6.0 EXPERIMENT-2A

Efficacy testing of CCPS and curcumin on arsenic-exposed liver slices maintained *in vitro* condition.

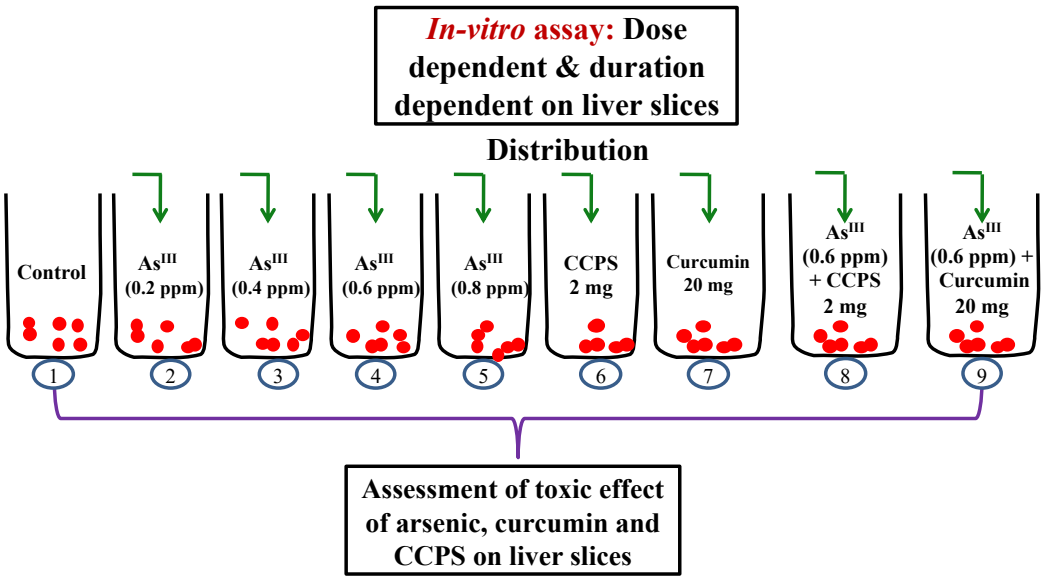
6.2A.1. Objective of the investigation

1. To identify the most effective dose of sodium arsenite on liver SOD and catalase *in vitro*.

2. To find out the efficacious role of CCPS and curcumin against sodium arsenite induced antioxidant status *in vitro*.

6.2A.2. Experimental design

Female Wistar rats were anesthetized by HCL ketamine. Finally, liver tissues were collected and transferred at -20^{0}C temperature. The liver slices were washed with Krebs solution and distributed in the following groups at 3 hrs and 6 hrs duration.



6.2A.3. Results

6.2A.3.1. Assay of antioxidant enzymes by spectrophotometry

Spectrophotometry data revealed that use of sodium arsenite at the dose of 0.2 and 0.4 ppm did not show any changes in the liver SOD and catalase activities (Table 6.1.). Whereas, treatment of 0.6 and 0.8 ppm sodium arsenite showed a significant diminution in liver SOD

and catalase activities (Table 6.1.). Treatment with only CCPS or curcumin or combination significantly increased the liver enzymes activities towards the control (Table 6.1.).

6.2A.3.2. Zymogram of antioxidant enzymes

Electrozymogram image marked that the fragmented bands of hepatic SOD and catalase expression were prominent in arsenicated group at the 0.6 and 0.8 ppm doses (Fig 6.1A and 1B). But, More fragmentation bands were developed in 0.6 ppm arsenic exposed group as compared to the untreated control group (Fig 6.1A and 1B). The fragmented nature of these bands was substituted by compact nature of bands when the arsenicated rats were treated with only CCPS or curcumin or its combination at the same time (Fig 6.1A and 1B).

6.0 EXPERIMENT-2B

Examine the direct combined effects of CCPS, curcumin and CCPS-curcumin on liver tissue antioxidant status against selective dose of sodium arsenite *in vitro*.

