Chapter 6

Overall Discussion

6.0. OVERALL DISCUSSION:

Arsenic could damage the ovarian and uterine tissue at the dose of 1.0 mg/100gm bodyweight. A higher amount of reactive oxygen species (ROS) was produced in arsenic fed group in association with toxicity in hepatic tissue and reproductive organs. Others also explored that arsenic exposure can enhance ROS generation (Dwivedi and Flora, 2015). Different biological actions of arjunolic acid and vitamin B_{12} were already established for their antioxidant property. Previous studies showed that the hepatic organ (Chattopadhyay et al., 2012) and uterine tissues were protected by the treatment with vitamin B_{12} from arsenic toxicity in female Wistar rats (Deb et al., 2018). In experiment I, we have been planned to establish whether arjunolic acid and vitamin B_{12} prevent arsenic-induced repro toxicity in a dose-dependent manner. In this experiment, we used three different doses of arjunolic acid i.e. 0.5 mg, 1.0 mg, and 1.5 mg, and three different doses of vitamin B_{12} i.e. 0.07 μg, 0.09 μg, and 0.1 μg per 100 gm body weight. Experiment II has been designed to find out the preventive (pre-treatment) role of arjunolic acid and vitamin B_{12} alone or jointly against arsenic-induced toxicity on the female reproductive toxicity. Experiment III has been considered to search out the protective (co-administration) role of arjunolic acid and vitamin B_{12} on arsenic-induced hazards alone or in combination in female reproductive organs. In experiment IV, we intended to focus the mode of action of arjunolic acid and vitamin B_{12} alone or jointly whether mitigate the uterine and ovarian disorders in a curative way (post-treatment) against arsenic-fed female rats. Experiment V has been designed to search out the direct events in hepatic tissue's and reproductive organs' oxidative stress i.e. direct effectiveness of arjunolic acid and vitamin B_{12} against arsenic toxicity by introducing *in vitro* assay system.

In arsenicated group, the higher level of uterine MDA and CD was associated with the lower level of ovarian and uterine superoxide dismutase, catalase, glutathione peroxidase, and peroxidase. Wang et al., 2006 established that arsenic in association with H_2O_2, the tri-valent form of arsenic produced several lipid peroxides and conjugated diene as the end products and gathered reactive oxygen species (ROS) (Wang et al., 2006). The dose-dependent, preventive, protective, and curative study executed by us showed that arsenic toxicity distorted the ovarian and uterine tissue by the production of excess amount of MDA and CD and reduced the intracellular enzymes SOD, catalase, GPx, and peroxidase. Dash et al also found the same result (Dash et al., 2018). Present study explored that arjunolic acid and vitamin B_{12} have the ability to reduce the level of MDA and CD through increasing the antioxidant enzymes activity. In experiment V, *in vitro* incubation with arsenic increased the ovarian, uterine, and hepatic MDA and CD levels that were indicative of the free radicals mediated hepatic damage as direct action. The production of ROS through oxidative injury develops hepatocellular deterioration as described by 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 as described by the introduction of *in vitro* assay system.

It was already explored that 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). Production of superoxide anions ($O_2^{\cdot-}$) and initiation of oxidative stress in association with arsenic reduces the mRNA expression of the SOD gene as explored by Rana et al., 2012. The production of H_2O_2 is obvious by the transformation of superoxide anion radicals. Here, arsenic increased the level of free radicals since it may be related to the reduced activity of this enzyme. The catalase activity was also decreased in arsenic-treated rats in our study. Hence,

