9.0. EXPERIMENT-5

Remedial effect of curcumin and CCPS against sodium arsenite mediated female repro-toxicity by *in-vivo.*

9.1. Objective of the investigation

1. In this experiment 20 mg/Kg BW of the curcumin and 2 mg/Kg BW of CCPS dose have been selected.

2. To explore the protective effect of curcumin and CCPS or curcumin-CCPS conjointly against arsenic mediated female repro-toxicity by co-administration study.

9.2. Experimental design

The female Wister rats of weight in between 110 ±10 gm were selected in this experiment. These rats were randomly divided into seven groups. The detailed procedure of this experiment has shown below:

Group-I: This group was control group with vehicle,

Group-II: Rats were treated with sodium arsenite (10 mg /Kg BW),

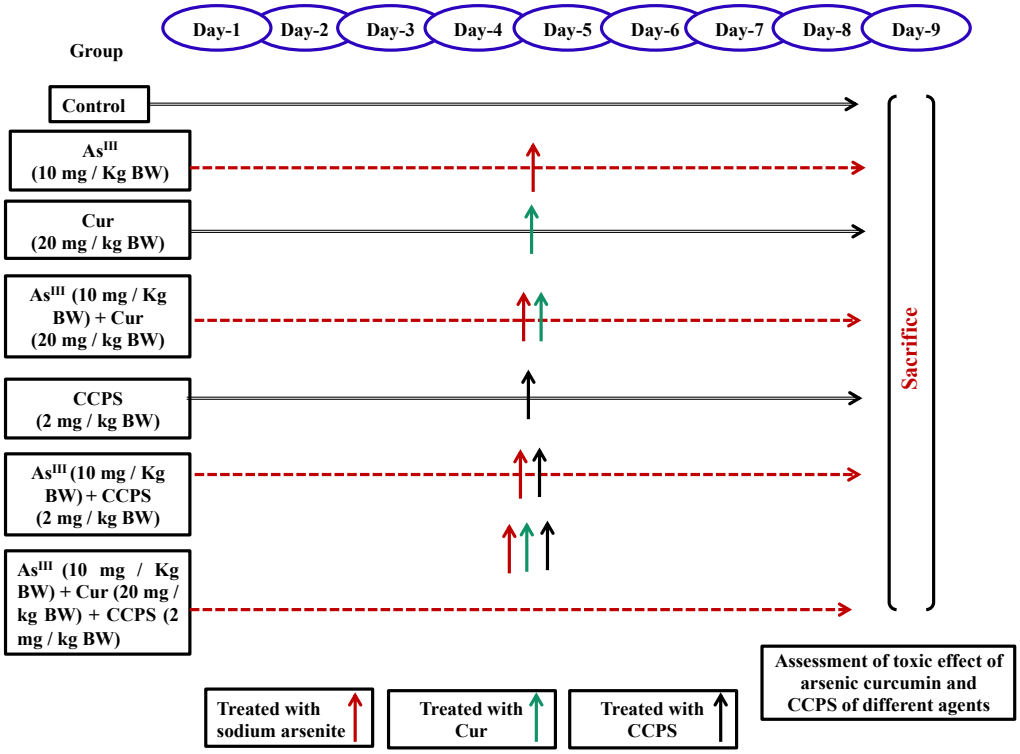
Group-III: Cur (20 mg/ Kg BW),

Group-IV: As^{III} (10 mg /Kg BW) plus Cur (20 mg/ Kg BW),

Group-V: CCPS (2 mg/ Kg BW),

Group-VI: As^{III} (10 mg /Kg BW) plus CCPS (2 mg/ Kg BW),

Group-VII: Cur (20 mg/ Kg BW) plus CCPS (2 mg/ Kg BW).



Seven group of rats were treated through oral gavage in co-administration mode for eight days. After 8 days, finally, body weights of these rats were recorded. Finally rats were anaesthetized by ketamine HCL. The collected samples were placed in separate bags and stored at -20^{0}C. Finally, these anaesthetized rats were euthanized using the over dose of barbiturate.

9.3. Results

9.3.1. Body growth and organ weights

Arsenic-treated rats gained their body masses than that of the control group (Table 9.1). A significant alteration was observed in uterine weight of arsenicated group when compared with control. Ovarian weights were notably increased in arsenic ingested groups when compared to the respective control group (Table 9.1). However, the co-treated curcumin and CCPS group significantly maintained the uterine weight. Both the curcumin and CCPS successfully protected rat's body weight along with uterine-ovarian weight in arsenicated group (Table 9.1).

9.3.2. Estrous cycle study

Control group of rats maintained the normal estrous cycle pattern whereas the regular pattern of estrous cycle was abolished in arsenic ingested rats (Fig 9.1). After 4 days the arsenication in rats shown a continuous diestrous stage. Normal estrous cycle pattern was reestablished when these rats were co-treated with curcumin and CCPS (Fig 9.1). Curcumin and CCPS in arsenicated group have exhibited a similar protective effect on the estrous cycle pattern (Fig 9.1).

9.3.3. MDA and CD assay

After arsenic treatment there no significant varriations were viewed in case of uterine MDA level (Fig 9.2). A remarkable elevation of the uterine CD in the uterine tissue was observed in arsenic ingested group in comparison with vehicle group. Also, co-treatment with the curcumin, CCPS alone and its combined mode CD level reduced significantly in arsenic ingested group (Fig 9.2). Here it was observed that uterine free radical product i.e CD production more successfully prevented when these rats were co-treated with curcumin and CCPS (Fig 9.2).

9.3.4. Uterine enzymatic antioxidant activity

Arsenic ingested group of rats has shown a significant reduction in the uterine SOD, catalase and peroxidase activities as compared to respective control (Fig 9.3A-3C). These uterine antioxidant enzymes activities significantly increased following co-administration of curcumin, CCPS and curcumin-CCPS in combination on arsenic ingested group (Fig 9.3A-3C). Further the above antioxidant enzymes were assessed by native gel electrophoresis. Electrozymographic study revealed that arsenic treated groups of rats had reduced uterine antioxidant enzymes expression (Fig 9.3D-3G). Above impairments of expression were significantly replaced by the betterment of the expression following CCPS and curcumin-CCPS co-treatment on arsenic ingested group (Fig 9.3D-3G).

9.3.5. Serum LDH study

As shown in Figure 9.4A, a significant elevation of serum LDH activity was observed by spectrophotometric analysis followed by presence of more dense band as detected electrozymographically (Fig 9.4B and 4C) in arsenic ingested group when compared with control. Above condition was natably recovered following the treatment with curcumin, CCPS and combine of curcumin-CCPS in arsenic ingested group (Fig 9.4A and 4C).