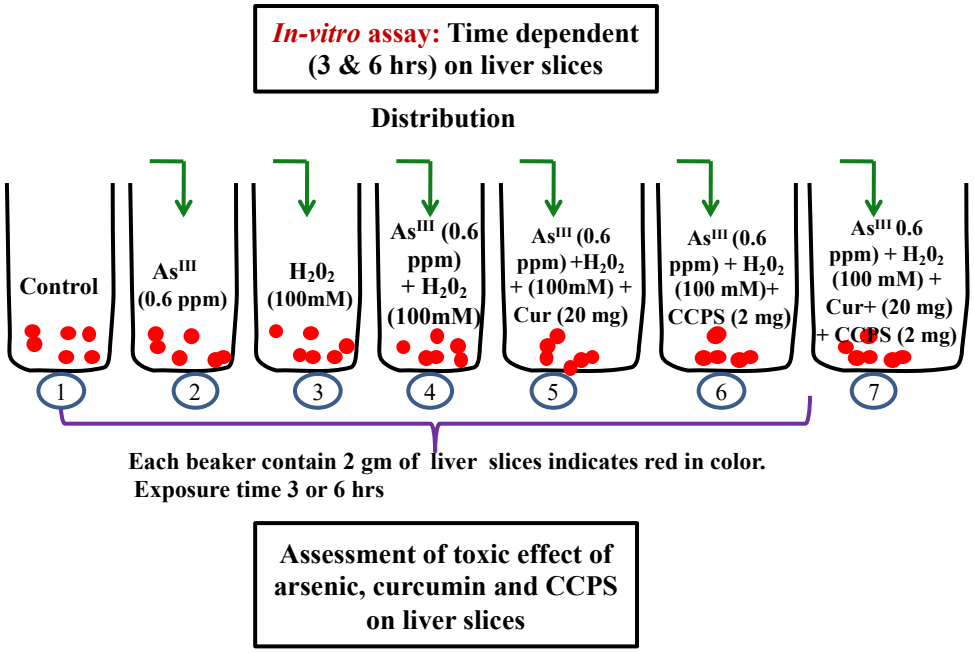
6.2B.1. Objective of the investigation

1. To search out direct efficacy of CCPS and curcumin on lipid peroxidation and SOD, catalase and peroxidase in liver tissue exposed to the selective dose of sodium arsenite.

2. To explore the direct effects of curcumin and CCPS on liver DNA damage induced by sodium arsenite *in vitro*.

6.2B.2. Experimental design

The analysis has been done using the collected liver slices. The liver slices were distributed in following groups.



6.2B.3. Results

6.2B.3.1. Assay of lipid peroxidation levels in duration dependent

Three (3) hrs or 6 hrs incubation of liver slices with arsenic showed significant increases in MDA level as compared to the control group (Table 6.2). The incubation of 3 and 6 hrs treatment with H_2O_2 alone and arsenic-H_2O_2 elevated the MDA level significantly (Table 6.2). Three hrs treatment did not show any significant variation in CD level but treatment with H_2O_2 alone or its combination restored CD in liver. Although 6 hrs incubation with arsenic or arsenic-H_2O_2 showed more significant changes in liver CD level than 3 hrs incubation (Table 6.2). The end products of lipid peroxidation state ware markedly reduced following the exposure with curcumin or CCPS as combination in the arsenicated group (Table 6.2). Strong improvement in the lipid peroxidation state was noted in a combined mode of curcumin-CCPS in arsenicated group (Table 6.2). Six hrs exposure of lipid peroxidation level in liver slices exhibited more significant effect than that of 3 hrs incubation in this case (Table 6.2).

6.2B.3.2. Status of antioxidant enzymes by spectrophotometry

The enzymatic SOD activity of liver was significantly reduced by the treatment with arsenic for 3 hrs and 6 hrs incubation as compared to the control group (Fig. 6.2A and 2B). Whereas, the SOD enzymatic activity was significantly declined at that time these slices were treated with H_2O_2 alone or its combination for 3 hrs and 6 hrs (Fig. 6.2A and 2B). No significant alteration was observed in the liver catalase activity after 3 hrs arsenic or H_2O_2 or its combination treatment (Fig 6.2C). However arsenic or H_2O_2 and its combination for 6 hrs showed significant diminution in the liver enzymatic activity of catalase (Fig 6.2D). Though curcumin or CCPS and its combination in arsenic-H_2O_2 challenged group did not show any significant changes in SOD and catalase enzymes antioxidant status for 3 hrs (Fig 6.2A-2C). Whereas curcumin or CCPS treatment for 6 hrs duration markedly recovered the SOD as well as catalase activities (Fig 6.2B and 2D).

6.2B.3.3. Zymogram of antioxidant enzymes

The zymographic analysis of liver slices was executed to get the expression of antioxidant enzymes status (SOD, catalase and peroxidase). Figure 6.3A-3E. illustrated reduced nature of SOD and catalase band expression following the application of arsenic, H_2O_2 and arsenic-H_2O_2 group in a time dependent fashion. Nevertheless, more diffuse band expression were noted in 6 hrs incubation groups than the 3 hrs (Fig 6.3A-3E). The diffusing bands were more amplified following 6 hrs incubation due to the treatment with curcumin, CCPS and combine mode of curcumin-CCPS in the arsenic-H_2O_2 group (Fig 6.3B, 3E). On the other hand the hepatic peroxidase expression was also reduced when tissue slices were incubated with arsenic, H_2O_2 and arsenic-H_2O_2 group for 3 hrs and 6 hrs (Fig 6.3G and 3H). The impression of the peroxidase enzyme was also reversed back when slices were treated with curcumin, CCPS and curcumin-CCPS combination *in vitro* manner (Fig 6.3G and 3H).

6.2B.3.4. LDH status

The zymogram study was performed to find out the tissue necrotic status (Fig 6.4A and 6.4B). A highly distinct LDH expression in liver was noted in arsenic H_2O_2 incubated group for 6 hrs duration (Fig 6.4B). More diffuse bands were noted in 6 hrs incubation group than that of 3 hrs duration. However, the elevated level of liver LDH impression was significantly dense following the treatment with curcumin, CCPS and jointly in the arsenic- H_2O_2 treated group (Fig 6.4A and 6.4B).

6.2B.3.5. DNA fragmentation assay

Higher degradation of hepatic DNA was viewed in arsenic and H_2O_2 exposed group as compared with control (Fig 6.5). But the present results showed that curcumin, CCPS and curcumin-CCPS in arsenic-H_2O_2 group were amplified the increasing of DNA degradation in hepatic tissue (Fig 6.5).

6.2B.3.6. Comet assay

The single-cell DNA study was performed in time-dependent mode (Fig 6.6A-5B). An increasing number of comets along with the elevation of tail length was noticed in only arsenic and H_2O_2 exposed tissue. This comet formation was noticeably larger when the rats were exposed with arsenic for 6 hrs. A small number of comet formations and reduction of tail length were observed following the treatment of curcumin or CCPS or combination in arsenic-H_2O_2 group (Fig 6.6A-5B).