there is a possibility of decreased levels of H_2O_2 occurrence in ovarian and uterine tissue in response to the reduced activity of catalase. In arsenic ingested groups, the activity of peroxidase was also diminished in present study. This is indicative of the accumulation of H_2O_2 in the uterus that is associated with programmed cell death (Christine et al., 2010). During preventive, protective, and curative treatment in our study, the weak expression of ovarian and uterine SOD was found in the sodium arsenite ingested group as appeared from the electrozymographic image with faint band strength of SOD in comparison to the vehicle-treated control group. In arsenicated group, the diminished activity of catalase in ovarian and uterine tissue showed the indistinct band density in electrozymogram. Low band density showed decreased H_2O_2 detoxification from the ovary and uterus. In these experiments, in arsenic-fed rats, the weak band of GPx was found may be due to the gathering of H_2O_2 in ovarian and uterine tissue at the time of programmed cell death and this was in agreement with the findings of others (Do et al., 2003). In the *in-vitro* assay, the reduction of SOD and catalase activities in ovarian, uterine, and hepatic tissue were found during incubation with sodium arsenite, H_2O_2, and arsenite plus H_2O_2 group in comparison with the control group as detected spectrophotometrically and electrozymographically. The activity of these enzymes was restored by the direct exposure of arjunolic acid and B_{12} in a duration-dependent manner (3 hrs and 6 hrs). In experiment I, in arsenic fed rats, arjunolic acid and vitamin B_{12} with different doses maintained the uterine antioxidant enzymatic activities and lipid peroxide end products production within a limit and this may be via their possible own intrinsic antioxidant properties. The pre-treatment, co-administration, and 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. Other researchers also reported that arjunolic acid (Sinha et al., 2008b) and vitamin B_{12} (Bhattacharjee et al., 2013) would also protect the enzymatic antioxidants activity and could prevent the level of lipid peroxide end products in arsenic ingested rats. Arjunolic acid is known to have its protection against antioxidant enzymatic activities through the production of a chelate complex with arsenic or removing excess ROS (Sinha et al., 2008b). In the way of diminishing the excess arsenic accumulation the significant role of vitamin B_{12} was also established (Bhattacharjee et al., 2013).

Serum lactate dehydrogenase (LDH) is a biomarker of cancer. In the cancerous cell, the level of serum LDH is higher than that of the normal cell (Zhang et al., 2015). In our present preventive, protective, and curative treatment, the manifestation of serum LDH showed its elevation in arsenic-treated rats as detected via electrozymogram. In the histological tissue sections of the uterus, the apoptotic tissue injury was found during the elevation of the serum LDH. LDH might play an important role in the changes of uterine fibrous serum through stimulating collagen production (Iglesias et al., 1988). During arsenication, cell transformation and apoptosis are obvious due to the high level of ROS generation (Zhang et al., 2015). From our earlier experiment, we established that arjunolic acid could reduce the serum LDH activity in arsenicated group and might diminish necrosis (Maity et al., 2018). In these experiments arjunolic acid, vitamin B_{12} alone, or in combination also diminished the expression of serum LDH as evaluated via electrozymography.

A group of worker established that DNA damage was introduced through the decreasing activity of SOD and catalase via ROS production and oxidative stress and that was substantiated by DNA smearing (co-administration) and single-cell DNA damage in the arsenic-treated rats (co-administration, post-treatment, and *in-vitro* treatment). This finding was corroborated with the

study of others (Wnek et al., 2011). The formation of ROS starts the DNA damage and it also suppresses the DNA repair system and repair of oxidative DNA injury as described by Kligerman et al., 2003. ROS is known to produce during oxidative stress, and deteriorates the structure of protein, lipid, and DNA (Liu et al., 2001). S-adenosyl methionine (SAM) the methyl donor is leading to DNA hypomethylation during arsenic metabolism. Due to the methylated form of arsenic, the SOD activity is altered that might further 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 their less methylated form (MMA^{III}). Hydroxyl radicals ($HO^{\cdot}$) are released by inorganic arsenic^{III} and DMA^{III}. Hydroxyl radicals damage the DNA extremely under the changed influence of SOD (Zamora et al., 2014). The DNA damage could further encourage apoptotic and necrotic tissue damage (Vermeulen et al., 2005). However, our present study explored a significant level of DNA damage. Arjunolic acid due to its powerful antioxidant, free radical scavenging, and metal chelating properties might prevent oxidative DNA injury in our experiments by reducing ROS production and was also explained by Manna et al., 2007. Vitamin B_{12} could maintain the methylation process by protecting the DNA repair system (Fenech, 1999) and this notion was supported from the results of the above mentioned experiments.