9.3.6. DNA and comet assay

Broken nature of uterine DNA was viewed in arsenic consumed rats as compared with control groups (Fig 9.5A). Curcumin and CCPS co-administration in arsenic exposed groups successfully mitigated the uterine damaging as documented by densitometric analysis (Fig 9. 5A). Fig 9.5B shows uterine DNA damage in arsenicated rats along with appearance of increased tail length as compared with the unexposed group. However, co-treatment of curcumin and CCPS on arsenic exposed group noticeably reduced the tail length (Fig 9.5B)

9.3.7. Serum vitamins and Hcy level

Arsenic ingested rats showed a deprination in the circulating level of serum vitamin B_{12} and also folic acid (Fig 9.6A and 6B) with an increase of serum Hcy level (Fig 9.6C) as compared to the control group. Co-treatment with curcumin and/or CCPS and combination fashion of curcumin-CCPS on arsenic ingested group significantly restored the level of above vitamins along with a decreased level of Hcy (Fig 9.6A-6C). However, curcumin and CCPS in arsenicated group showed a protective effect on the vitamins and Hcy level (Fig 9.6A-6C).

9.3.8. Ovarian steroidogenesis, gonadotrophins and estradiol level

In arsenic ingested group the activities of ovarian 17β-HSD and Δ^5, 3β-HSD (Fig 9.7A & 7B) were diminshed with a noteworthy decreased in the serum LH, FSH and estradiol when compared with the control group (Fig 9.7C, 7D and 7E). Co-treatment with curcumin, CCPS and combination fashion of curcumin-CCPS on arsenic ingestion group remarkably restored

of the above ovarian key regulatory enzymes activities and also reestablished the above level of gonadotrophins and estradiol (Fig 9.7A-7E).

9.3.9. Inflammatory markers and MT-1 level

An elevation of the inflammatory markers uterine NF-κB and IL-6, serum level of TNF-α and MT-1 was observed in arsenic ingested group (Fig 9.8A-8D). The diminution of the above inflammatory markers and MT-1 was noticed in following the co-treatment with curcumin or CCPS and combine of curcumin-CCPS in arsenicated rats (Fig 9.8A-8D). Curcumin and CCPS in arsenicated group significantly minimized the inflammatory response (Fig 9.8A-8D).

9.3.10. Ovarian and uterine histo-morphology

The numerous uterine layers were deteriorated and decreased the number of uterine secretory glands are shown in arsenic challenged rats as compared to the control rats (Fig 9.9A). The number of graafian follicles was reduced along with follicular atresia in the reproductive organ of ovaries of arsenic ingested group (Fig 9.9B). Still, co-treatment fashion with curcumin and/or CCPS on the arsenic ingested group re-established the ovaries and uterine horns from impairements of uterine layres, secretory glands in arsenic consumed rats (Fig 9.9A and 9.9B).

Table 9.1.

Groups	Body Weight (g)		Organo-somatic indices (g%)		Water intake (ml/100gm body weight)
	Initial	Final	Ovary in pair	Uterus	
Control	111±5.17	118±3.63	0.077±3.9	0.212±6	67.25±2.27
As^{III} (10 mg /Kg BW)	109±3.28	113±3.34	0.056±2.8*	0.157±20	77.25±4.48
Cur (20 mg /Kg BW)	117.4±8.15	122±8.4	0.083±5.5	0.208±34##	65.75±2.52#
As^{III} + Cur (10+20 mg /Kg BW)	115±7.47	119±8.29	0.079±5.9	0.203±35#	68.25±2.03
CCPS (2 mg /Kg BW)	116±1.67	122.6±0.45	0.085±3.6	0.217±1.7##	62±0.70#
As^{III} + CCPS (10+2 mg /Kg BW)	113.8±0.52	120.2±0.77	0.083±1.7	0.215±1.1##	62.5±1.02#
As^{III} + Cur + CCPS (10+20+2 mg /Kg BW)	115.2±0.52	118.2±0.52	0.078±1.14	0.214±1.1#	62±0.7#

Table 9.1. Protective effects of curcumin and CCPS on body mass and reproductive organs weight in arsenicated rats. Table represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test * indicate $p<0.05$ versus with vehicle whereas # and ## indicates $p<0.05$, $p<0.01$ versus As^{III} treatment.

Figure 9.1.

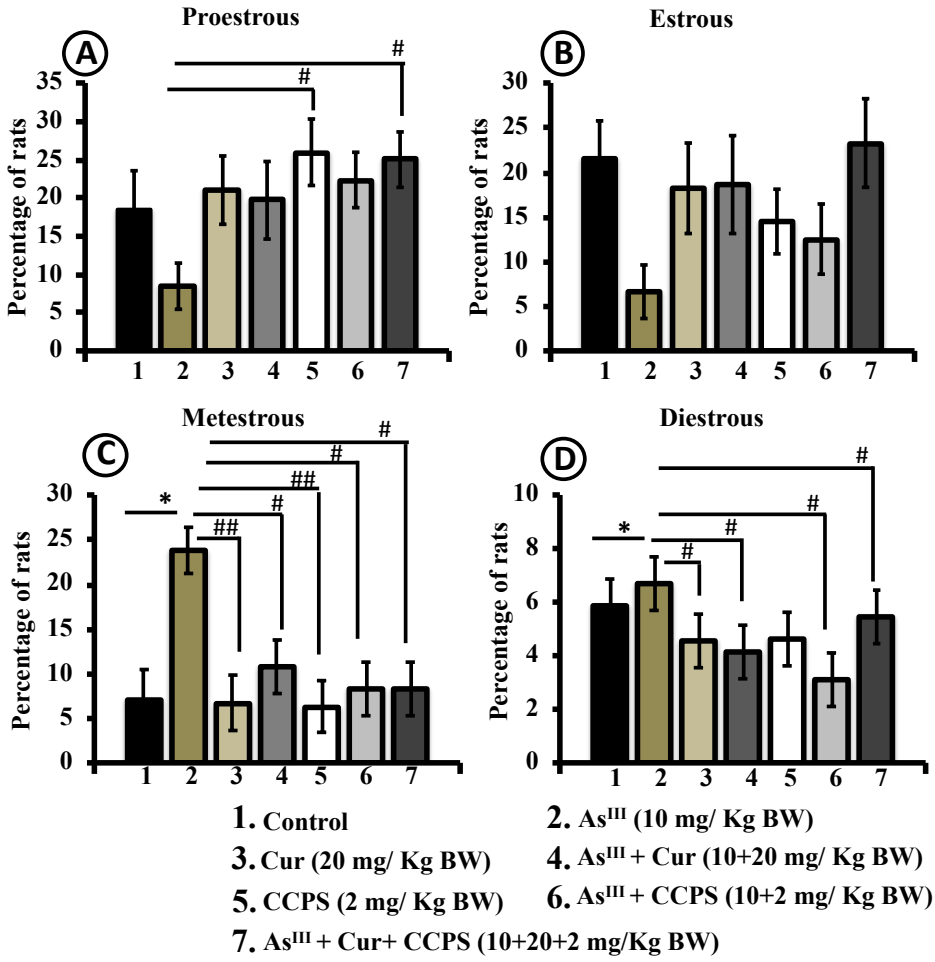


Fig 9.1. Protective effects of curcumin and CCPS on the pattern of estrous cycle against arsenic ingested different group of rats. Bar diagram represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test * indicate $p<0.05$ versus the control with vehicle, whereas # and ## indicate $p<0.05$, $p<0.01$ versus As^{III} treatment.

