

Spectrophotometric analysis explored that the activity of SOD, catalase, and GPx in ovarian and uterine tissue were decreased significantly in arsenic exposed rats in contrast to the control group (Fig. 4.2). Fig. 4.3 shown that the density of these enzymatic bands was diminished in arsenic ingested group in comparison to vehicle-treated control group significantly in ovarian and uterine tissue. While inactivating SOD the cysteine residue alteration of SOD in response to arsenic-mediated redox inequity might be possible (Ghosh et al., 2013). In the way of oxidative stress generation, the mRNA expression of the SOD gene was diminished by arsenic in response to the increased production of superoxide anion radicals (O2 ˙¯) (Rana et al., 2012). The superoxide anions were transformed into H_2O_2. The activity of SOD was reduced in arsenicated group showing the faint band (Fig. 4.3A and 4.3B) in this experiment. In arsenic fed group the catalase activity (Fig. 4.3C and 4.3D) were decreased. In ovarian and uterine tissue, the accumulation of H_2O_2 showed the diminished activity of catalase. Through the elevated deposition of H_2O_2 in arsenic ingested rats might reflect the weak expression of the GPx band (Fig. 4.3E and 4.3F) during programmed cell death (Do et al., 2003). The post-treatment with arjunolic acid and vitamin B_{12} alone or co-jointly had shown its significant ability to restore the activity of SOD, catalase, and GPx (Fig. 4.3).

5.4.1.3.5. Serum LDH status:

Electrozymogram of serum LDH was performed for the study of the necrotic nature of the tissue (Fig. 4.3G). The level of serum LDH was generally higher in necrotic cells than normal cells (Zhang et al., 2015). In arsenic fed group more intense band of serum LDH was seen (Fig. 4.3G). Apoptotic tissue lesions were found while there is an elevation in the serum LDH. Serum LDH encourages the fibrotic changes in the uterus via stimulating the collagen secretion (Iglesias et al., 1988). The release of the enzyme in serum was reduced following post-treatment of arjunolic acid and vitamin B_{12} as documented from the comparatively less dense band in these groups.

5.4.1.3.6. NPSH status:

The level of NPSH was decreased significantly in arsenic fed group than that of control (Fig. 4.2). Arjunolic acid and vitamin B_{12} alone or in combination had shown its ability to maintain NPSH level towards control.

5.4.1.3.7. Comet assay:

From the outcome of the comet assay the extruded nature of the broken uterine DNA from a bulk number of cells was clearly pictured in arsenic-treated rats (Fig. 4.4). The damage of the cellular DNA was found in arsenic group. The cellular DNA damage was repaired by arjunolic acid and vitamin B_{12} alone and in combination.

5.4.1.3.8. Esr 1, NF-κ B, Metallothionein, TNF alpha, and IL-6:

ELISA test demonstrated that ovarian estradiol receptor-1 level (Esr 1) was diminished significantly in arsenicated rats (Fig. 4.5). Whereas the level of uterine NF-κ B, hepatic metallothionein, serum TNF-alpha, and serum IL-6 were increased significantly in arsenic-treated group. Arjunolic acid and vitamin B_{12} alone or in combination noticeably reverted back the above effects on Esr 1 and pro-inflammatory cytokines and metallothionein (Fig. 4.5).

5.4.1.3.9. Ovarian and Uterine histopathology:

An elevation in the significant numbers of atretic follicles in arsenic exposed group in comparison to the control indicated the existence of ovarian follicular regression (Fig. 4.6A and Table 4.3). Arjunolic acid could significantly reduce the numbers of regressive follicles but there was no significant difference found in B_{12} alone and arjunolic acid plus B_{12} post-treated group (Table 4.3). Ovaries of arsenic-treated group also showed a significant decrease in the numbers of preantral and antral follicles. Arjunolic acid and B_{12} alone or its combination could effectively control the loss of growing and matured follicles by exerting its remedial effect through the minimization of the growth of regressing follicles (Fig. 4.6A and Table 4.3). Alone or jointly arjunolic acid and B_{12} could significantly restore the numbers of preantral follicles whereas arjunolic acid also significantly restored the numbers of medium antral follicles in arsenic intoxicated rats. Though B_{12} and arjunolic acid alone or in combination could also restore the numbers of medium antral follicles, small antral follicles, large antral follicles, and graafian follicles in arsenic fed rats but these changes were insignificant (Table 4.3).

Uterine tissue histology highlighted the degeneration of perimetrium, myometrium, and endometrium layers as indicated by reduced diameters of these layers followed by its remarkable thinning arsenicated rats (Fig. 4.6B) though myometrium showed an insignificant reduction of diameter. Uterine secretory glands were also lost in sodium arsenite exposed rats. Post-treatment of arjunolic acid and B_{12} remarkably modified the breadth of the uterine layers along with the restoration of the secretory glands (Fig. 4.6B and Table 4.3) but insignificant changes of diameter were observed when compared with arsenic-treated group (Table 4.3).

Table-4.1

	Body weight(g)		Organo-somatic indices(g)		Water intake (ml/100gm body weight)
	Initial	Final	Ovary in pair	Uterus	
Control	123.00±7.99	134.83±3.39	0.067±0.004	0.165±0.02	12.00±0.63
As^{3+}	124.16±6.05	135.00±4.71	0.066±0.003*	0.116±0.004*	13.90±0.57
AA	122.50±7.96	124.16±6.81	0.069±0.003	0.156±0.008	11.00±0.63
As^{3+}+AA	121.16±8.22	123.00±7.56	0.068±0.002	0.155±0.012†	11.60±0.82
B_{12}	123.40±7.15	134.00±8.17	0.067±0.004	0.126±0.003	12.00±0.63
As^{3+}+B_{12}	124.20±7.07	133.80±7.24	0.065±0.005	0.122±0.004	11.00±1.01
As^{3+}+AA+B_{12}	125.00±5.47	130.80±2.57	0.073±0.005	0.156±0.013†	12.80±0.76

Table 4.1: The data are mean ± SE, n=6, ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */† $p<0.05$.

Figure-4.1

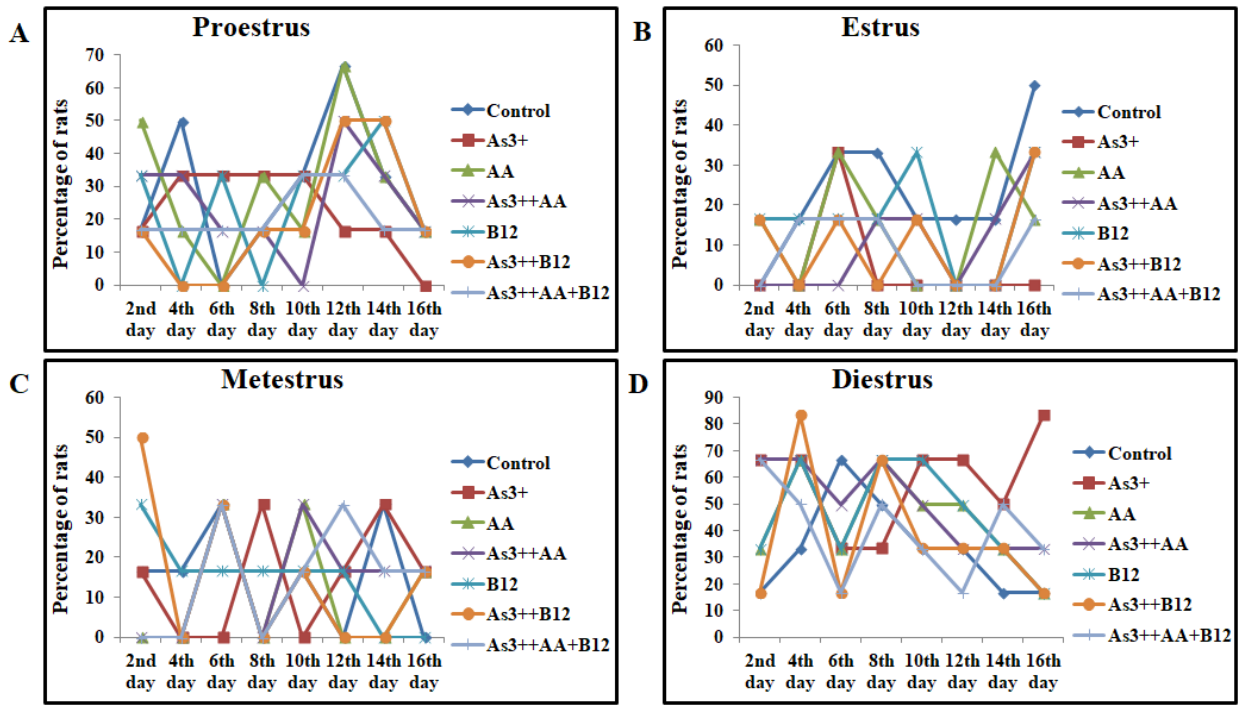


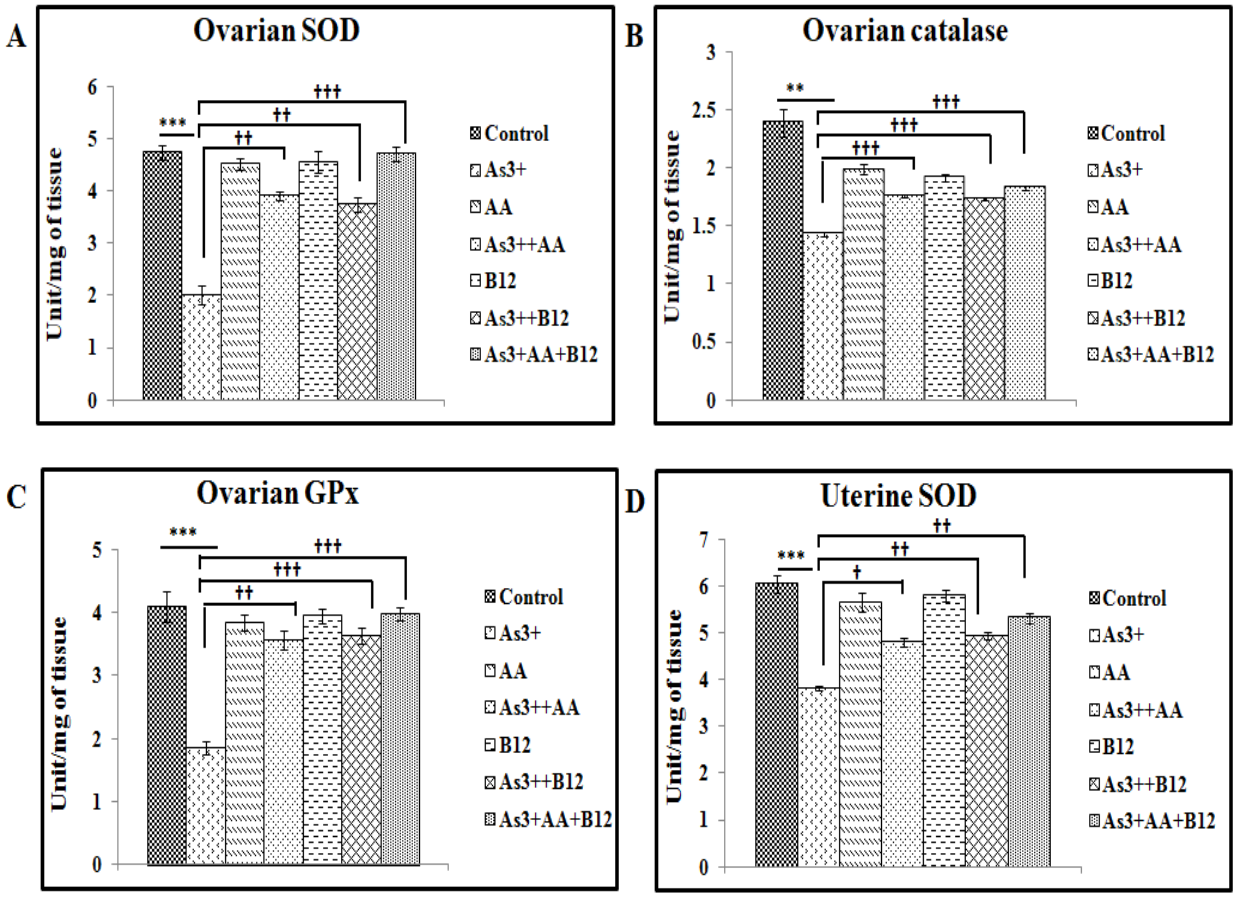
Fig. 4.1: Pattern of the estrous cycle. Persistent metestrus and diestrus were noticed in arsenic-treated rats after 6 days of treatment. This irregular pattern was further reversed back towards the regular pattern when arjunolic acid and B_{12} post-treated in arsenic-treated rats.