Table 6.1.

Group	SOD	Catalase
Control	13.91±1.05	45.17±1.45
As^{III} (0.2 ppm)	13.2±0.77	43.2±1.45
As^{III} (0.4 ppm)	10.35±0.84	40.2±2.35
As^{III} (0.6 ppm)	8.16±0.91*	25.4±1.54***
As^{III} (0.8 ppm)	5.27±0.63***	22.4±1.78***
CCPS (2 mg)	16.36±1.89	48.8±1.95
Cur (20 mg)	15.32±1.63	46.4±1.56
As^{III} + CCPS (0.6 ppm+2 mg)	13.6±1.34	47±1.92
As^{III} + Cur (0.6 ppm+20 mg)	12.16±1.16	45±2.33

Table 6.1. Effects of sodium arsenite on liver SOD and catalase activities in dose-dependent fashion. Table represents mean ± SE, N = 6. The data of the current investigation is analyzed using one-way ANOVA with Dunnett's Post Hoc t-test *, *** indicate $p<0.05$, $p<0.001$ versus control group with vehicle.

Figure 6.1.

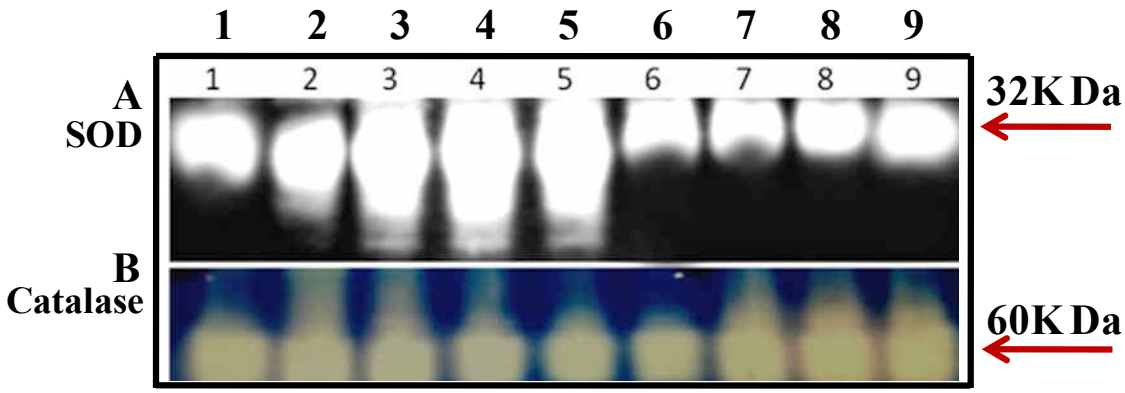


Fig 6.1. (A & B) represents the hepatic SOD and catalase expression on a native gel. Lane division: Lane 1 indicates the control group; Lane 2 as 0.2 ppm of As^{III}; Lane 3 as 0.4 ppm of As^{III}; Lane 4 as 0.6 ppm of As^{III}; Lane 5 as 0.8 ppm of As^{III}; Lane 6 as CCPS; Lane 7 as Cur; Lane 8 as As^{III} + CCPS and Lane 9 as As^{III} + Cur.

Table 6.2.

Group	MDA		CD	
Duration	**3hrs**	**6hrs**	**3hrs**	**6hrs**
Control	7.32±0.16	8.2±0.12	149.46±0.35	149.91±0.27
As^{III} (0.6 ppm)	8.48±0.23*	14.49±0.20*	165.73±0.31	314.33±0.81***
H_2O_2 (100 mM)	11.06±0.28***###	17.2±0.25***	182.2±0.08**	349.11±0.72***
As^{III} + H_2O_2 (0.6 ppm+100 mM)	13.18±0.19***###	18.82±0.28***#	200.13±0.19***##	420.89±0.60***###
As^{III} + H_2O_2 + Cur (0.6 ppm +100 mM+20 mg)	11.29±0.3***###	17.26±0.19***	189.09±0.36***#	231.22±0.715**#
As^{III}+ H_2O_2 + CCPS (0.6 ppm +100 mM+2 mg)	9.59±0.15***#	15.15±0.16***	170.44±0.35*	245.78±0.25**#
As^{III} + H_2O_2 + Cur + CCPS (0.6 ppm+100 mM + 20 mg+ 2 mg)	8.08±0.30	14.8±0.19***	160.15±0.18	211.57±0.54*###

Table 6.2. Protective effects of curcumin and CCPS on MDA and CD in hepatic tissues slices against arsenic exposed rats in duration dependent fashion. Table represents mean ± SE, N = 6. Presented values here are expressed by one-way ANOVA with Dunnett's Post Hoc t-test *, **, *** indicates $p<0.05$, $p<0.01$, $p<0.001$ versus control group with vehicle, whereas #, ##, ### indicate $p<0.05$, $p<0.01$ and $p<0.001$ versus As^{III} treatment.

Figure 6.2.