Figure 9.2.

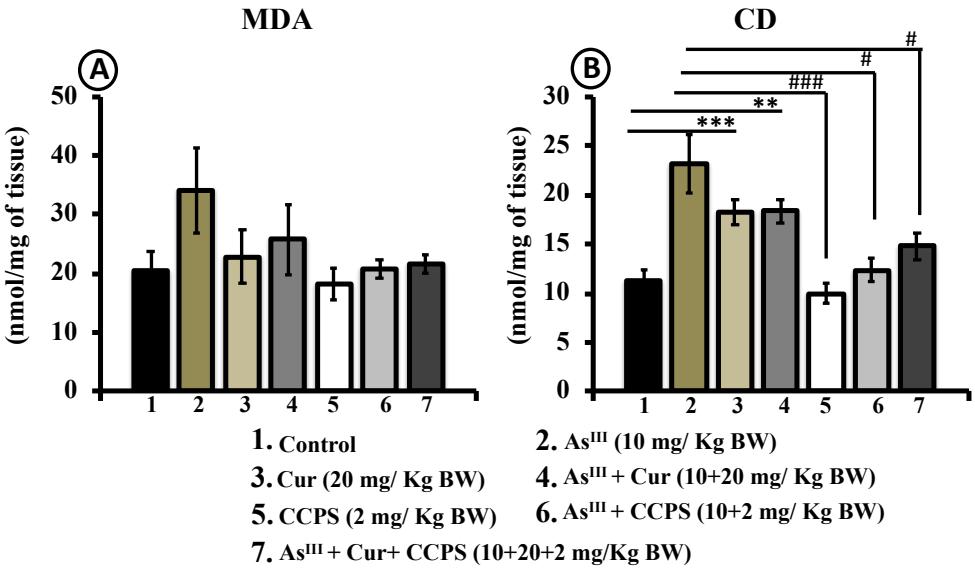
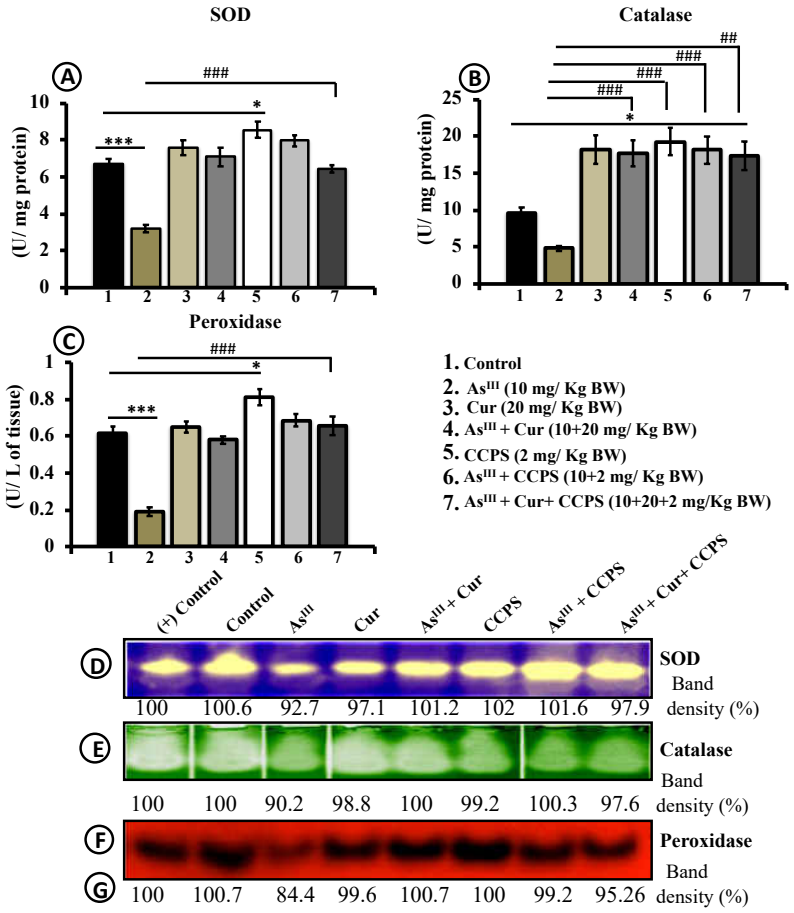


Fig 9.2. Protective effects of curcumin and CCPS on MDA and CD in uterine tissue against the arsenic challenged rats. Bar diagram represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test **,*** indicate $p<0.01$ and $p<0.001$ versus the control with vehicle, whereas # and ### indicate $p<0.05$, and $p<0.001$ versus As^{III} treatment.

Figure 9.3.

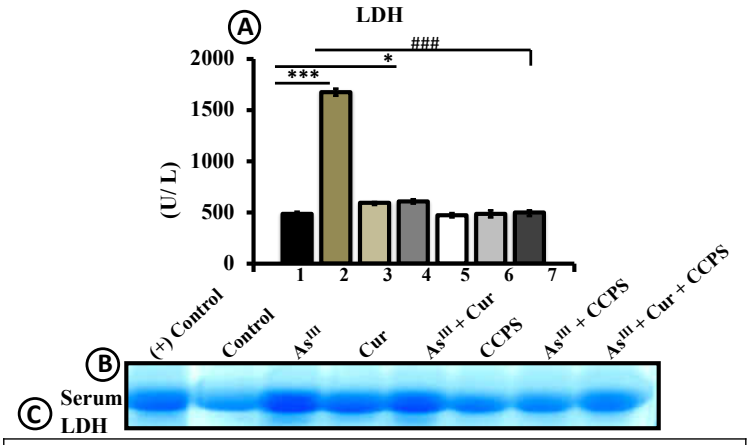


Statistical Comparison of the Band Density (%)

Uterine	Control	As^{III}	Cur	As^{III} + Cur	CCPS	As^{III} + CCPS	As^{III} + Cur + CCPS
SOD%	102.02± 0.77	94.14± 0.8***	99.62± 1.01#	102.05± 0.88###	101.33± 0.59###	101.74± 0.62###	98.89± 0.58#
Catalase %	100.29± 0.16	91.72± 0.49***	99.3± 0.21###	100.19± 0.22###	99.4±0. 17###	100.79± 0.24###	97.7± 0.21***###
Peroxidase%	100.89± 0.08	82.6±1. 42***	99.2±0. 31###	100.84± 0.31###	100.84± 0.31###	99.3±0. 16###	93.7± 1.3***###

Fig 9.3. (A, B & C) Spectrophotometric analysis showed the protective effects of curcumin and CCPS on uterine endogenous antioxidant enzymes activities against arsenic ingested different group of rats. Electrozymogram showed the protective effects of curcumin and CCPS on uterine expression of antioxidant enzymes. Table showing: (D) Band density (%) of SOD, catalase and PX respectively. Bar diagram represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test *, *** indicate $p<0.05$ and $p<0.001$ versus the control with vehicle, whereas # and ### indicate $p<0.05$ and $p<0.001$ versus As^{III} treatment.