Table-4.2

	Control	As^{3+}	AA	As^{3+}+AA	B_{12}	As^{3+}+B_{12}	As^{3+}+AA+B_{12}
MDA (nmole/gm)	36.52±4.75	48.3±3.26*	34.75±1.78	37.4±2.1†	37.52±0.69	38.15±0.76†	41.86±1.4
CD (nmole/gm)	8.29±0.51	20.29±6.55*	10.31±0.33	12.77±1.1†	8.73±1.21	9.01±1.63†	12.66±1.96

Table 4.2: Alone or in combination with arjunolic acid and vitamin B_{12} cure the uterine tissue by reducing MDA and CD levels in arsenic intoxicated rats. The data represents mean ± SE, n=6. ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */† $p<0.05$.

Figure-4.2


A
Ovarian SOD
Unit/mg of tissue
***
††
†††
Control
As3+
AA
As3++AA
B12
As3++B12
As3+AA+B12
B
Ovarian catalase
Unit/mg of tissue
**
†††
†††
Control
As3+
AA
As3++AA
B12
As3++B12
As3+AA+B12
C
Ovarian GPx
Unit/mg of tissue
***
†††
†††
††
Control
As3+
AA
As3++AA
B12
As3++B12
As3+AA+B12
D
Uterine SOD
Unit/mg of tissue
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††
†
††
Control
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AA
As3++AA
B12
As3++B12
As3+AA+B12

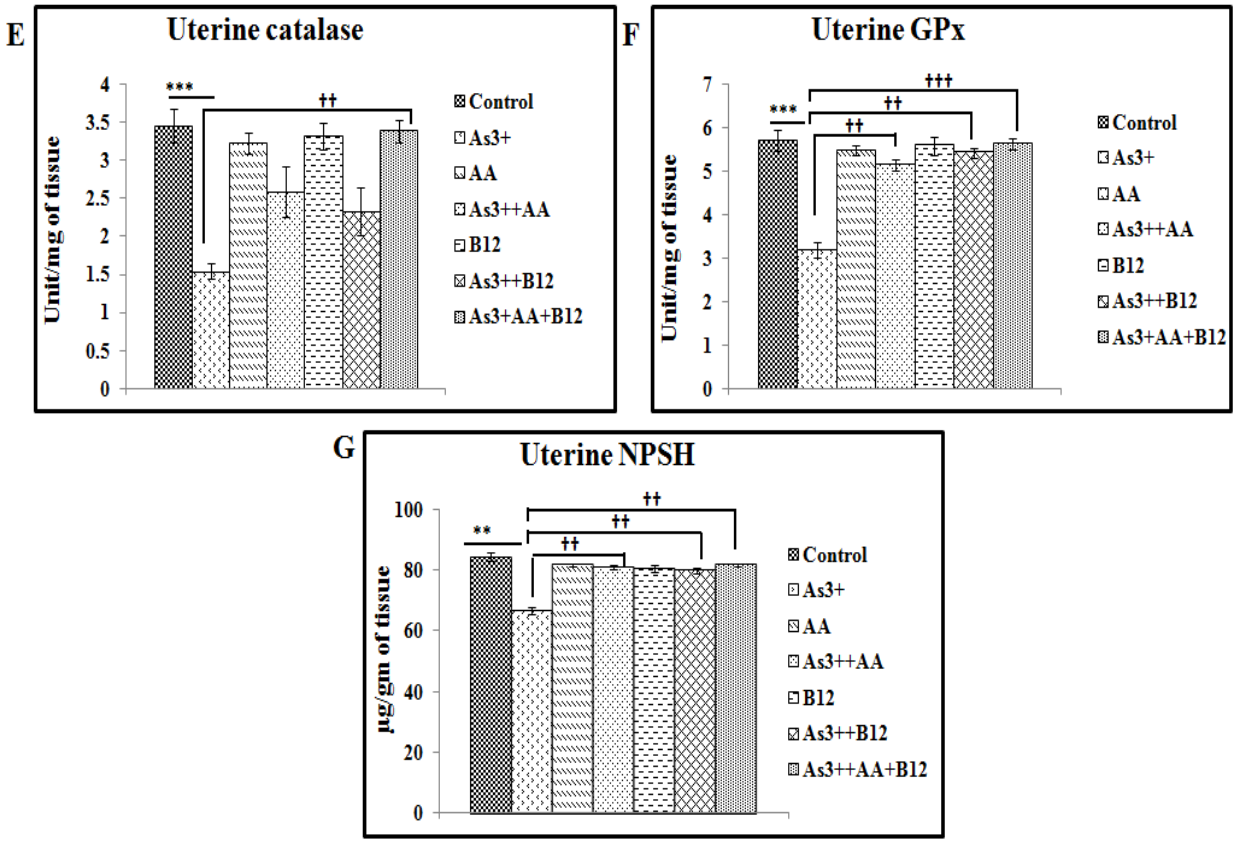
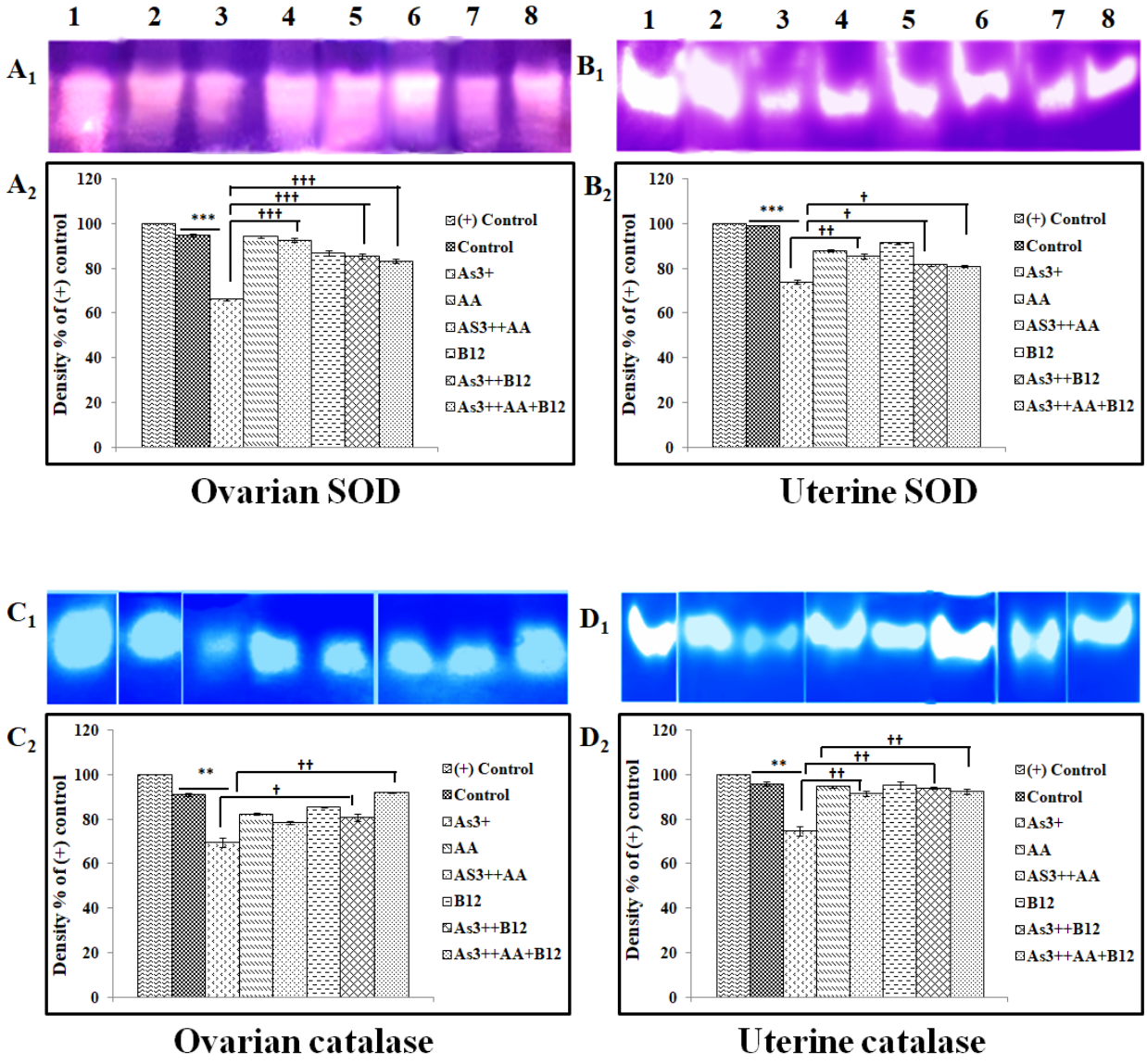


Fig. 4.2: Arjunolic acid and vitamin B_{12} shown their curative efficacy on ovarian and uterine SOD and catalase in arsenic intoxicated rats. Arjunolic acid and vitamin B_{12} post-treatment could maintain ovarian and uterine NPSH levels towards control in arsenicated rats. Data show mean ± SE, n=6. ANOVA followed by the post hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$.

Figure-4.3


1 2 3 4 5 6 7 8
A1
B1
A2
B2
Density % of (+) control
(+) Control
Control
As3+
AA
AS3++AA
B12
As3++B12
As3++AA+B12
Ovarian SOD
Uterine SOD
C1
D1
C2
D2
Ovarian catalase
Uterine catalase