The ovarian and uterine weights were diminished in arsenicated rats may be due to the low level of LH, FSH, and estradiol. Uterine weight is regulated by estradiol (Edman, 1983), whereas the weight of the ovary is regulated by gonadotrophins (Kulin and Reiter, 1973). Uterine malformation and diminution of the uterine somatic index were probably the outcome of downregulation of plasma estradiol signaling in arsenic-treated group which was further established by the reduced level of estradiol in arsenicated group in the present study. An

asynchronized estrous cycle pattern was observed by us as an outcome of constant metestrous or diestrous stage following 3 to 4 days of arsenic treatment. Following 3 to 4 days of the pre-treatment, co-administration, and post-treatment 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.

Previous studies showed that lower a dose of arsenic intoxication (0.4 ppm) for the duration of seven estrous cycles accumulated arsenic in uterine tissues (Chattopadhyay and Ghosh, 2010). The lower dose of arsenic treatment for 28 days showed the toxicity in different body parts (Chattopadhyay et al., 2012; Acharyya et al., 2015). In these experiments, we used a comparatively higher dose of arsenic (1.0 mg/100 gm body weight) for a short period with the lower dose of arjunolic acid (1.0 mg/100 gm body weight) and slightly higher doses of B_{12} (0.09µg/100 gm body weight) to investigate the preventive, protective, and curative effects of these bio-molecules on female reproductive organs. These doses were selected on the basis of the dose of arjunolic acid and B_{12} used by authors (Hemalatha et al., 2010; Vasanthi and Parameswari, 2012) (Mukherjee et al., 2006; Acharyya et al., 2015).

The water intake was higher in arsenic exposed group than that of other groups during the experiments (dose dependant, preventive, protective, and curative). Arsenic intoxication was also shown earlier 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. From the liver, the SGOT and SGPT are leaked into the serum due to inflammation. Hence, these hepatic enzyme levels were measured in our study from serum. In the co-administration study, the level of SGPT and SGOT were increased in arsenic-treated rats in comparison to the vehicle-treated control group. Pathological modification

in hepatic cells may be one of the important factors behind this change. The level of SGPT and SGOT was increased in serum may be due to hepatocellular necrosis. Henceforth, the increased permeability of the cell membrane may be one of the responsible factors behind the discharge of these enzymes into the blood stream (Vandenberghe, 1995; Rana et al., 1996). The level of these hepatic transaminases was diminished through the treatment with arjunolic acid and B_{12} alone or in combination in arsenicated rats.

From our previous studies, we observed that arsenic poisoning could diminish the activities of ovarian steroidogenesis (Chattopadhyay et al., 1999). Here, dose dependant, pre-treatment, co-administration, and *in-vitro* study also demonstrated that As^{III} could reduce the activities of these ovarian steroidogenic enzymes (Δ^5, 3β-HSD, and 17β-HSD). Decreasing level of ovarian steroidogenic enzymes could inhibit the level 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). Ovarian steroidogenic enzyme activities were suppressed due to the low level of serum LH and FSH and are also supported by the earlier study of Ghersevich et al., 1994. A higher concentration of ROS was shown to develop oxidative damage to the proteins with altering the estrogen signaling pathway as explored by Chatterjee and Chatterji, 2010. In arsenic-treated rats, arjunolic acid and vitamin B_{12} treatment diminished the level of ROS by refurbishing the activity of SOD and catalase in ovarian and uterine tissue in these experiments. Estrogen is known to regulate the activity of uterine GPx (Dimitrova et al., 2002). Estrogen has an important function for maintaining the activity of uterine peroxidase, and it recovers and propagates the endometrium layer of the uterus (DeSombre and Lyttle, 1979). Similar occurrence regarding GPx was noticed in our experiments. After ingestion of arsenic, the oxidative stress occurs in the uterus may be due to the endometrial ROS production (Chatterjee and Chatterji, 2010) which

may be supported by the low level of uterine SOD, catalase, GPx, and high level of uterine MDA and CD level in present study. There is a connection between the elevated amount of ROS and a lower level of estrogen following arsenication that further disrupts the endometrial cycle (Akram et al., 2010). In arsenic intoxicated rats, uterine cellular degenerations and indistinct uterine layers were found where the secretory cells were lost which was in agreement with the findings of others (Khorasani et al., 2011).