Figure 9.4.



Statistical Comparison of the Band Density (%)							
Ovarian	**Control**	**As^{III}**	**Cur**	**As^{III} + Cur**	**CCPS**	**As^{III} + CCPS**	**As^{III} +Cur + CCPS**
LDH %	77.80±0.77	114.93±1.54***	101.3±1.07***###	104.3±2.13***###	84.2±0.92###	81.2±1.15##	86.6±1.43**###

Fig 9.4. (A) Spectrophotometric measurement showed the protective effects of curcumin and CCPS on uterine tissue necrosis level (LDH) against arsenic ingested different group of rats. (B) Electrozymogram showed that the protective effects of curcumin and CCPS on uterine tissue necrosis activity (LDH). Figure showing: (C) Band density (%) of serum LDH status. Data represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test *,**, *** indicate $p<0.05$, $p<0.01$and $p<0.001$ versus the control with vehicle, whereas ##, ### indicate $p<0.01$and $p<0.001$ versus As^{III} treatment.

Figure 9.5.

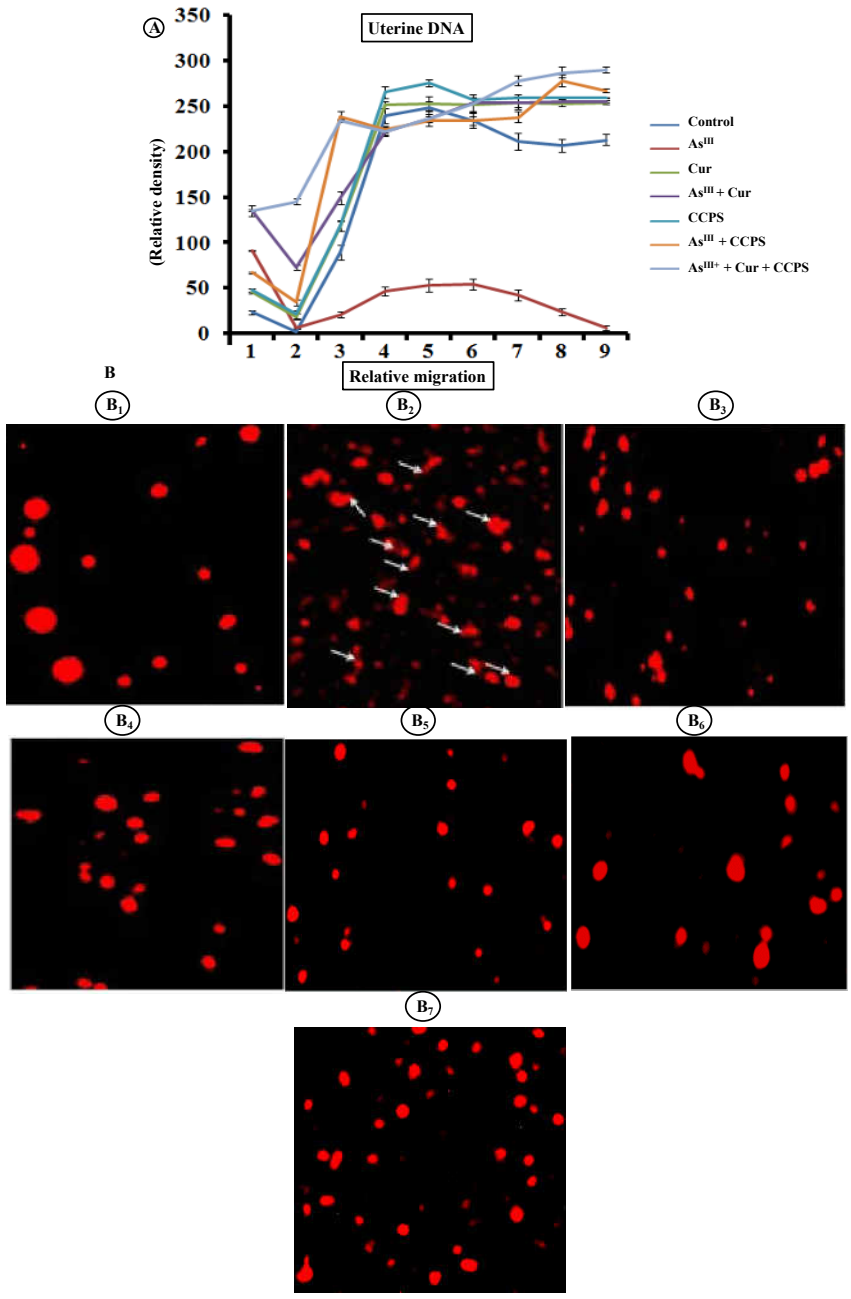


Fig 9.5. (A & B) Protective effects of curcumin and CCPS on uterine DNA damages against arsenic ingested different group of rats. Uterine DNA was evaluated by image J software. Figure B is showing the effect of curcumin and CCPS on DNA comet in uterine cells. Lane distribution; Lane B_1 indicate control with vehicle; Lane B_2 as As^{III}; Lane B_3 as Cur; Lane B_4 as As^{III} + Cur; Lane B_5 as CCPS; Lane B_6 as As^{III} + CCPS; Lane B_7 as As^{III} + Cur + CCPS.

Figure 9.6.