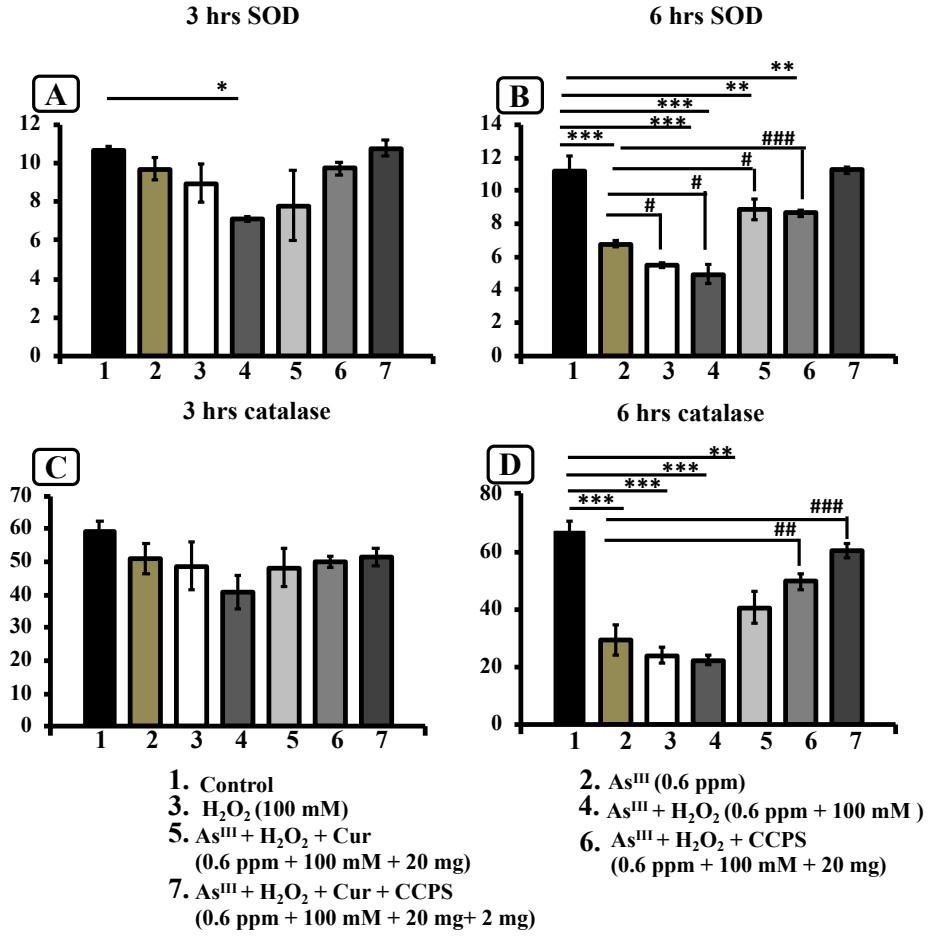


Fig 6.2. Protective effects of curcumin and CCPS on SOD and catalase level in liver tissue slices against arsenic exposed rats in duration dependent fashion. Data represent mean ± SE, N = 6. Presented values here are expressed by one-way ANOVA with Dunnett's Post Hoc t-test *, **, *** indicates $p<0.05$, $p<0.01$, $p<0.001$ versus control group with vehicle, whereas #, ##, ### indicate $p<0.05$, $p<0.01$ and $p<0.001$ versus As^{III} treatment.

Figure 6.3.