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). Arsenic can influence cell proliferation of porcine aortic endothelial cells (Barchowsky et al., 1999). The down-regulation of ERα was influenced by As^{3+} at mRNA and proteomic level and this could finally modulate estrogenic action (Chow et al., 2004). Our present study supported the above notion where post-treatment with arjunolic acid and vitamin B_{12} alone or co-jointly increased the estradiol receptor (ER-1) signaling and promoted the growth of ovary and uterus by maintaining the normal histo-architecture of ovary and uterus.

In arsenicated rats, the numbers of healthy follicles were diminished and the numbers of atretic follicles were elevated significantly may be because of the low level of plasma gonadotrophins and estradiol and that was earlier supported by others (Gore-Langton and Daniel, 1990). In pre-treatment, co-administration, and post-treatment studies, the ovarian oxidative stress occurred due to the consumption of arsenic and was also responsible for reduced level of ovarian SOD, catalase, and GPx in arsenicated group. The ovarian follicular deterioration was found in arsenic-treated rats. Oxidative stress developed because of the demolition of antioxidant enzymatic defenses in primordial and preovulatory follicles and was in agreement with the work of Tarin, 1996. The results of pre-treatment, co-administration, and post-treatment study were also similar

to the findings of others (Gore-Langton and Daniel, 1990; Maity et al., 2018) where arjunolic acid and vitamin B_{12} improved the different folliculogenesis of the ovary and decreased the follicular atresia. In arsenic intoxicated rats, vitamin B_{12} could elevate the ovulation process encouraging the development of the ovum as supported by other (Bennett, 2001). The development and proliferation of the uterine layers are maintained by the level of estradiol as described by Patil et al., 1998. The loss of uterine secretory cells developed in the uterine tissue. Arsenic-induced oxidative stress is known to degrade uterine morphology (Beltran-Garcia et al., 2000) in response to reduced uterine SOD, catalase, and GPx activity. In pre-treatment, co-administration, and post-treatment studies, the muscular layer of the uterus was thinned and responsible for the degradation of uterine cells via diminution of numbers of endometrial glands in arsenic-treated group. Earlier study suggested that in association with the rising level of ROS production, the endometrial cycle disruption was beginning via a low level of estrogen in arsenic ingested group (Akram et al., 2010). These types of ovarian-uterine disorders were inverted by the pre-treatment, co-administration, and post-treatment of arjunolic acid. Arsenic-induced apoptosis and necrosis in ovarian and uterine tissue were prohibited by this type of protective mechanism of arjunolic acid. Hence, arjunolic acid could recover the ovarian-uterine histo-architecture and uterine DNA damage in arsenic ingested rats. Excretion of arsenic is very important from the organs to diminish the ovarian, uterine stress and normalization of its morphology. Vitamin B_{12} acts as a co-factor for the synthesis of endogenous methionine from S-adenosyl homocysteine through the involvement of methionine synthase. For the elimination of arsenic, vitamin B_{12} may promote the methylation process through urine from the reproductive organs (Spiegelstein et al., 2003). Nevertheless, vitamin B_{12} bears significant role in preserving

the genetic materials and protecting the ovarian and uterine tissues from necrosis and probable carcinogenesis (Mukherjee et al., 2006).