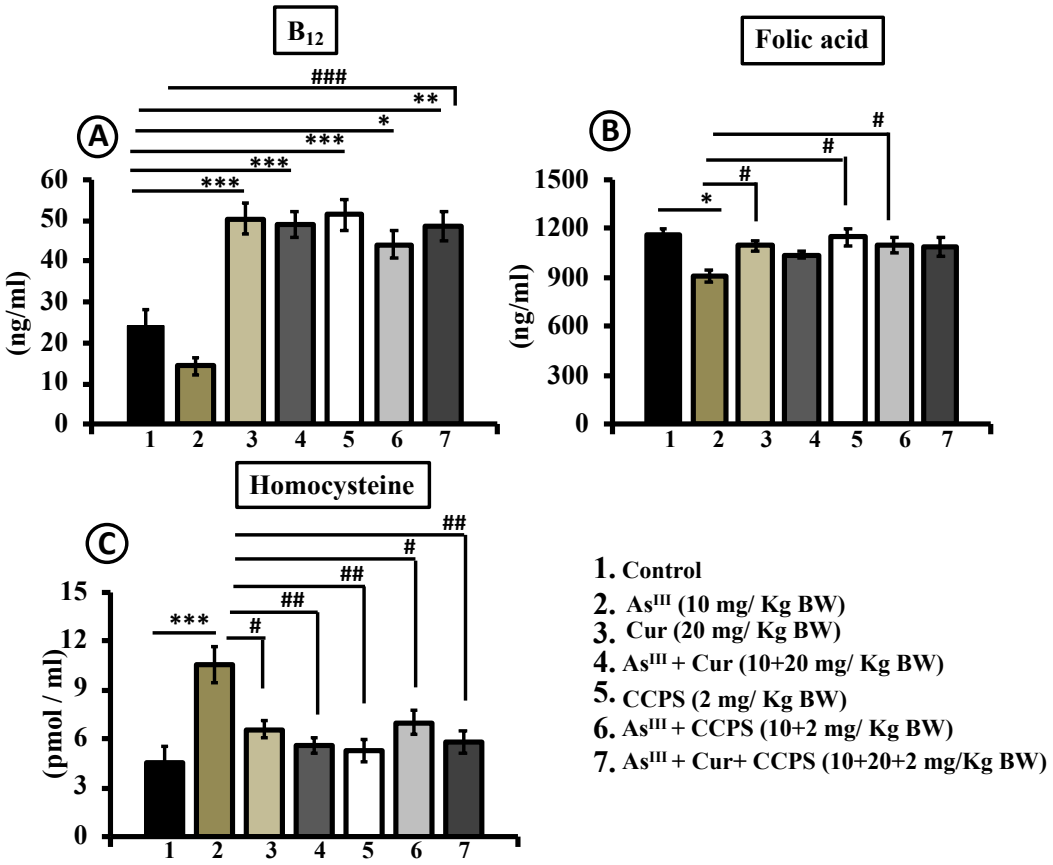


Fig 9.6. (A, B and C) Protective effects of curcumin and CCPS on serum vitamins and Hcy levels against arsenic ingested rats. Data represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test *, **, *** indicatesp<0.05, p<0.01 and p<0.001 versus the control with vehicle, whereas #, ## and ### indicate p<0.05, p<0.01, p<0.001 versus As^{III} treatment.

Figure 9.7.

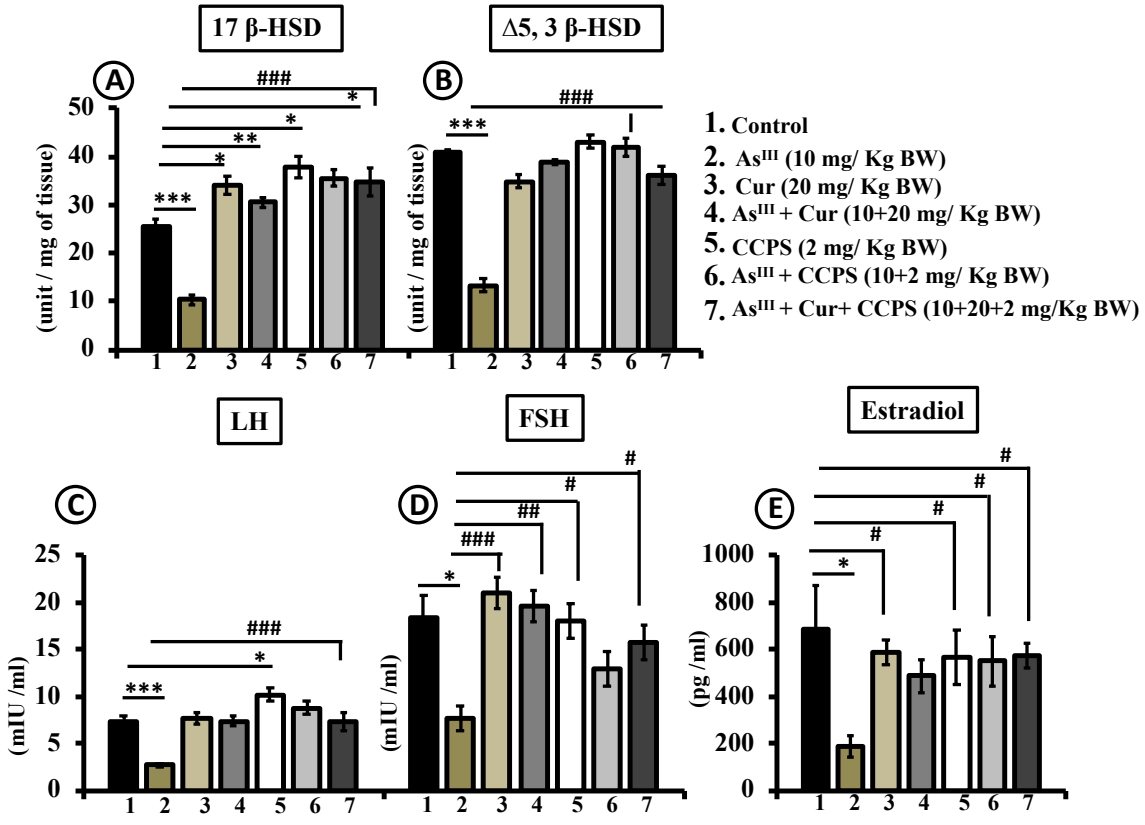


Fig 9.7. (A & B) Protective effects of curcumin and CCPS on ovarian steroidogenesis and gonadotrophins against arsenic ingested different group of rats. Data represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test *, **, *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ versus the control with vehicle, whereas #, ##, ### indicate $p<0.05$, $p<0.01$ and $p<0.001$ versus As^{III} treatment.

Figure 9.8.