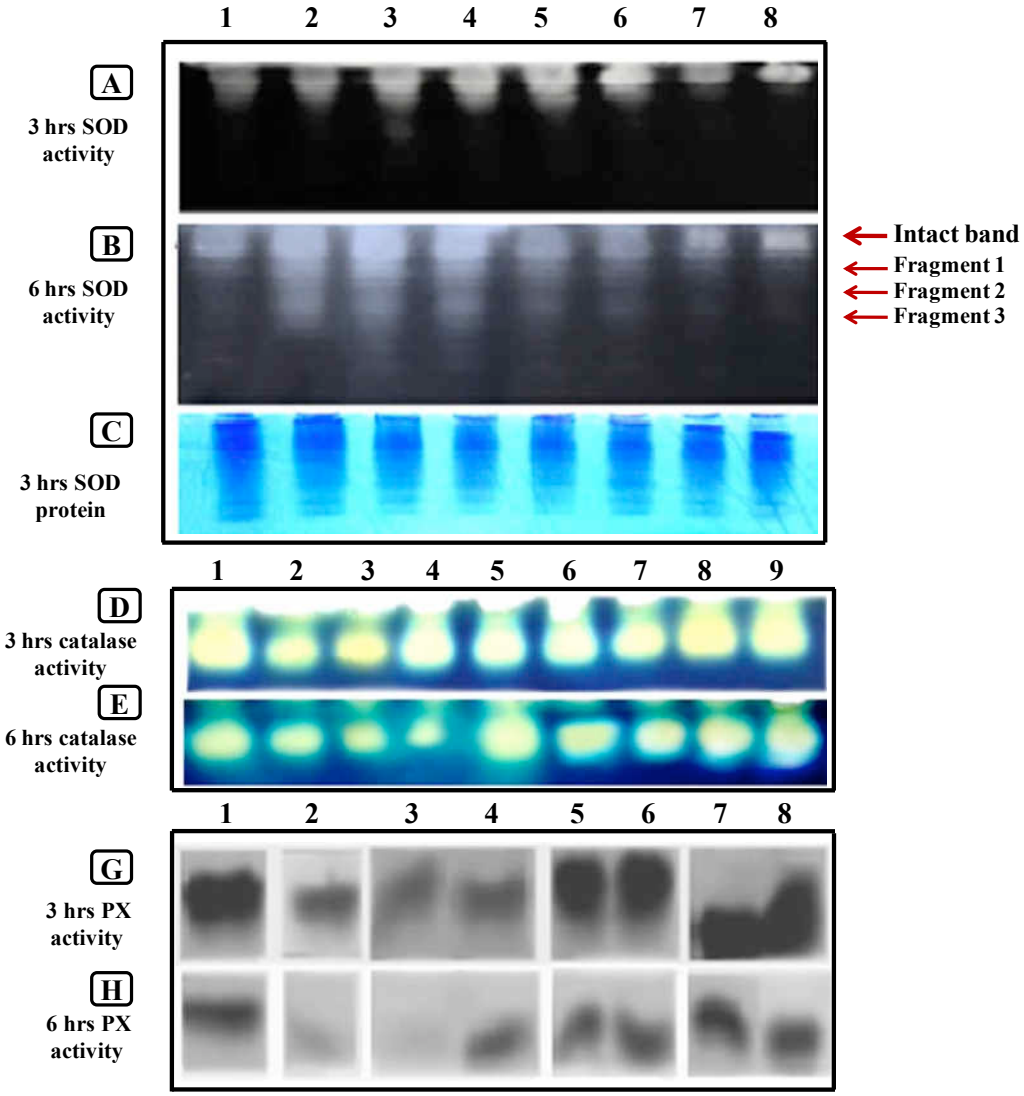


Fig 6.3. (A-H) indicates the SOD, catalase and Px expression in liver tissue on polyacrylamide gel. Lane division: Lane 1 indicates control group with vehicle; Lane 2 as As^{III}; Lane 3 as H_2O_2; Lane 4 as As^{III} + H_2O_2; Lane 5 as As^{III} + H_2O_2 + Cur; Lane 6 as As^{III} + H_2O_2 + CCPS; Lane 7, 8 as As^{III} + H_2O_2+ Cur + CCPS. Fig 2 denotes (D-H) the liver catalase and peroxidase expression on polyacrylamide gel. Lane division is following manner: Lane 1 is control group; Lane 2 as As^{III}; Lane 3 as H_2O_2; Lane 4 as As^{III} + H_2O_2; Lane 5 as As^{III} + H_2O_2 + Cur; Lane 6 as As^{III} + H_2O_2 + CCPS; Lane 7-9 as As^{III} + H_2O_2 + Cur + CCPS.

Figure 6.4.

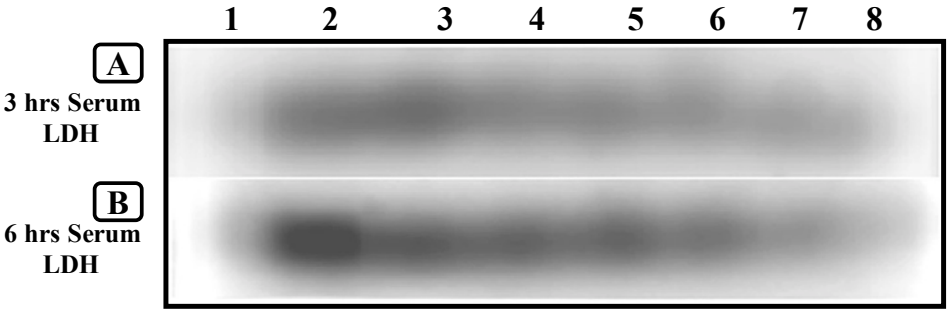


Fig 6.4 (A & B) denotes the activity of liver LDH. Lane division shown in following manner: Lane 1 is control group with vehicle; Lane 2 as As^{III}; Lane 3 as As^{III} + H_2O_2; Lane 4 as As^{III} + CCPS; Lane 5 as As^{III} + Cur; Lane 6 as As^{III} + H_2O_2 + CCPS; Lane 7 as As^{III} + H_2O_2 + Cur; Lane 8 as As^{III} + H_2O_2 + Cur + CCPS.

Figure 6.5.

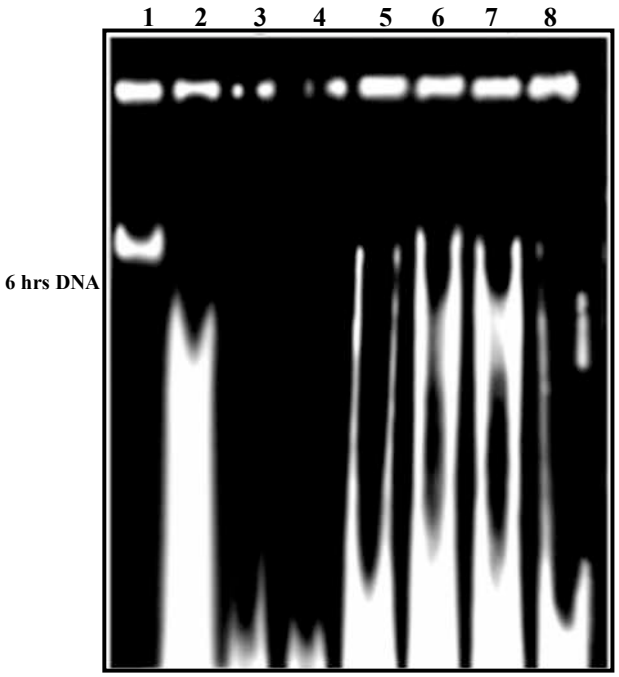


Fig 6.5. The effects of curcumin and CCPS or combination on DNA fragmentation against arsenic and H_2O_2 induced changes in hepatic slices. Lane division; Lane 1 indicates the control group with vehicle; Lane 2 as As^{III}; Lane 3 as H_2O_2; Lane 4 as As^{III} + H_2O_2; Lane 5 as As^{III+} + Cur; Lane 6 as As^{III} + CCPS; Lane 7 as As^{III} + H_2O_2 + Cur; Lane 8 as As^{III} + H_2O_2 + CCPS.