The results of preventive and protective treatment showed that the levels of vitamin B_{12} and folic acid were reduced and homocysteine was elevated in arsenic-treated group. The elevated level of serum LDH is associated with the low level of B_{12}-folate as studied earlier (Hoffbrand et al., 1966; Keskin and Keskin, 2015). To prevent the necrosis of reproductive organs, these two vitamins are important. The decreased levels of vitamin B_{12} and folic acid were associated with the reduced level of arsenic detoxification through the delay in biliary arsenic excretion (Kile and Ronnenberg, 2008; Hall et al., 2009). In the methylated form (As^{III}), the biliary emission of arsenic might be promoted by B_{12} and folic acid. 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). In polycystic ovarian syndrome, the increased homocysteine level is generally decreased by B_{12} and folic acid (Kilicdag et al., 2005). In these experiments, arjunolic acid and vitamin B_{12} showed its ability to suppress the elevated level of homocysteine probably via restoring the circulating level of B_{12} and folic acid (Jayarajah, 2005). Homocysteine lowering ability of B vitamins is already a proven fact (Jeremy et al., 2007).

In inorganic arsenic metabolism, the pentavalent form of arsenic is transformed into trivalent form. Methyl group is integrated with the As^{III} during the biomethylation process of inorganic arsenic metabolism (Cullen et al., 1984). Using one-carbon (1C) metabolism, S-adenosylmethionine (SAM) and methyltransferase enzyme play an important role in the detoxification of arsenic from the body through the modulation of the methylation process via methionine cycle. Our previous studies showed that the levels of vitamin B_{12} and folic acid in circulation were reduced in arsenic-fed rats (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 investigations again explored and referred that pre-treatment, co-administration, post-treatment with B_{12} might improve the status of SAM in arsenicated animals where arjunolic acid also served a critical role in trapping arsenic (Maity et al., 2018). In the co-administration study, the level of serum vitamin C was elevated 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. Rats are able to synthesize vitamin C, and it might be possible that the level of vitamin C was significantly higher in arsenicated rats due to the arsenic-induced oxidative stress. Actually, vitamin C in rats could maintain the homeostatic adjustment against oxidative stress primarily towards a limited extend but this vitamin alone cannot prevent the oxidative stress generation for later period most probably due to the alteration of other antioxidant and oxidative stress markers in due course of arsenic intoxication.

In post-treatment experimental study, we examined the serum level of inflammatory marker (NF-κB) and pro-inflammatory cytokines (TNF-α, and IL-6). In this study, following the increased level of serum LDH in arsenic-treated rats, the level of uterine NF-κB and serum TNF-α, or IL-6 were elevated. 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, the levels of uterine NF-κB and serum TNF- α, or IL-6 in arsenic ingested rats were inhibited by the post-treatment with arjunolic acid and B_{12} alone or jointly. Arsenic may endorse toxic effects in cells and tissues by influencing NF κB signaling pathway (Wei et al., 2016). Some early response genes closely connected 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) and

we obtained similar results in arsenicated group in comparison with the vehicle-treated control group. NF-κB plays a decisive role during the regulation of cell proliferation, differentiation, and transformation (Huang et al., 1999). In arsenic exposed group, the elevated level of cellular reactive oxygen species could encourage in elevated expression of NF κB by up-regulating nuclear transcription of NF κB and thereby accumulate the production of inflammatory cytokines was supported by Hu et al., 2002. The cell signaling pathways may be altered by arsenic through changing the level of NF κB (Kaltreider et al., 1999). The level of NF κB was increased in arsenic fed group than that of the control. Apoptotic cell death in JCS-16 leukemia cells was seen during arsenic intoxication where TNF alpha might play a pivotal role (Mak et al., 2002). It was established that in arsenic intoxicated rats, T helper cells apoptosis is accelerated by tumor necrosis factor receptor 1 (TNF-R1) (Yu et al., 2002). Low level of ROS production during apoptotic cell death by arjunolic acid could further suppressed the NF κB and TNF-α activation and was documented earlier (Manna et al., 2009). Through down-regulating the level of NF κB vitamin B_{12} could also reduce the level of TNF-α, though it has an anti-inflammation property (Veber et al., 2008). However, our present findings also revealed a down-regulation of pro-inflammatory cytokines following the post-treatment with arjunolic acid and B_{12} and these results are in agreement with the findings of the above group of workers.