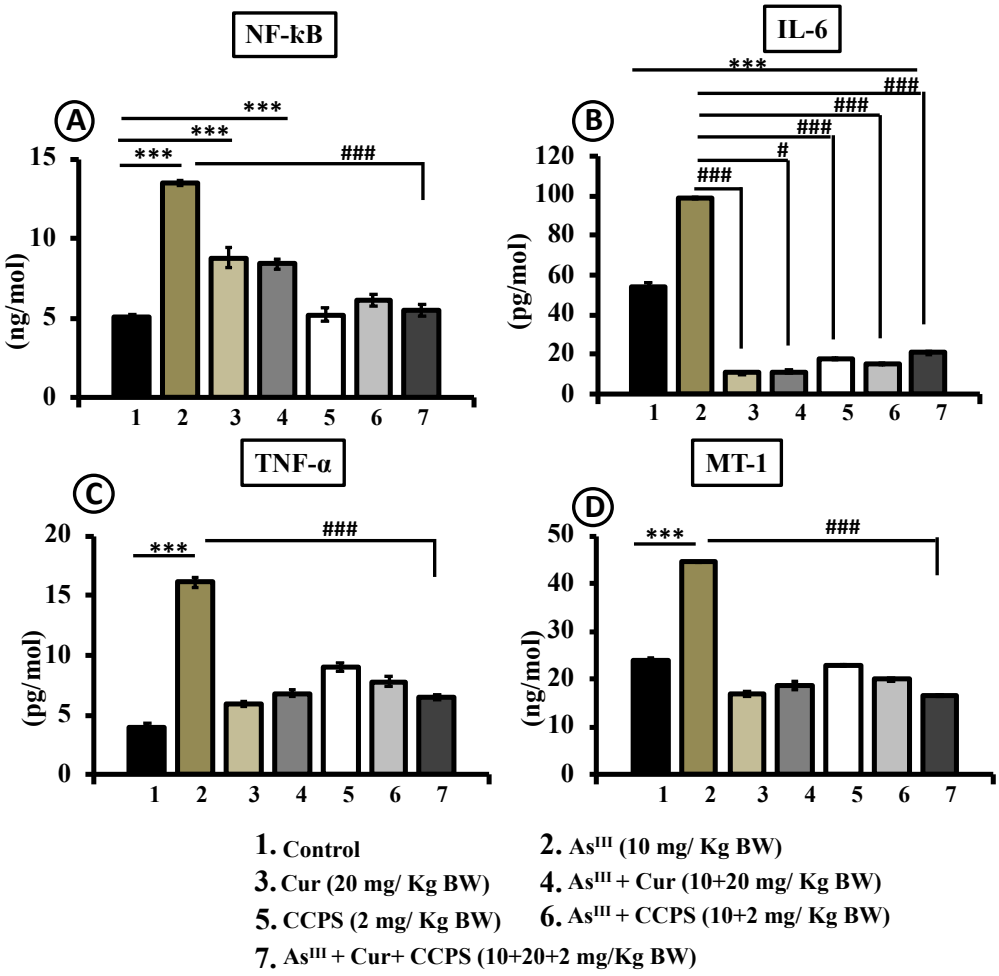
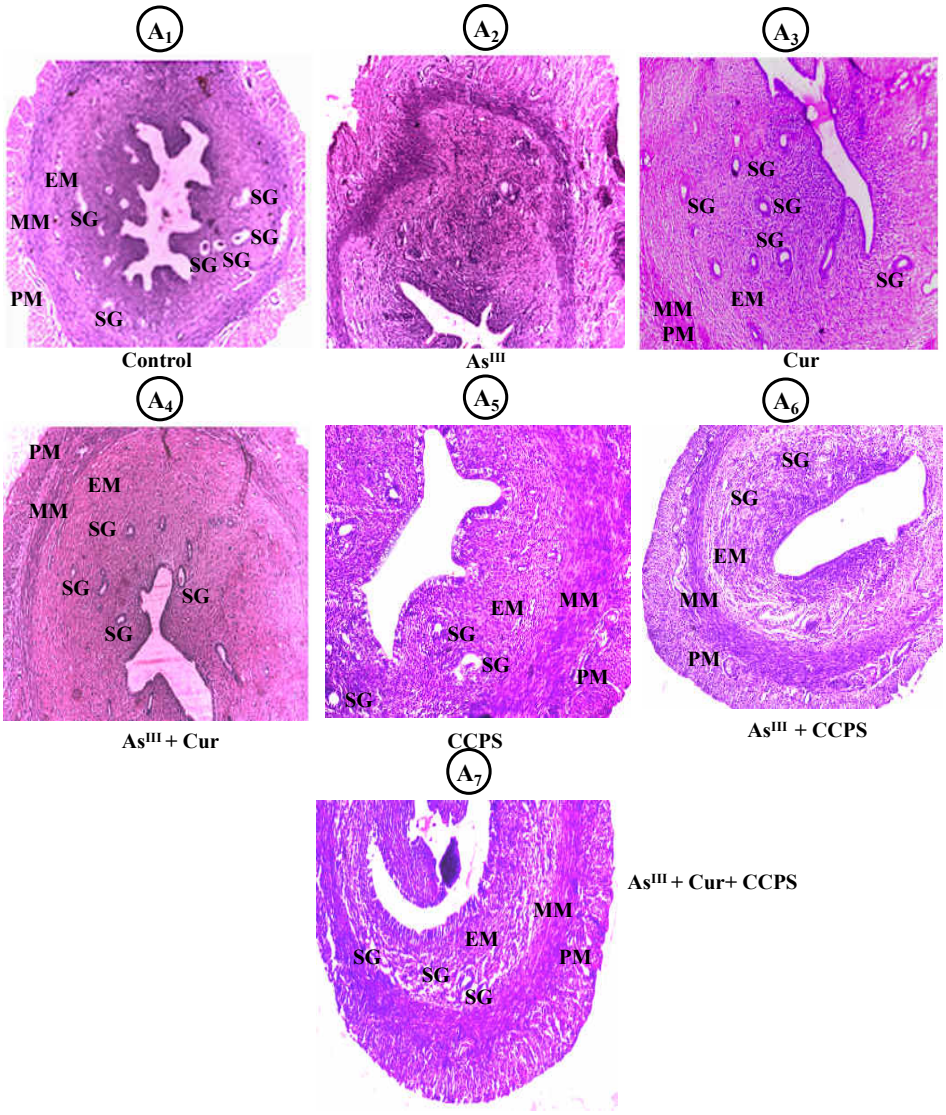


Fig 9.8. (A, B, C & D) Protective effects of curcumin and CCPS on pro-inflammatory markers against arsenic challenged group. All data represent mean ± SE, N = 6. Presented values here are expressed using one-way ANOVA with Dunnett's post Hoc t-test *, **, *** indicate $p<0.05$, $p<0.01$and $p<0.001$ versus the control with vehicle, whereas #, ## ### indicate $p<0.05$, $p<0.01$ and $p<0.001$ versus As^{III} treatment.

Figure 9.9A.


A1
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CCPS
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Figure 9.9B.