Figure 6.6.

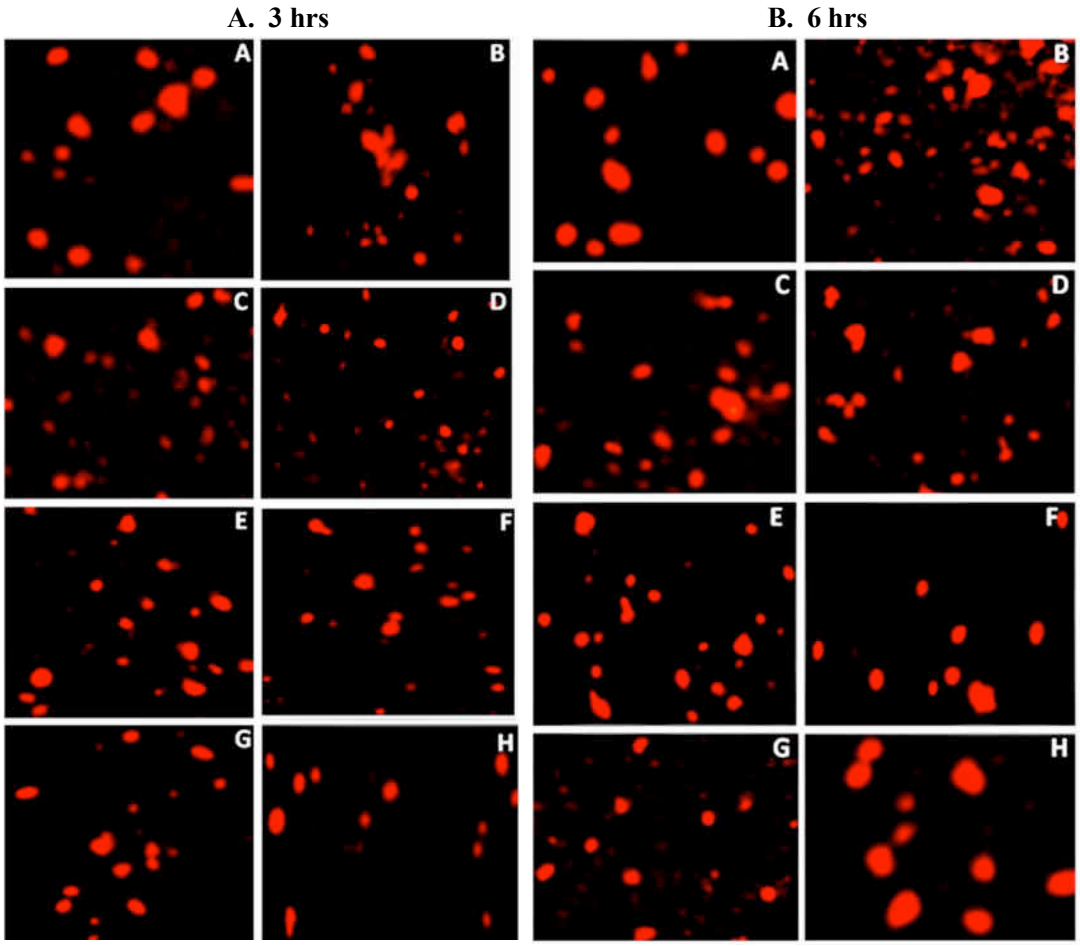


Fig 6.6. (A & B) The effects of CCPS and curcumin in liver cells on the single cell assay against arsenic and H_2O_2 induced changes in duration dependent fashion. Lane allotment; Lane A is control group with vehicle; Lane B as As^{III}; Lane C as H_2O_2; Lane D as As^{III} + H_2O_2; Lane E as As^{III} + Cur; Lane F as As^{III} + CCPS; Lane G as As^{III} + H_2O_2 + Cur; and Lane H as As^{III} + Cur + CCPS + H_2O_2.

6.4.Discussion

The liver is the most important metabolic dock of entry of arsenic. It is also major intention of arsenic toxicity. So in this experiment, it was planned to explore the efficacy of curcumin and pectic polysaccharide (CCPS) of *Momordica charantia* in the mitigation of biochemical hazards developed arsenic-exposed liver slices. In this study arsenic in association with H_2O_2 produced the ROS generation that further increased the formation of end products of lipid peroxidation and conjugated dienes. Increased ROS generation produced oxidative stress and damages the hepatic cells (Cardin et al., 2014). Arsenic could increase free radicals property (MDA and CD) (Maity et al., 2018). This evidence is similar to our present observation (Table 6.2). A significant reduction in liver SOD and catalase activities were observed in the arsenic exposed group in dose-dependent manner (Table 6.1). The electrozymogram analysis of the samples was done under native gel to judge the status of oxidative stress. The SOD bands strength in the liver was noticeably diffused in arsenic exposed group in a dose-dependent manner (Fig 6.1A). More diffused and fragmented bands were observed when liver slices were incubated for 6 hrs duration (Fig 6.1A). Actually, due to the arsenic exposure diminution of SOD activity produces the various downstream free radical products in response of reaction with H_2O_2 (Knoefler et al., 2013). The spectrophotometric and electrozymogram study revealed that the nature of SOD activities (Fig 6.2B) and band expression (Fig 6.3B) was more effectively noticed for the 6 hrs exposed liver tissue. In general, arsenic directly interacts to enzyme of the thiol group. The proof of interaction between arsenic and SOD has not been observed yet. Here As^{III} mediated inhibition of SOD may be the indirect effect of As^{III} which was helped to induce elevation of H_2O_2 levels (Umakoshi et al., 2009). The fragment of oxidized SOD could acquire an active form of α-amino acid and histidine residue. Besides it could form aromatic groups along with the charged and hydrophobic surface of its structure. The previous report suggested that