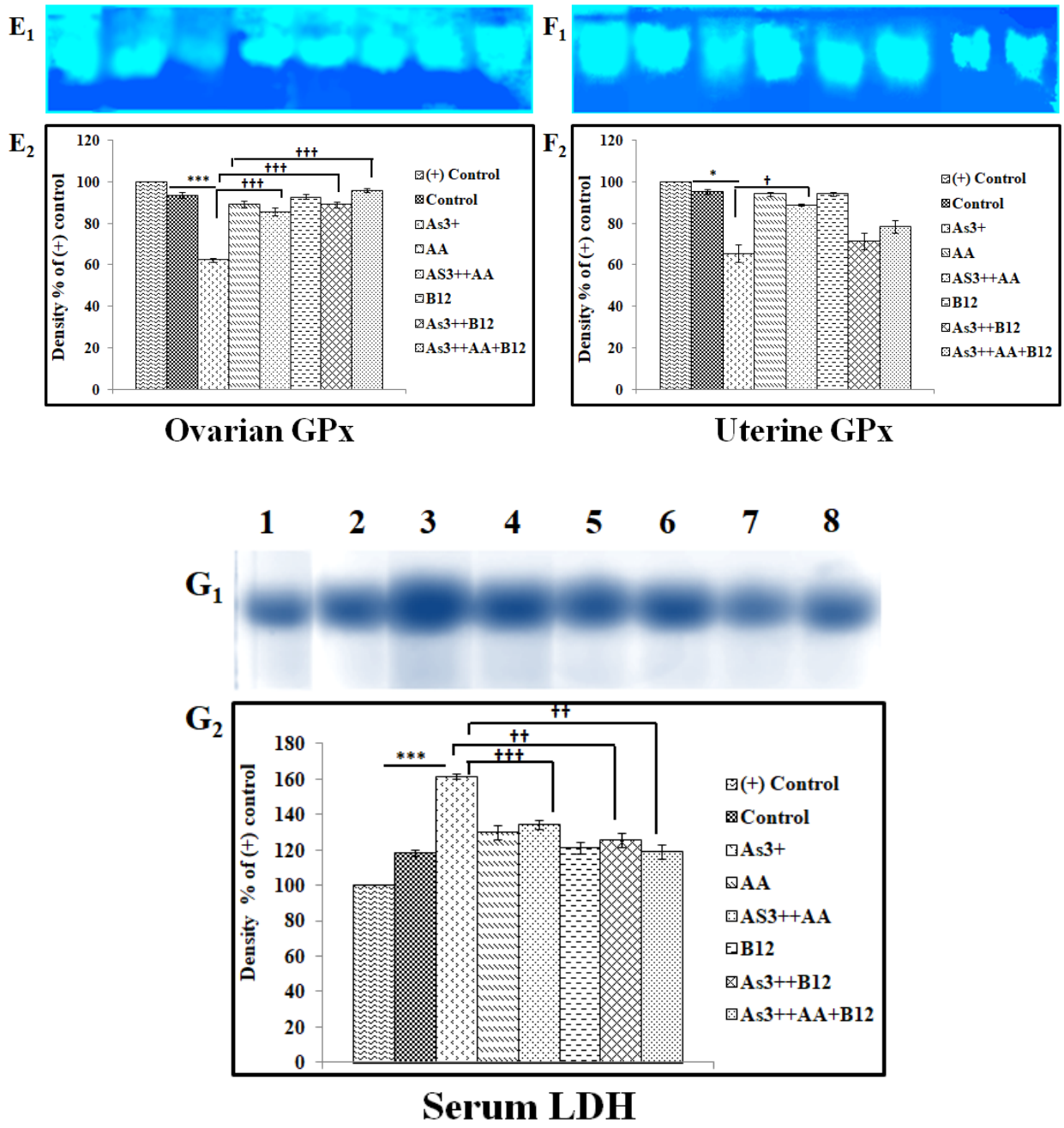


Fig. 4.3: (A, B, C, D, E, F, and G). On native gel the activity of SOD, catalase, and GPx were shown in ovarian and uterine tissue. Serum LDH activity was performed on an agarose gel to the extent of cellular damage. Lane distribution Lane 1: (+) control; Lane 2: control; Lane 3: As^{3+}; Lane 4: arjunolic acid; Lane 5: As^{3+}+arjunolic acid, Lane 6: $B_{12,}$ Lane 7: As^{3+}+B_{12}, Lane 8: As^{3+}+arjunolic acid+B_{12}. In each lane of 12% and 8% native gel, the same protein was

electrophoresed. The achromatic bands of SOD and GPx were shown after staining. The yellow bands of catalase were shown on the blue-green background of native gel. The densitometric analysis represents mean ± SE, n=6, ANOVA followed by the post hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **/††p<0.01, ***/†††p<0.001.

Figure-4.4

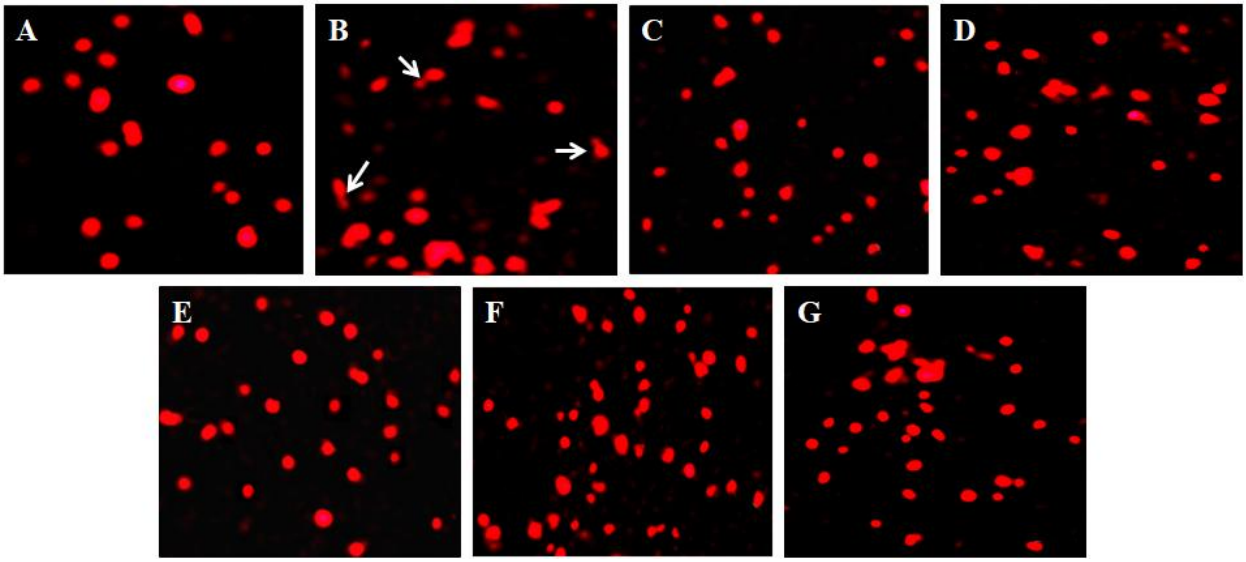


Fig. 4.4: Comet assay shows the curative role of arjunolic acid and B_{12}. Panel distribution; A: control, B: As^{3+}, C: arjunolic acid, D: As^{3+}+ arjunolic acid, E: B_{12}, F: As^{3+}+ B_{12}, G: As^{3+}+ arjunolic acid+ B_{12}.

Figure-4.5

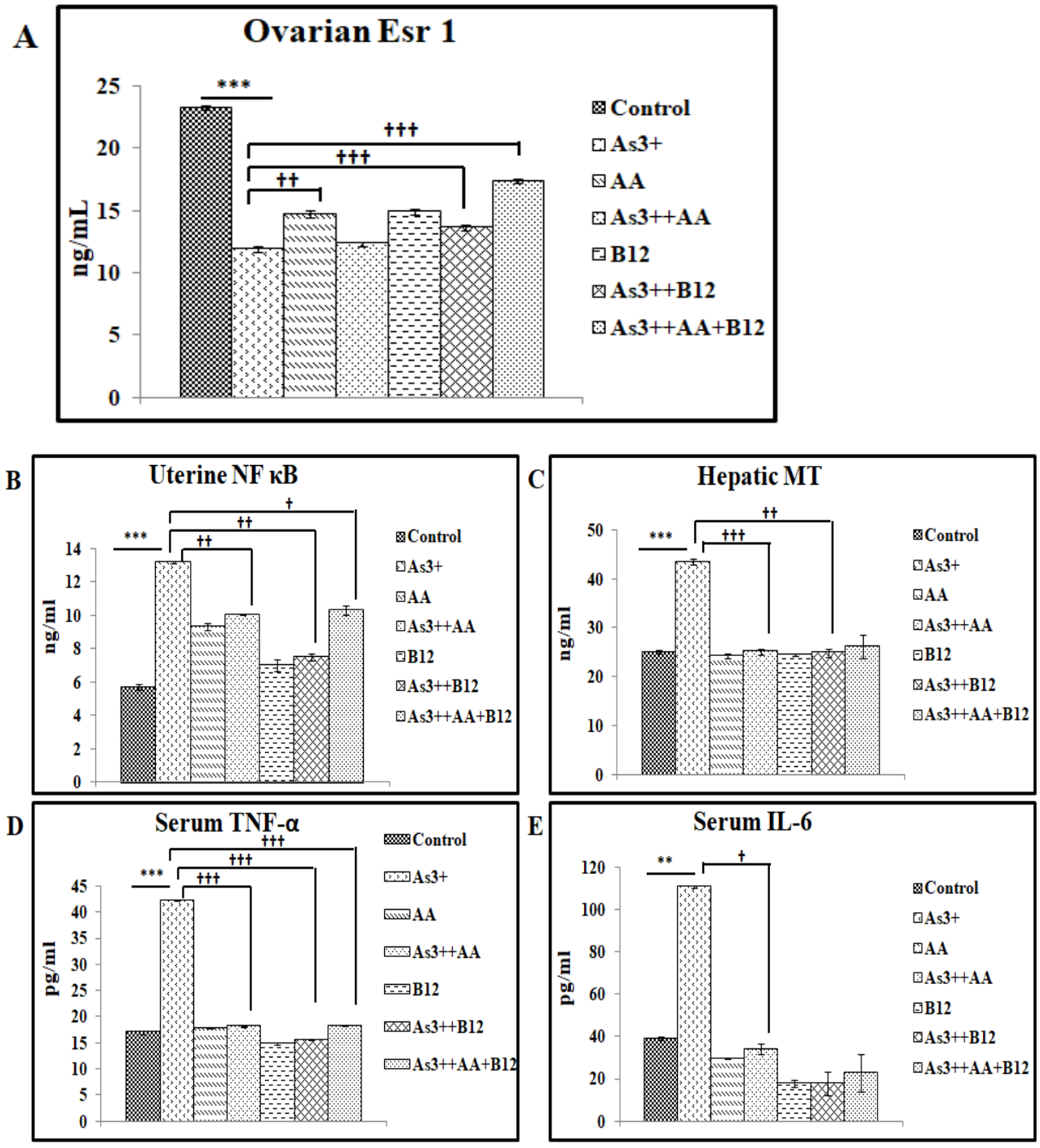


Fig. 4.5: Remedial effect of arjunolic acid and vitamin B_{12} on ovarian Esr-1, pro-inflammatory cytokines and metallothionein. The data represent mean ± SE, n=6. ANOVA followed by the post hoc

Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **/††p<0.01, ***/†††p<0.001.

Table-4.3

	SPAF	LPAF	SAF	MAF	LAF	GF	RF
Control	14.66±0.90	7.16±0.28	3.33±0.30	3.66±0.61	2.66±0.30	1.33±0.30	1.16±0.28
As^{3+}	2.83±0.72***	1.83±0.54**	1.66±0.51*	0.83±0.36*	0.83±0.28*	0.50±0.20*	7.50±0.90*
AA	10.33±0.45	8.16±0.54	4.16±0.28	3.66±0.30	1.50±0.31	1.16±0.28	1.33±0.30
As^{3+}+AA	8.83±0.54††	7.16±0.79†	3.50±0.39	2.83±0.28†	0.83±0.28	0.83±0.15	2.16±0.28†
B_{12}	11.66±0.50	7.16±0.43	5.16±0.28	1.83±0.28	1.33±0.30	1.33±0.30	2.16±0.28
As^{3+}+B_{12}	10.16±0.43††	6.33±0.69†	4.16±0.54	1.83±0.28	1.16±0.15	1.16±0.28	3.33±0.30
As^{3+}+ AA+B_{12}	10.66±0.45†††	5.66±0.45†	3.50±0.39	2.50±0.51	1.50±0.20	0.66±0.38	3.50±0.39

	Uterine diameter (µm)	Thickness of different layers in uterus (µm)		
		Endometrium	Myometrium	Perimetrium
Control	6086.33±149.55	261.62±23.27	88.41±6.95	138.28±10.59
As^{III}	5445.24±195.35	83.93±4.91**	52.05±9.9	71.33±7.51*
AA	6017.56±27.39	219.89±15.85	61.39±9.58	119.48±10.59
As^{III}+AA	5922.21±63.51	206.62±15.84††	55.05±9.06	112.18±10.14
B_{12}	5805.94±115.23	246.26±15.41	72.43±2.37	176.86±17.85
As^{III}+B_{12}	5782.00±111.14	236.81±14.84††	60.61±2.35	163.57±16.64†
As^{III}+AA+B_{12}	6140.53±136.71	198.79±12.44††	94.06±10.6	101.54±10.46

Table 4.3: Treatment with arjunolic acid and vitamin B_{12} alone or jointly changed arsenic-induced ill effects on ovarian follicle and uterine diameter. The follicles were classified as small

preantral follicles (SPAF) (< 94 μm), large preantral follicles (LPAF) (94–260 μm), small antral follicles (SAF) (261–350 μm), medium antral follicles (MAF) (351–430 μm), large antral follicles (LAF) (431–490 μm), graafian follicles (> 491 μm) on the basis of their morphology and diameter. Data represent mean ± SE, n=6, ANOVA followed by the post hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **/††p<0.01, ***/†††p<0.001.

