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INVESTIGATION OF OXIDATIVE POTENTIAL OF MAHASUDARSHAN CHURNA USING GOAT LIVER AS IN-VITRO EXPERIMENTAL MODEL

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ABSTRACT
The American Medical Research Community sounded a heavy metal warning against some herbo-mineral Ayurvedic formulations from Indian System of Medicine sold in United States of America. The products were rejected by Unites States Food and Drug Administration due to the presence of high level of lead, mercury and arsenic as impurities, marked as toxicity inducers. This work has been design to investigate whether the toxicity produced by *Mahasudarshan churna* is due to the results of lipid peroxidation, as oxidative degradation of phospholipids is one of the causes of drug-induced toxicity. The level of malondialdehyde, reduced glutathione and nitric oxide were estimated in control, drug-treated, drug-antioxidant treated and only antioxidant-treated group at two hours and six hours of incubation time in goat liver homogenates. Ascorbic acid was used to compare the oxidative potentials of *Mahasudarshan churna*. The level of malondialdehyde was found to be decreased in the drug-treated, drug-antioxidant treated and only antioxidant treated group where as the level of reduced glutathione and nitric oxide increased when compared to control. Above all the Drug-antioxidant treated group showed maximum anti-oxidant properties when compared to other groups. The study was designed to investigate the lipid peroxidation induction capacity of *Mahasudarshan churna* as consequences of its toxicity and found that lipid peroxidation is not the contributing factor.

INTRODUCTION

Ayurveda is a form of Indian system of medicine was practiced from ancient to heal or cure many acute or chronic diseases. Rasashastra which is a branch of ayurveda describes how metals, gems, minerals, and poisons can be used for manufacturing of ayurvedic formulations. These formulations are commonly known as herbo-mineral formulations. They are used to treat diseases results from the deficiency or imbalance of metallic micro nutrients in body [1-2]. Though it is believed that ayurvedic formulations are safe when compared to

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allopathic formulations, but due to lack of evidence based on its standards profile, quality and safety, it is not approved by the Food and Drug Administration (FDA) of United Sate (US) [3]. As herbo-mineral formulations contains heavy metals as an active ingredients it can potentially harm our vital body functions which may includes damage to the brain, kidney and lungs, when consumed more than the limit prescribe by World Health Organization (WHO).

The American Medical Research Community has sounded a warning against the herbal products of Indian system of medicine sold in US contain dangerous level of lead, mercury and arsenic as heavy metals. Herbo-mineral formulations like Mahasudarshan churna, bal guti, bal chamcha, bal soguthi, mahalakshmi vilas ras etc are rejected by US FDA as they produce mild to moderate toxicities [4-5].

A substance capable of producing therapeutic effects can yield undesired or adverse effects also. "Side effects" and "toxic effects" are the two dimensions of adverse effects. Toxicity is always dose-related, whereas side effect might not be dose-related [6]. Many mechanisms developed to illustrate drug-induced lipid peroxidation. Generation of peroxide free radicals during the therapy is one of them. Peroxide free radicals are oxygenated species generated due to the oxidation of membrane phospholipids [7-8].

As drugs are the potential candidate to induce lipid peroxidation, they might contribute to drug-induced toxicity as an effect of lipid peroxidation [9]. In this project work we are going to find out whether the toxicity produced by this formulations are due to the results of lipid peroxidation or not. As heavy metals have the tendency to produce peroxide free radicals when interact with lipids, antioxidant can be a useful tool to prevent drug induced lipid peroxidation [10-11]. Goat liver is selected as *in-vitro* model for induction of lipid peroxidation. Mahasudarshan churna, a herbomineral formation is selected as drug for the study. Ascorbic acid is selected as natural antioxidant.

Ascorbic acid is a monosaccharide found both in animals and plants. It works as a reducing agent [12-14]. Ascorbic acid was proved as an effective antioxidant to reduce the lipid peroxidation prompted by gentamycin, cisplatin, ceftazidime, flutamide, and tobramycin [15-19]. Estimation of MDA, GSH and NO was performed in goat liver homogenates. MDA is an oxidative by product of arachidonic acid. The level of MDA increases during lipid peroxidation. GSH and NO give protection against lipid peroxidation. The decreased level of GSH and NO than usual indicated the commencement of lipid peroxidation [20-25].