interruption of SOD activity occurs due to induced oxidative stress (Acharyya et al., 2015). The *in-vitro* study has shown that the liver catalase expression significantly diminished in the diffident dose (0.6 and 0.8 ppm) of arsenic treatment (Fig 6.1B). It is to be noted that the liver catalase expression has remarkably reduced and fainted band appear when the liver slices were treated at the dose of 0.6 ppm of arsenic for 6 hrs than that of 3 hrs (Fig 6.2C and 2D) (Fig 6.3D and 3E). Catalase dysfunction is mediated via the modulation of mRNA transcript expression (Wang et al., 2012). The active site of catalase may penetrate by metal or H_2O_2 and interact with the active site of asparagines and histidine of amino acids which causes activity modulation (Jakopitsch et al., 2003). Liver peroxidase expression was reduced when liver tissues were treated with arsenic and H_2O_2 in duration dependent mode (Fig 6.3G and 3H). This finding powerfully suggests H_2O_2 accumulation during the programmed cell death (Weydert et al., 2010). Ramses et al 2014 reported that curcumin is competent as a safe gourd effects against arsenic mediated hepato-toxicity by *in vitro* and *in vivo* model (Mishra and Palanivelu, 2008). *Momordica charantia* has antioxidant properties and protects the body from variable health disorders (Joseph and Jini, 2013). This study has shown that curcumin and CCPS renovated the hepatic damages. The curcumin and CCPS are known to be its positive action on the entire hepatic antioxidant enzyme activity (Menon and Sudheer, 2007; panda et al., 2015). Curcumin structure resembles the properties of polyphenolic compounds which contribute an important role by donating H-atom in the way of developing excellent antioxidant features of curcumin (AK and Gulcin, 2008). The presence of functional group of β-diketone in curcumin structure directly coupled with the methoxy and CH2 group that scavenge the free radicals (Esatbeyoglu et al., 2012). *Momordica charantia* contains both phenolic and flavonoids group. The phenolic group of *Momordica charantia* also can react with the free radicals. It also slows down the lipid peroxidation and antioxidant enzyme activities (Chaturvedi, 2009). Polysaccharide from *Momordica charantia* has a strong

scavenging activity (Tan and Gan, 2016). In this study curcumin, CCPS alone and combination treatment on the arsenic exposed group have significantly reduced lipid peroxidation in liver with higher CD level (Table 6.2). But, more strong improvement of the status of lipid peroxidation was observed in combination manner of remedy with curcumin and CCPS on the arsenicated group (Table 6.2). The fragmented impressions of these above antioxidant enzyme activities (SOD, catalase and peroxidase) have also reduced following the application of CCPS and curcumin in arsenic exposed group (Fig 6.3A-3H). In this study curcumin and CCPS alone or combined mode of treatment on arsenic and H_2O_2 (6 hrs) exposed group has increased the 12–27 and 42–56 folds of the above enzyme band strength (Fig 6.3B, 3D and 3H). Though the co-administration of curcumin and CCPS alone or combined mode successfully restrained the enzymatic antioxidant activities. It may be confirmed that CCPS and curcumin mostly played a critical role to recover the cells generation of the wide range of free radicals in liver. Our data also revealed that curcumin and CCPS averted the SOD fragmentation and completely inhibited the arsenic and H_2O_2 mediated liver tissue damages (Fig 6.3B). From this information it is postulated that, curcumin and CCPS possibly projected towards the recovery of the altered of cysteine residue of SOD in arsenic and H_2O_2 treated hepatocytes (Table 1, Fig 6.1A, 6.2B and 6.3B). Interestingly it is found that the catalase expression remarkably restrained following the treatment of curcumin and CCPS (Fig 6.3D and 6.3E). The catalase band has more prominent impression 6 hrs exposure of arsenicated hepatic tissue (Fig 6.3E). It may be assumed that in hepatic tissue the alteration of H_2O_2 detoxification possibly helps to decrease the catalase activity. The electrozymographic data has shown that the liver peroxidase expression has remarkably increased and amplified the band intensity by the treatment with curcumin and CCPS (Fig 3G and 3H). It is now confirmed that *in vitro*-study for 6 hrs shown more precise effect than that of 3 hrs exposure in this regard (Fig 6.3H). The *in-vitro* study shown that the