In this experiment, the level of metallothionein (MT) is raised in comparison to the vehicle-treated control group in the hepatic tissue of arsenic-treated rats. Bhattacharya and Bhattacharya reported that arsenic intoxication was the cause of enhanced level of MT in the liver (Bhattacharya and Bhattacharya, 2007). Our result was also similar with their investigation in this regard. 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 our study, the production of ROS and apoptosis was recovered initially probably by the elevated amount of MT1 expression in arsenic exposed group. The expression of MT acts on the cellular defense mechanism and protects the cells from reactive intermediates to arsenic toxicity (Bi et al., 2004).

In arsenicated group, the free radicals production was reduced by arjunolic acid. In arsenic-treated rats, the regular estrous cycle revival implies the potentiation of estradiol signaling through the treatment with arjunolic acid and was supported by Bennett, 2001. In arsenicated rats, arjunolic acid protected the ovarian and uterine oxidative stress, apoptotic, and necrotic injury probably via defending the antioxidant status against oxidative stress as well as inflammatory response which was also established by us earlier (Maity et al., 2018). Containing two vicinal equatorial –OH groups, arjunolic acid can bind with arsenic.

The OH groups of arjunolic acid might be involved with As (III) (with a lone pair of electrons of oxygen) for the development of a five-membered chelate complex (Maity et al., 2018). However arjunolic acid prohibits oxidative damage. The chelate complex eradicates free toxins through reducing oxidative damages. For the free radical scavenging activity of arjunolic acid one carboxylic hydrogen atom of the chelate complex is accountable (Sinha et al., 2008b). The carboxylic hydrogen atom which is present 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 can alter the arsenic-induced transformation through chelation therapy in various cell signaling pathways (Manna et al., 2007).

The anti-inflammation property of vitamin B_{12} can alter 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 occupied for its direct reaction with reactive oxygen and nitrogen species (Veber et al., 2008). Vitamin B_{12} down-regulates the transcription factor NF-kB and deteriorates the nitric oxide synthesis and encourages oxidative phosphorylation (Wheatley, 2007).

In the present study cytokines were released in arsenic-treated rats because of an imbalance between antioxidants and oxidative stress markers. The level of cobalamin reduction was earlier established as the cause of inequity between cytokine and epidermal growth factor (Scalabrino et al., 2008).The deficiency of vitamin B_{12} can accumulate the H_2O_2 in the uterus and decreases the activity of SOD (Jia and Domenico, 2010).

During the deficiency of B_{12} severe oxidative stress is induced and cellular redox homeostasis is ruined (Bito et al., 2017). Vitamin B_{12} could diminish the lipid peroxide end product level. Vitamin B_{12} can recover the activity of SOD, 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. Vitamin B_{12} has a direct relation to the methylation pattern of DNA (Friso and Choi, 2002).

We can conclude from the results of experiment I, 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) and 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. The above-mentioned doses are the critical dose of arjunolic acid and B_{12} that can effectively refurbish sodium arsenite induced alterations of uterine oxidative stress and ovarian

steroidogenesis through the restoration of lipid peroxidation, activities of enzymatic antioxidants, and steroidogenic enzymes.

The results of preventive treatment showed that in the restoration of body growth, organs weight, antioxidant status of ovary and uterus, arjunolic acid and B_{12} alone has beneficial preventive effects against arsenic-induced female repro-toxicity in Wistar rats. The ovarian as well as uterine histoarchitechtures are improved by these bio-molecules. Possibly through the elimination of arsenic from organs and lowering of lipid peroxide end products and serum LDH, the degradation of the tissues of these sex organs were prevented through apoptosis and necrosis. Arsenic may be removed from the ovary and uterus via methylation process where methionine plays an important role. The above study will be helpful for the preventive treatment strategy development in the arsenic affected people. Further study is necessary for this field.