Figure-4.6

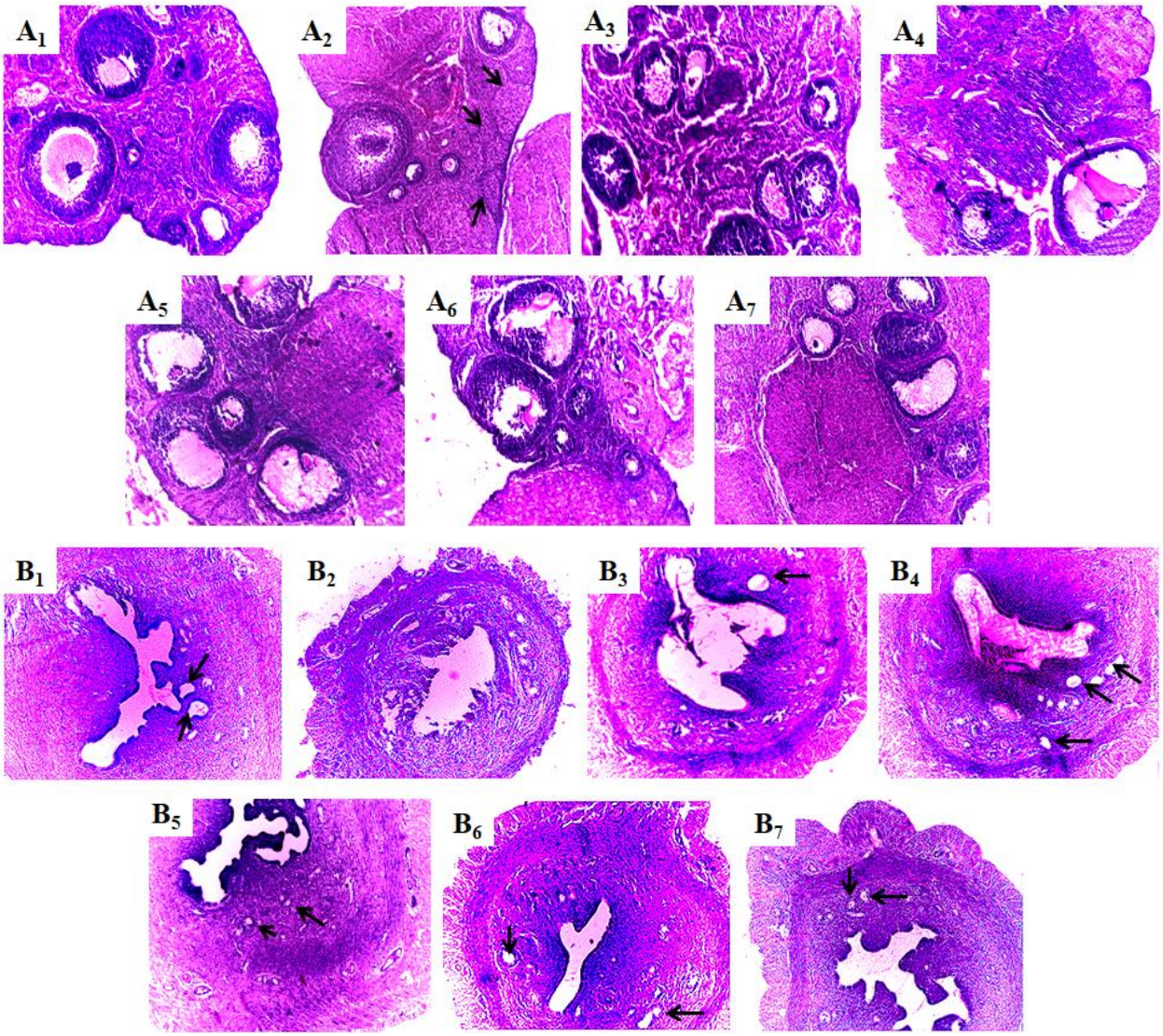


Fig. 4.6: (A and B) 5.0 µM thick tissue of ovary and uterus were sectioned. After staining with eosin and hematoxylin (Harris) the section was monitored under the microscope. (A) Ovarian tissue focused follicular atresia following arsenic treatment and the ameliorative effect of arjunolic acid and B_{12}. Panel distribution; A_1: control, A_2: As^{3+}, A_3: arjunolic acid, A_4: As^{3+}+ arjunolic acid, A_5: B_{12}, A_6: As^{3+}+ B_{12}, A_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate follicular atresia.

(B) The secretory glands were lost in arsenic-treated group and the remedial effect was seen following arjunolic acid and B_{12} post-treatment. Panel distribution; B_1: control, B_2: As^{3+}, B_3: arjunolic acid, B_4: As^{3+}+ arjunolic acid, B_5: B_{12}, B_6: As^{3+}+ B_{12}, B_7: As^{3+}+ arjunolic acid+ B_{12}. Arrows indicate secretory glands.

5.4.1.4. DISCUSSION:

Uterine and ovarian tissue damage was shown due to treatment with arsenic at the dose of 10 mg/kg body weight. Present results showed the antioxidant property of arjunolic acid and vitamin B_{12} with different biological actions. Previous reports show that arjunolic acid and vitamin B_{12} could protect the hepatic organ (Chattopadhyay et al., 2012) and uterus (Deb et al., 2018) in arsenic intoxicated female rats. In our earlier studies, we discussed about the preventive role of arjunolic acid that hampers the arsenic-induced uterine dysfunction with oxidative stress in female rats (Maity et al., 2018). In this study, we intended to focus that how the arjunolic acid and vitamin B_{12} alone or jointly protect the uterine and ovarian disorders in curative mode (post-treatment) against arsenic-induced female rats.

The elevated ROS production could reduce the intracellular antioxidant enzymatic activity due to the toxicity of arsenic. These results could correlate with the previous findings (Dash et al., 2018). The post-treatment with arjunolic acid at the dose of 1.0 mg/100gm body weight and vitamin B_{12} at the dose of 0.09μg / 100gm bodyweight alone or jointly could protect the ovarian and uterine antioxidant enzymatic activities as documented by the electrozymographic analysis (Fig. 4.3). Comparing the other studies, the inhibition of the activities of SOD, catalase, and GPx were protected by arjunolic acid (Sinha et al., 2008b) and vitamin B_{12} (Bhattacharjee and Pal, 2014b) in arsenic fed rats. Producing chelate complex with arsenic or removing excess ROS, arjunolic acid could protect the antioxidant enzymatic activities (Sinha et al., 2008b). Vitamin B_{12} may play as an antioxidative agent through diminish the excess arsenic accumulation (Bhattacharjee et al., 2013).

Kligerman et al showed that DNA damage was initiated in arsenic-treated rats through the formation of ROS that suppresses the DNA repair system and repair of oxidative DNA injury

(Kligerman et al., 2003). Due to its powerful antioxidant, free radical scavenging and metal chelating properties arjunolic acid could prevent oxidative DNA injury by reducing ROS production (Manna et al., 2007). Vitamin B_{12} could maintain the methylation process through protecting the DNA repair system (Fenech, 1999).

A useful biomethylation process is imperative in the transformation of the pentavalent form of inorganic arsenic to trivalent form by the addition of the methyl group with the trivalent form of arsenic in the human body (Cullen et al., 1984). This methylation process promotes detoxification of arsenic from the body where one-carbon (1C) metabolism in association with methyltransferase and S-adenosylmethionine (SAM) contribute significantly. The level of vitamin B_{12} and folic acid in circulation is reduced in arsenic fed rats that showed in our previous studies (Maity et al., 2018). Vitamin B_{12}, a co-factor of the methionine cycle in association with folic acid maintains the synthesis of endogenous methionine from S-adenosylhomocysteine (Sahin et al., 2003). Our present investigation again explored that post-treatment with B_{12} in arsenicated animals might improve the status of SAM where arjunolic acid also served a critical role in trapping arsenic (Maity et al., 2018).

In the present experiment, we also used a comparatively higher dose of arsenic (1.0 mg/100 gm body weight) for 8 days with the lower dose of arjunolic acid (1.0 mg/100 gm body weight) (Hemalatha et al., 2010; Vasanthi and Parameswari, 2012) and slightly higher doses of B_{12} (0.09µg/100 gm body weight) (Mukherjee et al., 2006; Acharyya et al., 2015) to examine the post-treatment effects of these bio-molecules on female reproductive organs.

Arsenic, an endocrine-disruptor is known to show its mimetic, agonistic, and antagonistic role in the modulation of the hormone action based on the level of endogenous estrogens (Stoica et al., 2000). Cell proliferation of porcine aortic endothelial cells is influenced by arsenic (Barchowsky

et al., 1999). As^{3+} could influence the down-regulation of ERα at mRNA and proteomic level and this could finally modulate estrogenic action (Chow et al., 2004). Maintaining the normal histo-architecture of ovary and uterus, post-treatment with arjunolic acid and vitamin B_{12} alone or co-jointly increased the estradiol signaling receptor (ER-1) and promoted the growth of ovary and uterus in this experiment (Fig. 4.2).

The numbers of atretic follicles are elevated significantly (Table 4.2) and the healthy follicles numbers are diminished in arsenic treated rats because of the low level of plasma gonadotrophins and estradiol (Gore-Langton and Daniel, 1990). Due to the consumption of arsenic the ovarian oxidative stress occurs (Sun, 1990) showing the reduced level of ovarian SOD, catalase, and GPx in arsenicated group in this experiment (Fig. 4.3). The ovarian follicular degradation is found in arsenic fed rats. Oxidative stress arises in primordial and preovulatory follicles because of the demolition of antioxidant enzymatic defenses (Tarin, 1996). The present results of this experiment are also comparable with the findings of others where arjunolic acid and vitamin B_{12} improved the different folliculogenesis of ovary and decreased the follicular atresia (Gore-Langton and Daniel, 1990; Maity et al., 2018). Encouraging the development of ovum vitamin B_{12} could elevate the ovulation process in arsenic intoxicated rats (Bennett, 2001). The level of estradiol maintains the uterine layers development and proliferation (Patil et al., 1998). The uterine tissue injury started through the initiation of the loss of uterine secretory cells. Uterine morphological deterioration may be found due to the arsenic induced oxidative stress (Beltran-Garcia et al., 2000) in response to reduced uterine SOD, catalase, and GPx activity. In arsenic-treated group the muscular layer of the uterus is thinned and the degradation of uterine cells, diminutions of endometrial glands were found (Fig. 4.6B). In association with the raising level of ROS production, the endometrial cycle disruption is beginning via the low level of estrogen in

arsenic ingested group (Akram et al., 2010). From our previous experiment, we proved that arjunolic acid could reduce the serum LDH activity in arsenicated group by decreasing the necrosis (Maity et al., 2018). In this experiment arjunolic acid, vitamin B_{12} alone or in combination diminished the level of serum LDH through electrozymography (Fig. 4.3). These types of ovarian-uterine disorders are inverted by the post-treatment of arjunolic acid. This type of protective mechanism of arjunolic acid prohibited the arsenic-induced apoptosis and necrosis in ovarian and uterine tissue. Hence, in arsenic ingested rats the ovarian-uterine histo-architecture and uterine DNA damage are recovered by the treatment with arjunolic acid.

To diminish the ovarian, uterine stress and normalization of its morphology, excretion of arsenic is very important from the organs. Vitamin B_{12} acts as a co-factor for the synthesis of endogenous methionine from S-adenosyl homocysteine through the involvement of methionine synthase. Vitamin B_{12} may promote the methylation process for the elimination of arsenic through urine from the reproductive organs (Spiegelstein et al., 2003). However, vitamin B_{12} is important for preserving the genetic materials and protecting the ovarian and uterine tissues from necrosis and probable carcinogenesis (Mukherjee et al., 2006).