liver DNA was damaged at higher concentration of arsenic (0.6 ppm) (Fig 6.5). Interestingly, the liver DNA was drastically altered when tissues were treated with H_2O_2 and arsenic-H_2O_2. To confirm this we further performed single cell comet assay (Fig 6.6). The broken nature of with increasing tail of comet was also noted in arsenic or H_2O_2 and arsenic-H_2O_2 group ((Fig 6.6). Several studies explained that arsenic helped to increase the formation of reactive oxygen species (ROS) causing oxidative DNA damages. For example single-strand breaks (SSBs) and that can be followed to double-strand breaks (DSBs) during replication, inhibition of DNA (Rossman and Klein, 2011). DNA is continually interacted by the reactive species. DNA and guanine lesions are the most abundant. DNA lesion is represented by the formation of 8-OH-G which was one of the major oxidative DNA products (Valko et al., 2005). Guanine has the least oxidation potential it can be modified easily by the reactive species (Jena, 2012). Hydroxy-2′-deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2′ -deoxyguanosine (8-oxodG) are the predominant forms of free radical mediated lesions, and is now contributed as biomarker favouring oxidative stress (Valavanidis et al., 2009). It was also revealed that the reduction of antioxidant enzyme expression has directly correlated with the oxidative stress and the DNA damages contributed by ROS (Celino et al., 2009). Necrotic status of tissues was examined out by zymogram study of LDH (Fig 6.4A and 4B). It is to be noted that hepatic LDH expression was stimulated when tissues were treated with arsenic and H_2O_2 in duration dependent manner (Fig 6.4A and 4B). Liver LDH expression was consequently higher following 6 hrs exposure (Fig 6.4B). Possible initiation of apoptotic tissue lesions may be recognized by the up regulated LDH status in liver cells. These findings is also similar with the findings of investigators (Weinstein et al., 2014). Curcumin helps to reduce formation of 8-hydroxy-20-deoxyguanosine and enhance the capacity of DNA repair (Roy et al., 2011). The extract Momordica fruit could help to reduce the carcinogen-induced liver lipid peroxidation and lymphocytes DNA damage (Rahman, 2007). Our result showed

that the hepatic DNA degradation successfully reduced uses these two in the arsenic/H_2O_2 treated group (Fig 6.5). It was entirely vetoed the single cell damages with the curcumin and CCPS for 3 or 6 hrs duration (Fig 6.6A and 6B). Our data also revealed that the combined mode of treatment in arsenic treated group has more powerfully renovated the liver LDH expression than that of curcumin and CCPS when applicated alone. From this above investigation, it is hypothesized that curcumin and CCPS are useful to protect the necrotic and apoptotic liver tissues. This is also corroborated with other investigations (Itokawa et al., 2008). In the present study first time we demonstrated the promising role of curcumin and CCPS in hepatic tissue slices against arsenic intoxicated rat model. From the present investigation it may conclude that the two products curcumin and CCPS have successfully contributed to mitigating higher concentration of arsenic and H_2O_2 induced liver ailments. Here we assumed that curcumin and CCPS structure have a unique chelating property that may directly interact with arsenic and H_2O_2 *in vitro* condition. However, curcumin and CCPS markedly exhibited for the direct and better protective effect in hepatic tissue following 6 hrs in vitro exposure with arsenic.

Figure 6.7

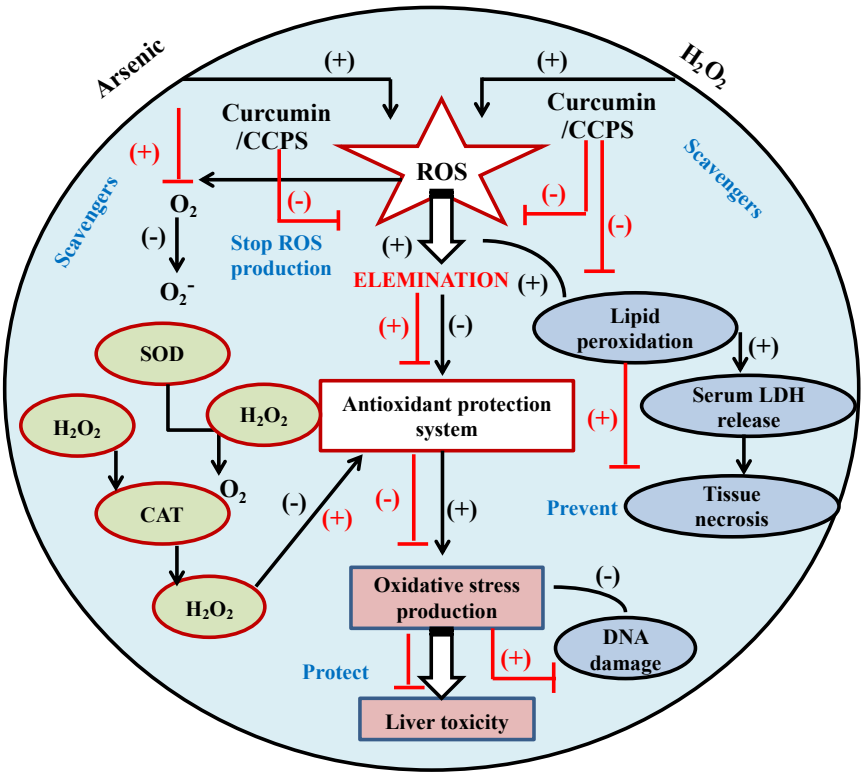


Fig 6.7 Schematic diagram denotes the hypothetical mechanism action of curcumin and CCPS against arsenic/H_2O_2 induced liver toxicity. Black colour **(+)**, **(-)** sign and black line represents the stimulatory and as well as inhibitory effect of arsenic/H_2O_2 respectively. Red colour **(+)**, **(-)** sign and red line indicates the stimulatory and inhibitory effect of curcumin and CCPS respectively.