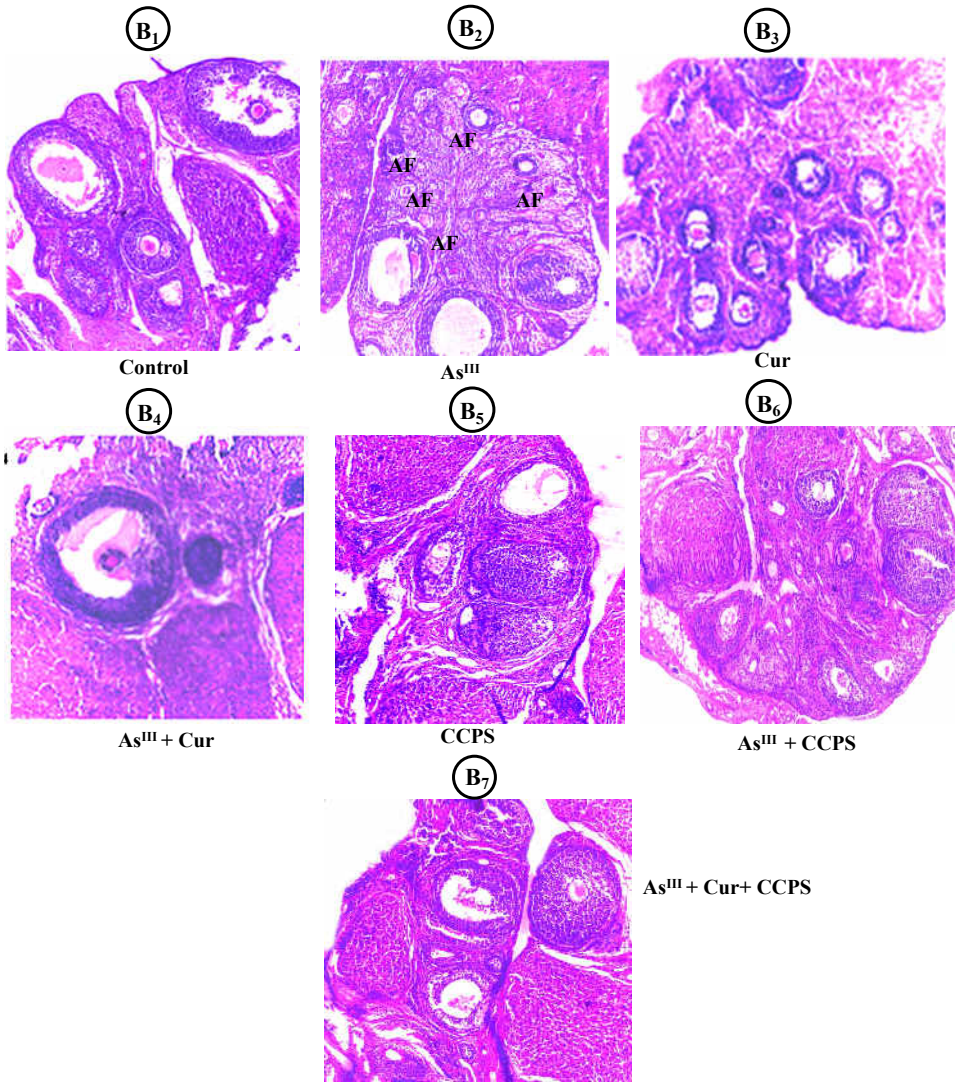

Fig 9.9A and 9B. Represent the protective effects of curcumin and CCPS in uterine and ovarian cell morphology against arsenic ingested different group of rats. Fig. 9A shows the uterine cell morphology. Here PM, MM EM denotes Perimetrium, Myometrium and Endometrium of the uterine layers and SG denotes the secretary glands. Arsenic treatment rats degenerates these layers but CCPS treatment rat recovered the three uterine layers. Fig. 9B shows the ovarian cell morphology. Here, AF denotes the atretic follicle. Numbers of atretic follicles here increased in arsenicated group.

9.4. Discussion

In this study, the arsenic noticeably induced the injury of uterus and ovary. Previous study has been reported that oxidative stress is increased due to arsenic in the female reproductive organs (Wang et al., 2017). Arsenic causes uterine and ovarian cell degeneration which by the over production of lipid peroxidation followed by a down regulation of antioxidant enzymatic activities. These reports are similar with the results of the present study (Fig 9.2 and 3). The intracellular enzymes SOD, catalase and peroxidase exert the first line of cellular protection. These enzymes play crucial role to diminish the toxic effects of the lipid peroxidation state (Ray and Husain, 2002). Arsenic exposure also elevates the serum lactate dehydrogenase (LDH) that indicating the possible risk of cellular necrosis and malignancy (Karim et al., 2010). A significant elevation of serum LDH activity in arsenic ingested rats was found in this study (Fig 9.4A). Higher expression of LDH following arsenic application has also been noted. (Fig 9.4B). It may be assumed that arsenic up-regulates serum LDH and protects uterine tissue from necrotic toxicity by minimizing the leakage of the LDH from tissue (Hoffbrand et al., 1966). Chronic arsenic exposure induces ROS coupled with the oxidative stress which promotes the uterine necrotic (Ciaccio et al., 1998) and apoptotic alteration induction of DNA damage (Li et al., 2002). The present investigation also reflects arsenic in rats noticeably prompted the uterine DNA damages (Fig. 9.5A) and single cell degradation (Fig 9.5B). Arsenic is know to promote the hypo-methylation of uterine DNA. It is also recognized to methyl giver of arsenic metabolism via SAM (Reichard and Puga, 2010). Arsenic and also its methylated form cause the modification of SOD activity which is one of the ways to develop the tissue necrotic and apoptotic death (Jomova et al., 2011). S-adenosyl methionine (SAM) is known to be a weak alkylating agent. It bears a methyl group that transferrs to the DNA during the non enzymatic-catalytic reaction and thereby generates the mutagenic lesions in DNA (Rydberg and Lindahl, 1982). Arsenic and also its methylated

form take part in the enhancement of the superoxide radical and hydroxyl radical and diminuation of SOD activity (Faraci and Didion, 2004). Generally, arsenic is detoxified by the methylation process of methionine cycle following the addition of SAM. The methylation of arsenic in methionine cycle uses vitamin B_{12}as well as folic acid for the protection of endogenous methionine. Hence, B_{12}act as a co-factor of methionine synthesis and folic acid that contributes to the methyl groups' 5-methyl tetra hydrofolate (Gamble et al., 2006; Ma et al., 1999). In addition, arsenic metabolism via one-carbon of SAM produces the s-adenosyl-homocysteine and methylarsonic acid which contributes in the production of homocysteine (Hall and Gamble, 2012). So, removal of arsenic is essential to restore SAM. Folic acid and homocysteine metabolism are also important for the protection of endogenous methionine level. In this study it was observed that the serum folic acid and vitamin B_{12} significantly reduced by arsenic (Fig 9.6A and 6B). At the same time it increased Hcy level (Fig 9.6C). It is also posible that higher level of Hcy suppresses follicular developments and oocytes maturation. Hyper-homocystemic condition could also decrease estradiol production that causes infertility. In addition arsenic also decreases the ovarian steroidogenic (Fig 9.7A and 7B) activities followed by lower level of gonadotrophins LH, FSH (Fig 9.7C and 7D). Continuous diestrous phases we noted in the arsenic exposed group that also causes the interaption of normal physiological process of uterus (Fig 9.1C and 1D). Previous study has been reported that arsenic suppresses mature follicular formation with existing follicular artesia which might be the response of the elevation of Hcy (Kanakkaparambil et al., 2009). Peroxyl and superoxide radical formation occur in hyper-homocystemic condition following the inhibition of cellular enzymatic antioxidants (Weiss, 2005). From these information it could be understood that over production of Hcy reduces arsenic elemation in methylated form. In addition the circulating levels of folate and B_{12} along with Hcy were altered due to arsenic ingestion and plays critical in the progression of tissue necrosis in uterine organ.