Serum level of inflammatory marker (NF-κB) and pro-inflammatory cytokines (TNF-α, and IL-6) were examined by us in this present experimental work (Fig. 4.5). In this study, the level of uterine NF-κB and serum TNF-α, or IL-6 were elevated (Fig. 4.5) following the enhanced level of serum LDH (Fig. 4.3) in arsenic-treated rats. Comparing the results of this study with Das et al., it is clear that a high dose of arsenic is responsible for the elevation of cytokines through raising the level of collagen deposition in hepatic tissue (Das et al., 2005). In this experiment, post-treatment with arjunolic acid and B_{12} alone or jointly could inhibit the elevation in the levels of uterine NF-κB and serum TNF- α, or IL-6 in arsenic ingested rats (Fig. 4.5). Influencing NF

κB signaling pathway arsenic may endorse toxic effects in cells and tissues (Wei et al., 2016). Some early response genes closely associated with inflammatory responses, cell growth, cell cycle progression, and neoplastic transformation, are the limiting factor behind the elevation of NF κB (Chen and Shi, 2002) in arsenicated group in comparison with vehicle-treated control group (Fig. 4.5). During the regulation of cell proliferation, differentiation, and transformation NF-κB exhibits a critical role (Huang et al., 1999). The enhanced level of cellular reactive oxygen species in arsenic exposed group could encourage to express NF κB by upregulating the nuclear transcription of NF κB and thereby accelerating the production of inflammatory cytokines (Hu et al., 2002). Arsenic could alter the cell signaling pathways by changing the level of NF κB (Kaltreider et al., 1999). In arsenic fed group the level of NF κB is increased than that of the control. During arsenic intoxication; apoptotic cell death in JCS-16 leukemia cells was seen where TNF alpha may play a pivotal role (Mak et al., 2002). Apoptosis of T helper cells in arsenic intoxicated is accelerated by tumor necrosis factor receptor 1 (TNF-R1) (Yu et al., 2002). Reducing ROS production during apoptotic cell death, arjunolic acid could suppress the NF κB and TNF-α activation (Manna et al., 2009). Though Vitamin B_{12} has an anti-inflammation property, it can also reduce the level of TNF-α through down-regulating the level of NF κB (Veber et al., 2008). However, our present findings also revealed a downregulation of proinflammatory cytokines following the post-treatment with arjunolic acid and B_{12} and these results are in agreement with the findings of above group of workers.

In the hepatic tissue of arsenic-treated rats, the level of metallothionein (MT) (Fig. 4.5) is raised in comparison to the vehicle-treated control group in this experiment. Our result is also similar with the investigation of Bhattacharya and Bhattacharya, who showed that arsenic intoxication is the cause of enhanced level of MT in the liver (Bhattacharya and Bhattacharya, 2007). Arsenic

synthesizes MT that is rich in high cysteine and acts as a neutralizing nucleophilic equivalent and it also binds with heavy metals (Agarwal and Bhattacharya, 1990). In arsenic intoxicated group, the production of ROS and apoptosis were initially recovered probably by the elevated amount of MT1 expression. The expression of MT acts on the cellular defense mechanism and protects the cells from reactive intermediates to arsenic toxicity (Bi et al., 2004).

Arjunolic acid can reduce the free radicals production in arsenicated group. The regular estrous cycle revival implies the potentiation of estradiol signaling by the post-treatment of arjunolic acid in arsenic-treated rats (Bennett, 2001). The ovarian and uterine oxidative stress, apoptotic and necrotic injury might be recovered by the treatment with arjunolic acid in these rats due to the built-up of a defense mechanism against oxidative stress as well as inflammatory response (Maity et al., 2018). Two vicinal equatorial –OH groups of arjunolic acid might be the targeted bind sites for arsenic. The OH groups of arjunolic acid might be accompanying with As^{III} (with a lone pair of electrons of oxygen) to develop five-membered chelate complex. The chelate complex may further eradicate free toxins and minimize oxidative damages. One carboxylic hydrogen atom of the chelate complex is accountable for free radical scavenging (Sinha et al., 2008b). The presence of carboxylic hydrogen atom in arjunolic acid is the cause of DPPH (2,2-diphenyl-1-picryl hydrazyl) radical scavenging activity. Arjunolic acid contains poly hydroxyl groups and therefore, the oxidization of arjunolic acid is imperative to interact with ROS in the way of achieving an uninterrupted cellular antioxidant system. Arjunolic acid has been also documented for a probable chelating action with arsenic to maintain the standard regulation of various cell signaling pathways (Manna et al., 2007).

Anti-inflammatory property of vitamin B_{12} leads to the alteration of oxidative stress (Wheatley, 2007). Glutathione sparing effect of B_{12} could reduce oxidative stress by involving the signaling

molecules that encourage methionine synthase activity (Veber et al., 2008). Vitamin B_{12} is also implicated for its direct reaction with reactive oxygen and nitrogen species (Veber et al., 2008). The transcription factor NF-κB is down-regulated by vitamin B_{12} that could decline nitric oxide yield and encourage oxidative phosphorylation (Wheatley, 2007). Cytokines release following arsenication in animals implies an imbalance between antioxidants and oxidative stress markers. The reduced level of cobalamin is the cause of inequity between cytokine and epidermal growth factor (Scalabrino et al., 2008). A deficiency of vitamin B_{12} leads to the accumulation of H_2O_2 in the uterus and finally decreases the activity of SOD (Jia and Domenico, 2010). Under B_{12} deficient conditions severe oxidative stress is induced and cellular redox homeostasis is abandoned (Bito et al., 2017). Vitamin B_{12} could diminish the lipid peroxide end product level. Vitamin B_{12} also contributes in restoring the activity of SOD and catalase in arsenicated rats (Bhattacharjee and Pal, 2014b). Reducing DNA disintegration and promoting histological outlook of the female tissues B_{12} limits apoptotic changes and cellular damages of ovary and uterus. Moreover, B_{12} is also implicated for its direct involvement in the methylation pattern of DNA. (Friso and Choi, 2002).

In conclusion, during the maintenance of normal growth, organ weight, antioxidant status and histoarchitecture of ovary and uterus B_{12} exhibited better effects in post-treated conditions than that of arjunolic acid. Vitamin B_{12} and arjunolic acid act as a safeguard to mitigate the inflammatory response of the arsenicated cells. Arsenic removal from the organs perhaps protects the apoptotic and necrotic tissue deterioration. B_{12} as a component of the methylation process probably eliminates arsenic from the body where methionine plays an important role. The results of the present experiment however explored a probable curative role of B_{12} and

arjunolic acid where these two may be the preferred choices as remedies against sodium arsenite induced female reproductive organs' anarchy.

5.5. EXPERIMENT-V

5.5.1. *In vitro* studies with Arjunolic acid and vitamin B_{12} of sodium arsenite treatment in mature Wistar rat:

5.5.1.1. Aims and Objectives:

This experiment has been designed to search out the events in hepatic tissue and reproductive organs and effectiveness of these bio-molecules against arsenic toxicity on hepatocelluar oxidative stress and female reproductive health hazards maintaining *in vitro* assay system in mature Wistar rats.

5.5.1.2. Treatment of tissue maintaining *in vitro* assay system:

The rats were anesthetized by intramuscular injection of ketamine HCl at the dose of 24 mg/kg body weight following the ethical guidelines of CPCSEA and finally euthanized by the intramuscular injection of an overdose of ketamine. The ovarian, uterine, and hepatic tissues were collected from the anesthetized rats. Through placing into a separate sterile bag the slices were transferred in an insulated ice container having − 20 °C temperature to the laboratory. Then weighed the samples and washed them with ice-cold Krebs's solution. The samples were allocated into different groups.

Groups I Control group,

Group II As^{3+} treated group (0.02 mg/0.09 g uterine slices and 0.02 mg/2 g liver slices),

Groups III H_2O_2 treated group (100 mM /0.09 g uterine slices and 100 mM/2 g liver slices),

Groups IV As^{3+} + H_2O_2,

Groups V As^{3+} + Arjunolic acid (0.02 mg/0.09 g uterine slices and 0.02 mg/2 g liver slices),

Groups VI As^{3+} + B_{12} (0.0018µg/0.09 g uterine slices and 0.0018µg/2 g liver slices),

Groups VII As^{3+} + H_2O_2 + Arjunolic acid,

Groups VIII As^{3+} + H_2O_2 + Arjunolic acid + B_{12}.

5.5.1.3. Results:

5.5.1.3.1. Observation of Lipid Peroxidation End Products:

The level of ovarian, uterine, and hepatic MDA and CD were enhanced significantly in arsenic, H_2O_2, and As^{3+}+H_2O_2 group when compared with the control value of 3 and 6 hrs incubation. (Fig. 5.1). But 6 hrs incubation showed more significant results than that of 3 hrs incubation. Results showed that the level of hepatic MDA and CD were reversed by the treatment with arjunolic acid and B_{12} alone and or in combination following 3 and 6 hrs incubation.

5.5.1.3.2. Effect of Arjunolic acid and vitamin B_{12} on Antioxidants Status:

The activities of ovarian, uterine and hepatic SOD and catalase were significantly diminished in arsenic, H_2O_2, and As^{3+}+H_2O_2 treated group than that of control following 3 and 6 hrs incubation (Fig. 5.2) but 6 hrs incubation had shown more inhibitory effect on these enzymatic activities. Electrozymographic documentation showed that the expression of ovarian, uterine, and hepatic tissue SOD and catalase were also decreased following the treatment with arsenic, H_2O_2, and As^{3+}+H_2O_2 treated group in 3 and 6 hrs incubation (Fig. 5.2). However, the activities of these enzymes were restored and reversed back significantly towards control following the treatment with arjunolic acid, B_{12} alone, or jointly for 6 hrs *in vitro* incubation.

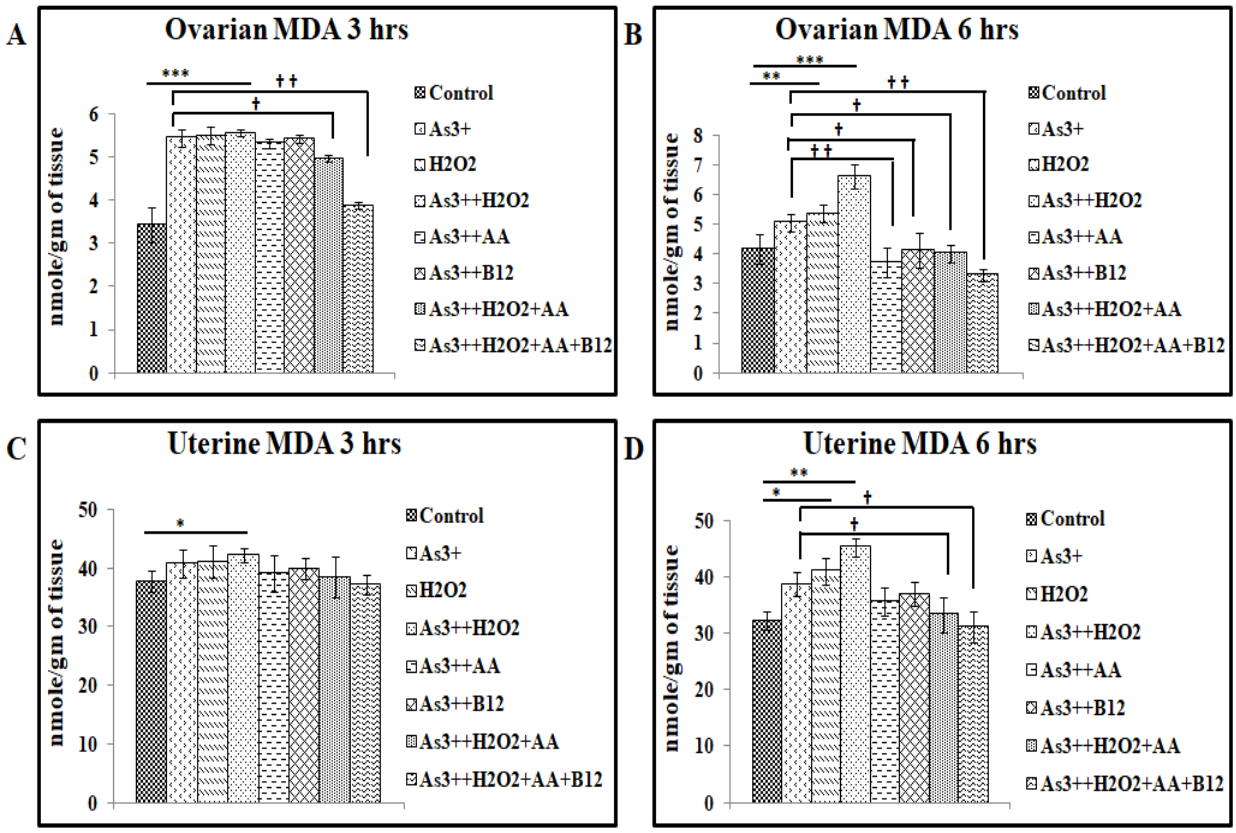
5.5.1.3.3. Ovarian steroidogenesis level:

The activity of ovarian steroidogenic enzymes i.e. Δ^5, 3β-HSD, and 17β-HSD was decreased in arsenic, H_2O_2, and As^{3+}+H_2O_2 treated group than that of control more significantly following 6 hrs incubation. Arjunolic acid and B_{12} had the ability to refurbish the level of ovarian steroidogenic enzymes towards normalcy (Fig. 5.3).