The results of protective treatment explored that the genetic constituent and cellular synthetic mechanism can be protected by the co-treatment with arjunolic acid and B_{12} alone or in combination. In this experiment, vitamin B_{12} and folate might be protecting the necrotic tissue deterioration and it might be feasible from the possibility of arsenic removal from ovary and uterus. In the reconstruction of tissue structural materials and amino acid pool, this type of protective action is helpful. Arjunolic acid and B_{12} have a crucial role as active exogenous nutrients in the way of the protection of arsenic-induced ovarian-uterine toxicity and carcinogenicity. Other hand, arsenic is usually removed from the body via methylation process where methionine has an important role. Probably, arjunolic acid traps As^{III} due to its chelating property. Arjunolic acid may increase the bioavailability of vitamin B_{12} and folate which further improves arsenic detoxification. Therefore, oxidative stress which was induced by arsenic is

diminished in ovarian and uterine tissue. In the arsenic affected population, the above study will be helpful for the protective treatment strategy. Extensive study is essential in this field.

Curative treatment is more effective than those of preventive and protective treatment. In this experiment, the results prove that arjunolic acid and vitamin B_{12} have the ability to maintain the normal reproductive function in arsenicated female rats. This experiment showed that arjunolic acid and B_{12} had better beneficial curative effects in the renovation of body growth, organ weight, antioxidant status, and histoarchitechture of ovary and uterus in post-treated condition. Vitamin B_{12} and arjunolic acid act as safeguard to diminish the inflammatory response of the arsenicated cells. Perhaps, the apoptotic and necrotic tissue deterioration are mitigated by arsenic removal from the organs. B_{12} as a component of the methylation process probably eliminates arsenic from the body where methionine plays an important role. However, the results of this experiment 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.

Although some arguments came out from the outcome of above experiments. First argument is the level of MDA (1.23-1.81x) and CD (1.18-3.45x) in different experiments were increased in different ratio in spite of treatment with same amount of arsenic. From the results of our different experiments, it was apparent that changes of MDA and CD levels occurred at a different ratios. Such changes in ratio in different experiments are not clearly understood. It may be possible that the variation in ratio of MDA and CD in different experiments may be due to the biological systemic variation between rats or may be due to the seasonal variation during the experimental run. Second argument is that in experiment II, figure-3, in densitometric analysis, for uterus SOD and GPx, combined treatment of rat with AA and B_{12} decreased the protein level nearly to the

arsenic-treated group. Similar phenomenon was found for ovarian GPx. These findings are in contrary to the claimed result. Actually, the experiment-II is a pre-treatment study to find out the preventive effect of AA and B_{12}. Hence, the change in the nature of the experimental condition probably allowed the rat for biological adaptation of parameters though this type of incidence is yet to be understood properly from the current study. During this duration, arsenic was not given to them. The rats were fed with arsenic for the last eight days. For this reason, the combinational treatment with AA and B_{12} might not affect the uterine SOD and GPx protein and ovarian GPx protein against arsenic. Probably this is the reason for such type of occurrence of result.

From the results of *in-vitro* assay, we can conclude that arjunolic acid and vitamin B_{12} alone or jointly can remove the toxicity with a higher concentration of arsenic and H_2O_2 from systems in a short duration (3 and 6 hrs). The exposure of arjunolic acid and or vitamin B_{12} restored the ovarian, uterine, and hepatic MDA and CD levels, ovarian, uterine, and hepatic SOD and catalase activity, ovarian steroidogenic enzymes, and hepatic cellular DNA damages. Arjunolic acid and or vitamin B_{12} perhaps protected the apoptotic degradation in tissue due to the elimination of arsenic and or H_2O_2 mediated oxidative stress from the organs. Arjunolic acid and vitamin B_{12} alone or in combination were also accounted for direct effect in ovarian and uterine tissue at 6 hrs duration but had an indirect effect in hepatic tissue at 3 and 6 hrs duration.

Nevertheless, the noninvasive treatment strategy with arjunolic acid and B_{12} against arsenic-induced repro-toxicity highlighted an indirect action during the refurbishment of female reproductive function by the involvement of hypothalamico-pituitary-ovarian axis as indicated from the results of *in vivo* experiment-I-IV; although *in vitro* study (Experiment-V) indicated the possible direct action of arjunolic acid and B_{12} too against arsenic.