Arsenic mediated heper-homocysteinemia also causes uterine inflammation following the elevated expression of inflammatory markers and pro-inflammatory cytokines (Poddar et al, 2001). An increasing level of ROS due to arsenic exposure suppresses the oxidization of NF-κB activation in different cells and DNA binding capability of NF-κB (Fiers et al., 1999). Our study also confirmed that, the inflammatory factors uterine NF-κB, serum TNF-α and IL-6 increase in arsenic-challanged rats (Fig 9.8A-8C). During the apoptosis, the IKK-NF-κB pathway is helpful for maintenance of MT-1 level in cells and stops the accumulation of ROS in cells (Peng et al., 2007). It is also documented that a higher level of MT-1 was reflected in arsenic ingested rat (Bhattacharya and Bhattacharya, 2007). We also found the changes in MT-1 levels in similar fashion (Fig 9.8D). The MT-1 is synthesized in the most sensitive organ such as liver. The MT-1 has played a crucial role in heavy metal homeostasis and detoxified the metal from the body following its self defense mechanism. MT-1 contributes zinc to serve a free radical scavenging activities during the detoxification of metal (Verderame et al., 2017). Present investigation reflects the possibilities of curcumin and CCPS renovation of the uterine and ovarian functional disorganization because of its several interacting properties with free radicals arsenic. Curcumin and CCPS is known to have positive action on the activity of antioxidant enzymes (Panda et al., 2015; Larasati et al., 2018). Curcumin structure also contains the polyphenolic and the functional group of β-diketone and both are also coupled with the methoxy (R-O-CH_3) and CH_2 group. The presence of these groups in the curcumin makes enable its scavenging free radicals (Esatbeyoglu et al., 2012). CCPS also has a stronger free radical scavenging activity. It is to be noted that the curcumin and CCPS co-application decreased ROS production. Uterine SOD, catalase and peroxidase activities were counteracted by arsenic and CCPS in arsenic treated rats (Fig 9.3A-3G). In addition present study established that curcumin and CCPS counteracted the production of uterine MDA and CD levels (Fig 9.2A and 2B) in arsenicated group. These

results were found to be same with earlier findings (Yang and Shu, 2015). It was found that, curcumin and CCPS also limits the generation of tissue necrotic marker serum LDH (Fig 9.4A-4C). In addition curcumin and CCPS treatment significantly protected the DNA degradation of uterine tissue (Fig 9.5A) and the single cell DNA damages in response to oxidative damages (Fig 9.5B). From the above results it has been predicted that the curcumin and CCPS could successfully improve the uterine damages following the minimization of the oxidative stress and tissue necrosis marker. This nature of improvement may also be successfully initiated by several ways. Curcumin and CCPS treatment on arsenic ingested rats controls uterine and ovarian damages via improving the circulating vitamin levels. Curcumin and CCPS in arsenicated rats successfully diminished Hcy level following the improvement of the vitamin B_{12}-folate levels (Fig 9.6) (Marcus et al., 2007). Hence it is speculated that curcumin and CCPS like herbal could effectively maintain the absorption rate of these vitamins to increase the bioavailibility. Nevertheless, it is also hypothesized that curcumin and CCPS exert its pivotal action on hypothalamic pituitary-ovarian axis to improve ovarian steroidogenesis via gonadotrophins LH and FSH in the arsenic ingested rats (Fig 9.7A-7E). Aktas et al 2012 has been reported that, the curcumin also prevent the ovarian follicular artesia from ROS mediated apoptosis. Present study documated that curcumin and CCPS contributes in mproving the uterine and ovarian histo-architectures (Fig 9.9A and 9B). Oral application of arsenic when coupled with curcumin and CCPS. It minimizes follicular artesia and protects the uterus from degenaration (Fig 9.9A). Curcumin and CCPS also diminished the steroidogenic enzyme 3β HSD and 17β HSD activities. Hence, it is confirmed that the curcumin and CCPS have a direct effect on hypothalamic pituitary-ovarian axis. Curcumin and extracted polysaccharide from CCPS are known to be projected positive action on different inflammatory markers (Esatbeyoglu et al., 2012; Panda et al., 2015). Curcumin suppresses NF-κB activation and controls other inflammatory markers to defend the cell from

inflammation (Li et al., 2016). On the other hand CCPS could also suppress the inflammatory markers NF-κB, TNF-α, and IL-6 via NF-κB signalling pathway (Mohammad et al., 2016). Similar instant was documated in curcumin and CCPS co-treated arsenic ingested rats where we also found the suppression of NF-κB, TNF-α and IL-6 (Fig 9.8A-8C).

We conclude that oral application of curcumin and CCPS alone or in combination played a critical as well as protective role against arsenic induced female repro-toxicity. Here it is hypothesized that curcumin bears a unique chelating property by which it may chelate with arsenic and detoxify arsenic from the tissues by methylation process. On the otherhand CCPS has galacturonate acid residue in its structure and that may interact with the arsenic during its detoxification (Fig 9.10).

Figure 9.10.

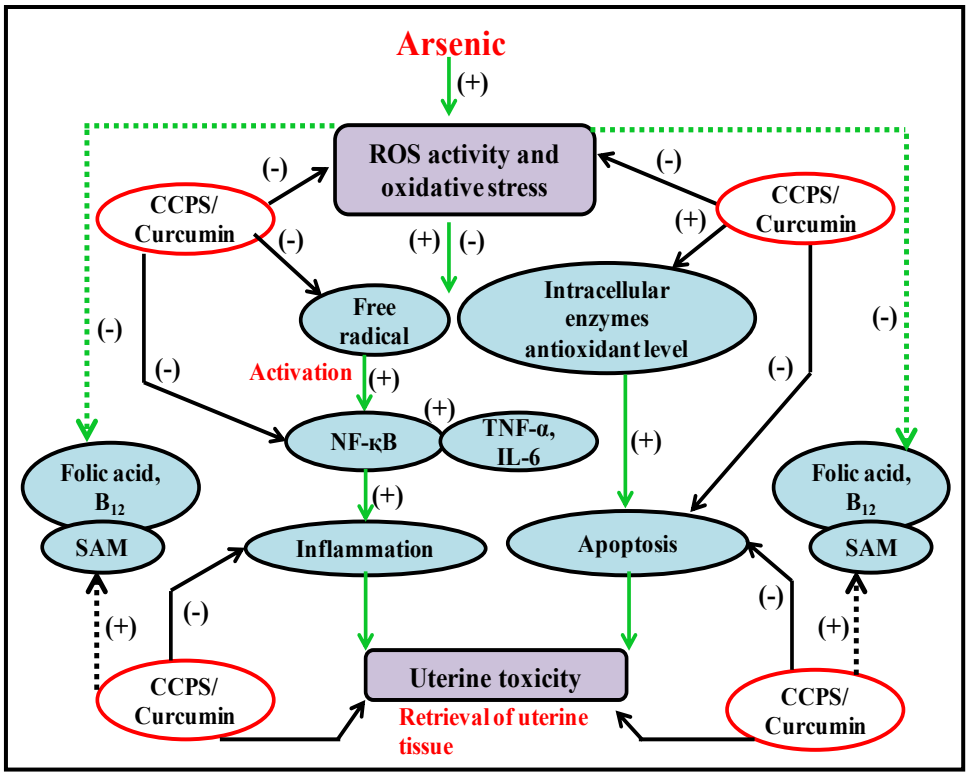

Fig 9.10. Schematic scheme represent the hypothetical mechanism of curcumin and CCPS alone or combination action against arsenic caused uterine toxicity. Black colour **(+)**, **(-)** sign and green line indicate stimulatory and inhibitory effects of arsenic respectively. Black colour **(+)**, **(-)** sign and black line indicates stimulatory and inhibitory effects of curcumin and CCPS respectively.