5.5.1.3.4. Comet assay:

The cellular DNA damage was shown as comet form in arsenic, H_2O_2, and As^{3+} + H_2O_2 group. The results of this experiment showed that arjunolic acid and B_{12} could limit the single-cell DNA damage in hepatic tissue in an *in-vitro* assay (Fig. 5.4).

Figure-5.1



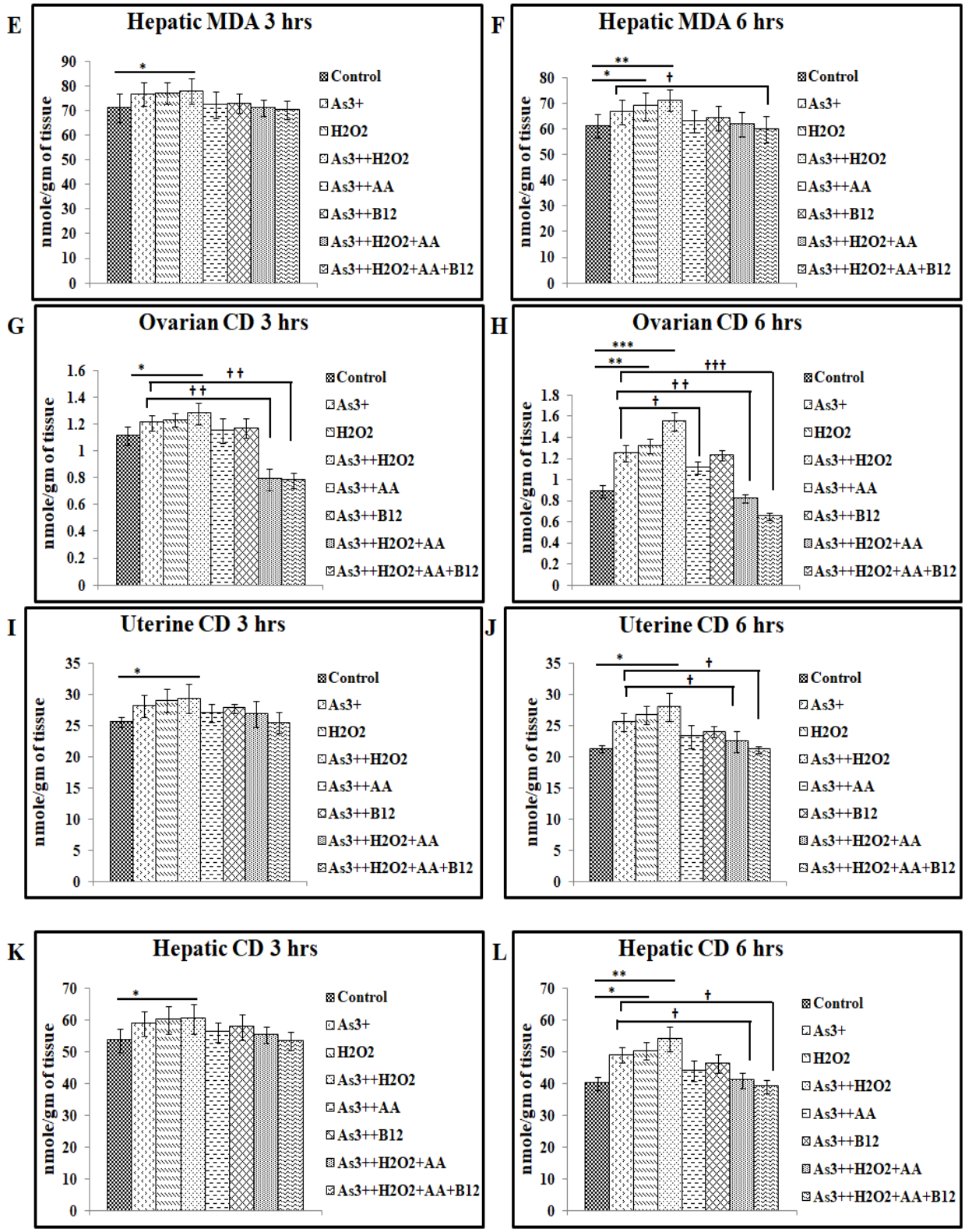
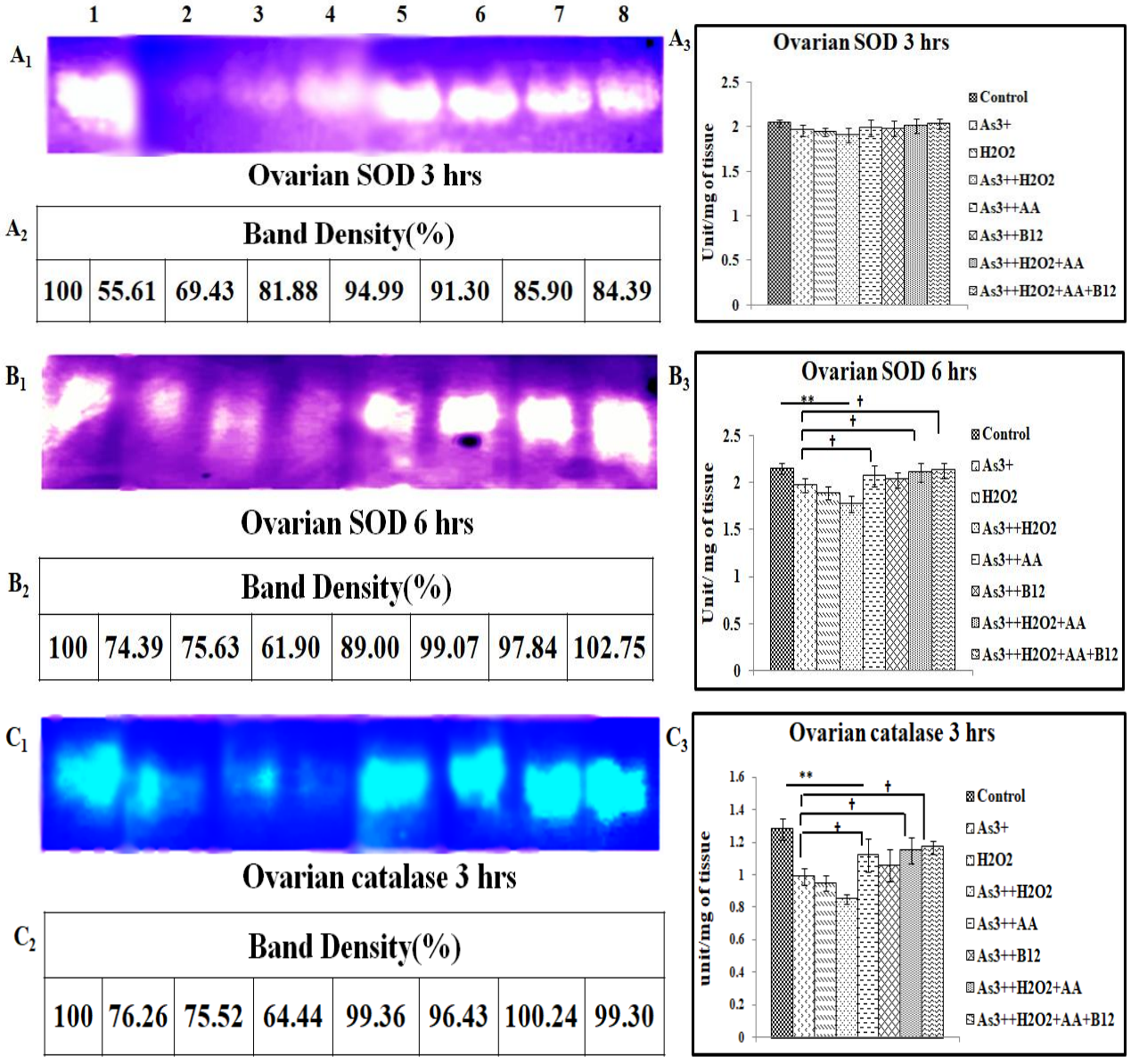


Fig. 5.1: (A, B, C, D, E, F, G, H, I, J, K, and L) Each bar represents mean ± SE, n= 6. ANOVA

followed the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **p<0.01.

Figure-5.2



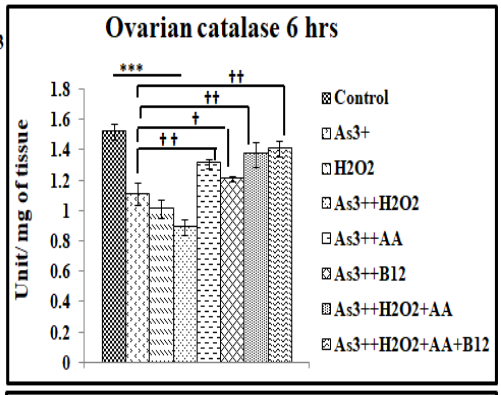
D_1

Ovarian catalase 6 hrs

D_2

Band Density(%)							
100	62.84	70.26	69.34	91.27	100.80	124.50	124.07

D_3



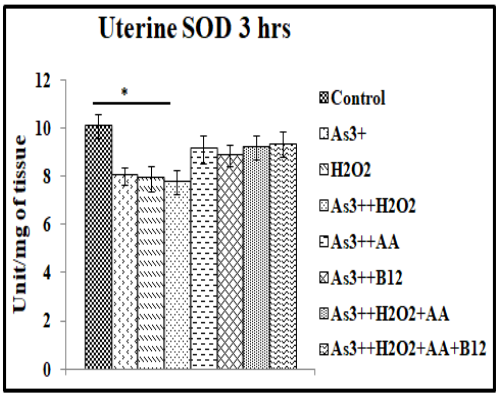
E_1

Uterine SOD 3 hrs

E_2

Band Density(%)							
100	72.06	82.87	80.20	96.13	93.69	95.02	87.70

E_3



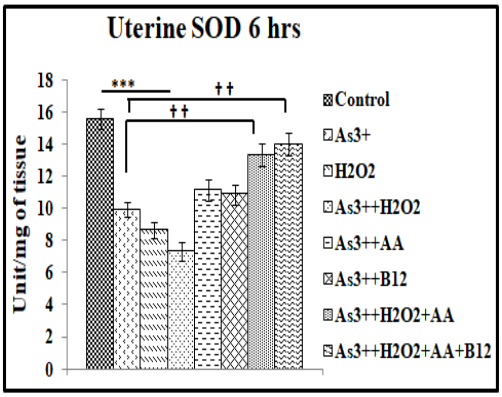
F_1

Uterine SOD 6 hrs

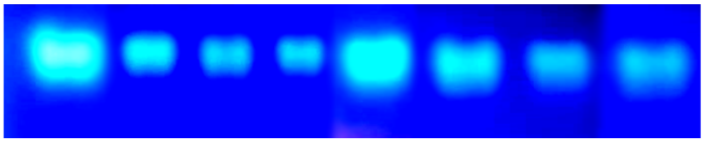
F_2

Band Density(%)							
100	72.26	84.29	81.13	119.58	103.70	92.41	108.17

F_3



G_1

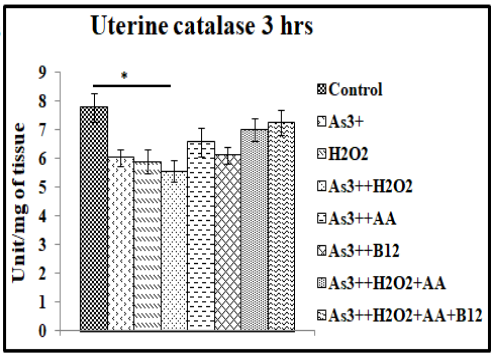


Uterine catalase 3 hrs

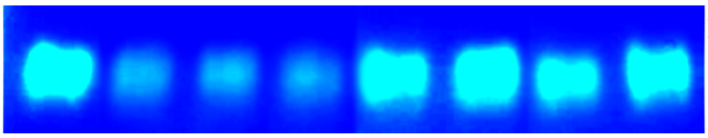
G_2

Band Density(%)							
100	69.20	64.12	62.38	89.81	80.12	75.43	76.72

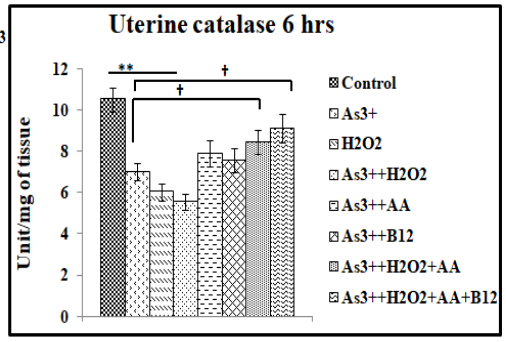
G_3



H_1



H_3



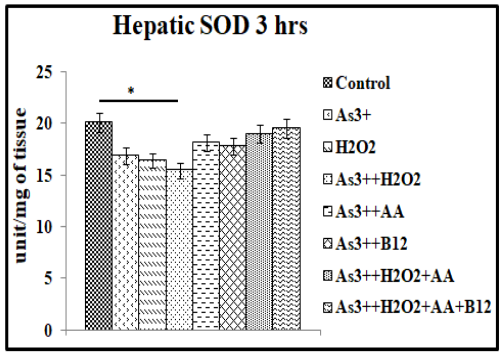
Uterine catalase 6 hrs

H_2

Band Density(%)							
100	75.13	74.79	72.99	98.87	100.11	93.79	96.52

I_1

I_3



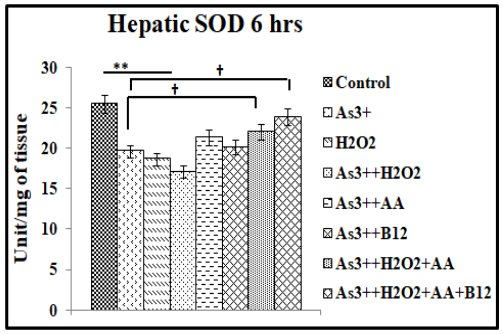
Hepatic SOD 3 hrs

I_2

Band Density(%)							
100	96.04	98.65	98.92	100.28	100.15	101.46	101.79

J_1

J_3



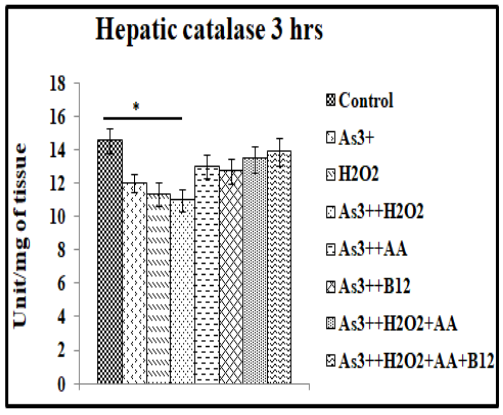
Hepatic SOD 6 hrs

J_2

Band Density(%)							
100	92.68	95.05	97.73	100.33	101.06	99.97	100.96

K_1

K_3



Hepatic catalase 3 hrs

K_2

Band Density(%)							
100	85.69	91.54	92.18	95.06	99.69	90.73	92.73

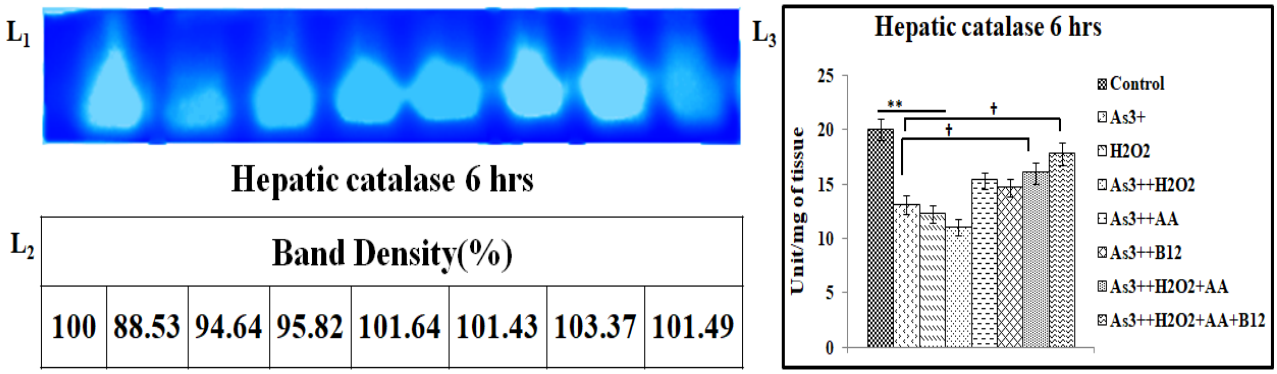
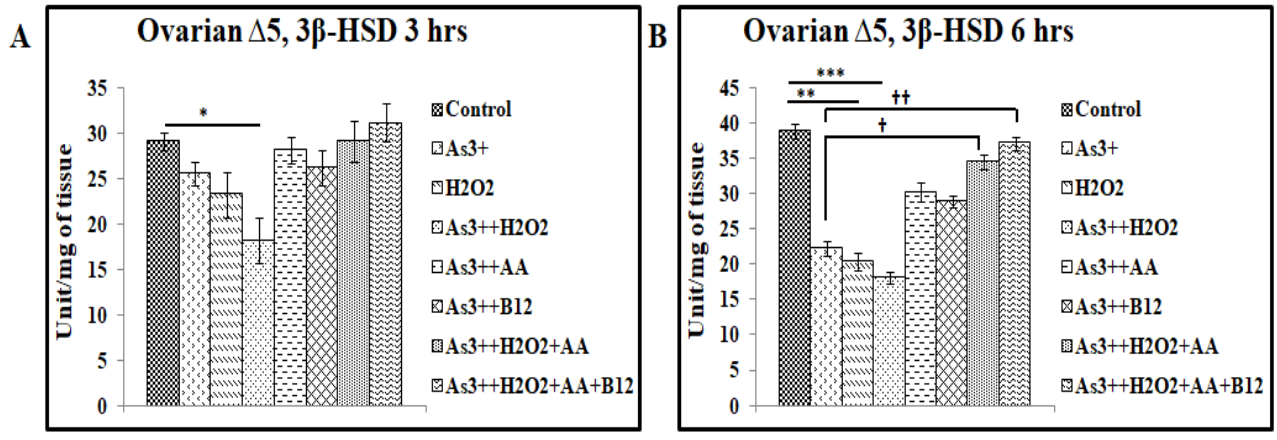


Fig. 5.2: (A, B, C, D, E, F, G, H, I, J, K, and L) SOD and Catalase 3 hr and 6 hr activity in ovarian, uterine, and hepatic tissue on a native gel. Lane distribution Lane 1: Control; Lane 2: As^{3+}; Lane 3: H_2O_2; Lane 4: As^{3+}+ H_2O_2; Lane 5: As^{3+} + arjunolic acid; Lane 6: As^{3+} + B_{12}; Lane 7: As^{3+}+H_2O_2+ arjunolic acid, Lane 8: As^{3+}+H_2O_2+ arjunolic acid +B_{12}. The tabular form represents the percentage of band density through densitometric analysis of electrozymogram. Each bar represents mean ± SE, n = 6. ANOVA followed the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **/††p<0.01, ***p<0.001.

Figure-5.3



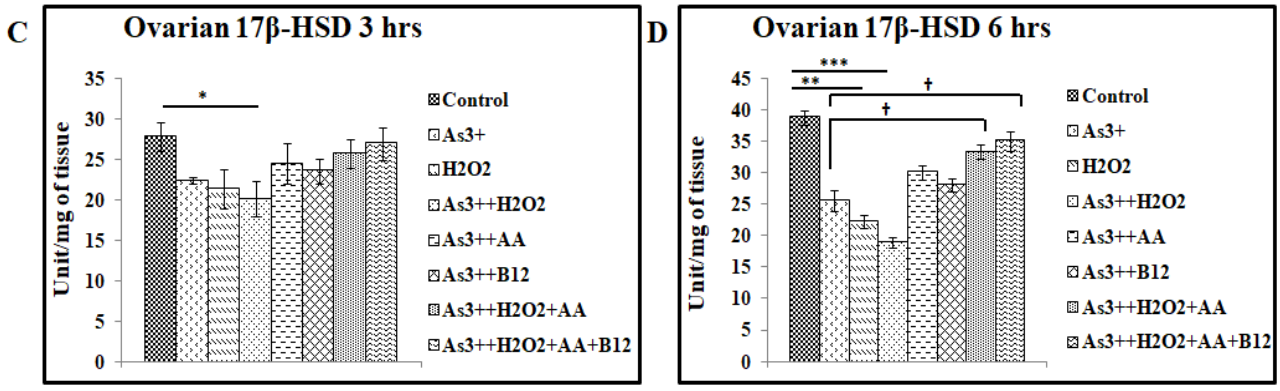
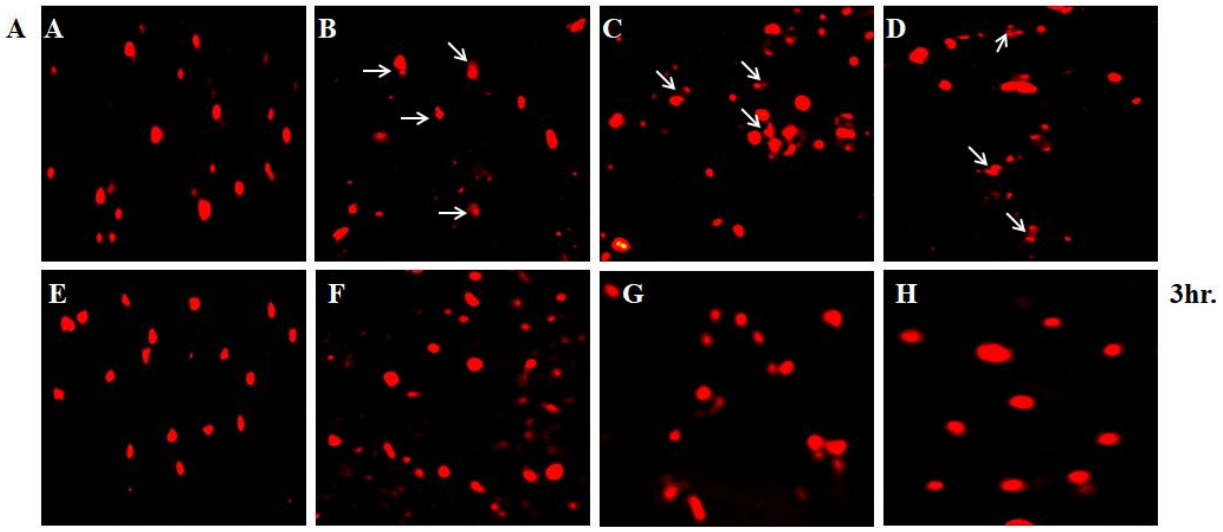


Fig. 5.3: (A, B, C, and D) Each bar represents mean ± SE, n = 6. ANOVA followed by the Post Hoc Dunnett test. * indicates the comparison between control and rest of the groups while † indicates comparison with arsenic-treated groups. */†p<0.05, **/††p<0.01, ***p<0.001.

Figure-5.4



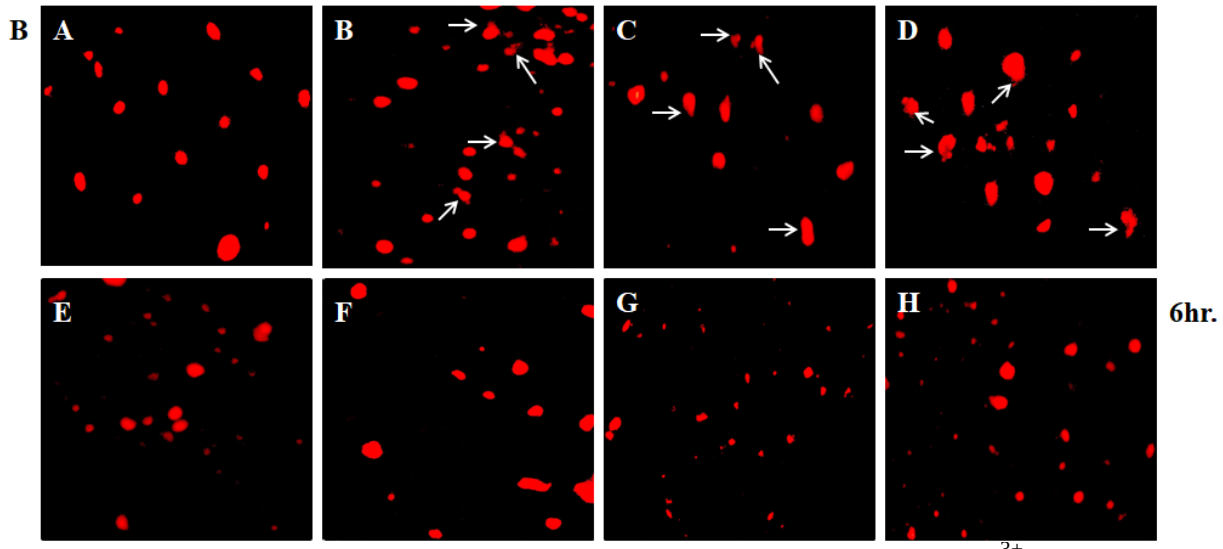


Fig. 5.4: (A) 3hr & (B) 6 hr Lane distribution; Lane A: Control; Lane B: As^{3+}; Lane C: H_2O_2; Lane D: As^{3+} + H_2O_2; Lane E: As^{3+} + arjunolic acid; Lane F: As^{3+} + B_{12}; Lane G: As^{3+}+H_2O_2+ arjunolic acid, Lane H: As^{3+}+H_2O_2+ arjunolic acid +B_{12}. Arrows indicate comet form.

5.5.1.4. DISCUSSION:

In association with toxicity in hepatic tissue and reproductive organs, a higher amount of reactive oxygen species (ROS) is generated in arsenic group. The generation of ROS is enhanced by arsenic exposure (Dwivedi and Flora, 2015). In vitro incubation with arsenic elevation in the ovarian, uterine, and hepatic MDA and CD level (Fig. 5.1) is indicative of the free radicals mediated hepatic damage as direct action. As^{3+} when reacting with H_2O_2 produces ROS and lipid peroxides and conjugated dienes as the end products (Wang et al., 2006). ROS is generated through the oxidative injury that develops hepatocellular deterioration (Cardin et al., 2014). Arjunolic acid and vitamin B_{12} showed direct action on the liver to improve the oxidative damage that occurred by arsenic *in vitro*.

The activity of SOD and catalase was reduced in arsenic, H_2O_2, and arsenic plus H_2O_2 group than the control group in ovarian, uterine, and hepatic tissue spectrophotometrically. The activity of these enzymes was restored by the direct exposure of arjunolic acid and B_{12} (Fig. 5.2) in duration-dependent manner (3 hrs and 6 hrs). We also demonstrated native gel electrophoresis of these enzyme proteins to evaluate the expression of these proteins and the extent of oxidative stress in ovary, uterus, and liver (Fig. 5.2). In arsenic, H_2O_2, and its combination group reduced the expression of SOD protein as documented from the appearance of faint band in ovary, uterus, and liver (Fig. 5.2) in 3 and 6 hrs duration. The strong band strength of SOD in uterine slices returned through the exposure of arjunolic acid and B_{12} towards control tissue slices (Fig. 5.2). Due to the inactivation of SOD, the superoxide anion radicals were enhanced that produced frequent downstream radical products in association with H_2O_2 (Watanabe et al., 2003; Knoefler et al., 2013). Arsenic had also direct interaction with the thiol residue of the enzyme. Hence, the inactivation of SOD in arsenic exposed group was found because of the effect of arsenic that led

to elevate H_2O_2 level in arsenic-treated group. Previous reports showed that modification of cysteine residues is responsible for the exhaustion of SOD and its subsequent activity in response to the exposure of arsenic and H_2O_2 that leads to oxidative stress (Acharyya et al., 2015). Others found that accumulation of H_2O_2 may be the possible cause of insufficiency of vitamin B_{12} that reduces the activity of SOD (Jia and Domenico, 2010). Alone or jointly arjunolic acid and vitamin B_{12} could restore the activity of SOD. However, the restoration of arsenic induced free radicals and cysteine modification were possible through 3 and 6 hrs direct exposures of arjunolic acid and vitamin B_{12} alone or in combination (Fig. 5.2). The activity of catalase was diminished significantly in arsenic, H_2O_2, and arsenic plus H_2O_2 exposed ovarian, uterine, and hepatic slices spectrophotometrically (Fig. 5.2) along with its expressional motif in duration-dependent manner. A faint band of catalase enzyme in arsenic/H_2O_2 indicates the reduced expression of this antioxidant enzyme (Fig. 5.2) in ovary, uterus and liver. The reappearance of a strong band in arsenic/H_2O_2 exposed uterine and hepatic slices exposed to in this *in vitro media* containing arjunolic acid and B_{12} could suggest a noticeable recovery of this protein in duration-dependent manner (Fig. 5.2). Decreased activity of catalase signifies that the detoxification of H_2O_2 from tissue was hindered from ovarian, uterine, and hepatic tissue. The exclusion of catalase activity due to the exposure of arsenic is indicative of intracellular ROS accumulation (Binu et al., 2017). The reduced activity of catalase was arbitrated via arsenic through the modulation of the catalase manifestation at the mRNA transcription level (Yang et al., 2012). In the active site of catalase metal or H_2O_2 might be pierced. During the changes in activity of catalase metal or H_2O_2 could interact with amino acids asparagine and histidine in its active site (Jakopitsch et al., 2003). It has been showed that vitamin B_{12} can recover the activity of SOD,

catalase through diminishing the level of lipid peroxide end product in arsenicated group (Bhattacharjee and Pal, 2014b).

Data of this experiment showed that the activities of ovarian steroidogenic enzymes were decreased in arsenic, H_2O_2, and its combination group in 3 and 6 hrs (Fig. 5.3). These enzymes were coupled with the regulation of gonadotrophin hormones and estradiol. Decreasing level of ovarian steroidogenic enzymes could inhibit the levels of estradiol (Hinshelwood et al., 1994). The serum levels of LH and FSH could regulate the activities of Δ^5, 3β HSD and 17β HSD (Odell et al., 1963). Following an environment of a low level of plasma gonadotrophins the activities of ovarian Δ^5, 3β-HSD and 17β-HSD were reduced (Ghersevich et al., 1994). The activities of these ovarian steroidogenic enzymes were repaired by the exposure of arjunolic acid and or vitamin B_{12} may be due to the direct association of these two in maintaining a comparatively free oxidative stress in the cells due to the restoration of SOD and catalase.

Inactivation of SOD and catalase promotes DNA damage by the accumulation of ROS (Wnek et al., 2011) and we demonstrated single-cell DNA damage (Fig. 5.4 A and B) in this experiment in a duration-dependent fashion. Elevating the ROS production arsenic could exert oxidative DNA with single-strand breaks (SSBs) and double-strand breaks (DSBs) through replication, prevention of repairing system of DNA, increasing mutagenesis and carcinogenicity (Rossman and Klein, 2011). ROS was produced due to the oxidative stress that alters protein, lipid, and DNA structure (Liu et al., 2001). S-adenosyl methionine (SAM) a methyl donor that leads the hypomethylation of DNA during the metabolism of arsenic. The methylated form of arsenic could develop tissue death via apoptosis that transforms the activity of SOD (Jomova et al., 2011). Hypomethylation was the result of the oxidative DNA breakage. The inorganic arsenic^{III} and DMA^{III} are more cytotoxic than its less methylated form (MMA^{III}). Inorganic arsenic^{III} and

DMA^{III} release more hydroxyl radicals ($HO^{\cdot}$). Due to the production of hydroxyl radicals, injury of DNA was materialized through the influence of SOD activity (Zamora et al., 2014). The damage of DNA could persuade apoptotic tissue damage (Vermeulen et al., 2005). In this experiment, the single-cell DNA damage was vetoed through the treatment with arjunolic acid and B_{12} alone or jointly in 3 and 6 hrs in hepatic slices (Fig. 5.4). The cellular damage of DNA in hepatic slices was found in arsenic and or H_2O_2 as the increase in the numbers of comet. However, and the protection was possible through the treatment with arjunolic acid and B_{12} alone or jointly that shown in Fig. 5.4 (A and B) may be due to the reduced oxidative stress by these two.

From our previous experiment, we found that the development of free radicals can be prohibited by the treatment with arjunolic acid in arsenicated group. Therefore, the antioxidant status was protected through the treatment with arjunolic acid and that might counteract the apoptotic injury and ovarian and uterine oxidative stress (Maity et al., 2018). Having two vicinal equatorial –OH groups arjunolic acid can bind with arsenic. The OH groups of arjunolic acid may be involved with As^{III} (with a lone pair of electrons of oxygen) for the development of a five-membered chelate complex. However the oxidative damages are reversed by arjunolic acid. One carboxylic hydrogen atom of the chelate complex between As^{3+} and arjunolic acid may be accountable to eliminate the free toxins through reducing the oxidative damage (Sinha et al., 2008b). Arjunolic acid alters arsenic induced transformation through chelation therapy in various cell signaling pathways (Manna et al., 2007).

B_{12} can reduce oxidative stress by involving glutathione sparing effect via changing the signaling molecules that can encourage the methionine synthase activity and it has also direct action with reactive oxygen and nitrogen species (Veber et al., 2008). During B_{12} deficiency, severe

oxidative stress is induced, and cellular redox homeostasis is ruined (Bito et al., 2017). It can forbid the apoptotic changes and cellular damages via averting the DNA disintegration. Vitamin B_{12} has a direct relation on the methylation pattern of DNA of genes (Friso and Choi, 2002).

From the above results of *in-vitro* assay, we can conclude that arjunolic acid and vitamin B_{12} alone or jointly can remove the toxicity with higher concentration of arsenic and H_2O_2 from systems in a short duration (3 and 6 hrs). Ovarian, uterine, and hepatic MDA and CD levels, ovarian, uterine, and hepatic SOD and catalase activity, ovarian steroidogenic enzymes, and hepatic cellular DNA damages are restored through the exposure of arjunolic acid and or vitamin B_{12}. Due to the abolition of arsenic and or H_2O_2 mediated oxidative stress from the organs, arjunolic acid and or vitamin B_{12} perhaps protect the apoptotic deterioration in tissue. Alone or jointly arjunolic acid and vitamin B_{12} have also accounted for direct effect in ovarian and uterine tissue at 6 hrs duration but have indirect effect in hepatic tissue at 3 and 6 hrs duration.