MATERIALS & METHODS

Materials

N-1-napthylethylenediamine dihydrochloride, thiobarbituric acid, 5,5'-Dithiobis-(2-nitrobenzoic acid); ascorbic acid, reduced glutathione; 1,1,3,3-tetraethoxypropane; trichloroacetic acid, sodium hydroxide, potassium dihydrogen phosphate and sodium nitrite were purchased from Loba Chemie Private Limited, India. Mahasudarshan churna of Shree Baidyanath Ayurved Bhawan Pvt. Ltd. were purchased from local ayurvedic medical store, India. Analytical grade reagents were used in this work.

Methods

Preparation of goat liver homogenate [16]

Goat liver was obtained from Raipur Municipal Corporation (RMC) authorized slaughterhouse. It was selected due to its availability and similarities with the human live. The goat liver was harvested and cut into pieces with a sharp knife. Then the pieces were dipped into a germ-free conical flask previously filled with phosphate buffer (pH 7.4) solution. Before preparing the homogenates, the liver pieces were removed from the container and rinsed well with newly prepared phosphate buffer solution of pH 7.4. After that, the liver was ground into homogenates in the ratio of 1 g/ml of same phosphate buffer. The homogenate was then divided into four parts of equal volume and treated as mentioned below.

One of the portions was treated with 1 ml of distilled water and marked as a control group (C). The second portion of the homogenate was treated with Mahasudarshan churna at a dose of 55.12 mg/g of tissue homogenates. This group was marked as drug-treated group (D). The third portion was treated with Mahasudarshan churna and ascorbic acid at a dose of 55.12 mg/g and 0.166 mg/g of tissue homogenates respectively. It was kept as a drug- and antioxidant-treated group (DA). The fourth portion was incubated with ascorbic acid at a dose of 0.166 mg/g of homogenate and kept as an antioxidant-treated group (A). Then the samples were shaken for 4 h using an orbital shaker.

Estimation of MDA level from the tissue homogenate samples

The extent of peroxidation of lipids in the samples was determined by measuring the level of MDA using thiobarbituric acid (TBA) method [26]. The extent of MDA in the samples was estimated at 2 h and 4 h of incubation. After each specified hour of incubation 2.50 ml of an incubated mixture was poured into the centrifuge tubes (5 centrifuge tubes for each set, i.e. for C, D, DA, and A) and 2.50 ml of 10 % w/v TCA solution was mixedwith each tube to precipitate protein. The samples were then centrifuged at 3000 r.p.m for 30 min, and the supernatant was separated through filtration.

Then 2.50 ml of the filtrate was taken in a stoppered glass tube (5 stoppered glass tubes for each set, i.e. for C, D, DA, and A). 5.00 ml of 0.002 M thiobarbituric acid (TBA) solution was added to each tubes. The volume was adjusted to 10.00 ml by the addition of distilled water. The tubes were then kept on a water bath and boiledtill a pink colour developed.The absorbance of solutions were measured and recorded at 530 nm against a solution prepared by 5.00 ml of TBA solution and 5.00 ml of distilled water. Shimadzu UV-1800 Double Beam Spectrophotometer was used to measure the absorbance. The concentration of MDA present in the test samples were calculated from the standard calibration curve.

1,1,3,3-Tetrahydroxy propane (TEP) was used to construct the standard calibration curve. A series of standard solution were prepared by mixing 5.00 ml of freshly prepared TBA solution with several aliquots of standard TEP solution in graduated tubes. The volume of the tubes was then made up to 10.00 ml with distilled water. The tubes were then boiled in a steam bath form about 30 min, cooled, and their absorbances were recorded at 530 nm. The blank solution was prepared by mixing 5.00 ml of TBA solution and 5.00 ml of distilled water. Obsersed absorbances were plotted against concentrations to obtain the best-fit equation i.e. y=0.006x, where y=absorbance, x=concentration. The value of R^2 was found to be 0.992 & standard error mean (sem)=0.015.

Estimation of NO level from the tissue homogenate samples

Estimation of NO was done as mentioned in Griess's method [27-28]. The level of NO in the samples was determined at 2 h and 4 h of incubation. After each specified hour of incubation 2.50 ml of an incubated mixture was poured into the centrifuge tubes (5 centrifuge tubes for each set, i.e. for C, D, DA, and A) and 2.50 ml of 10 % w/v TCA solution was added to each tube for the precipitation of protein. The samples were then centrifuged at 3000 r.p.m for 30 min, and the supernatant was separated through filtration.5.00 ml of the filtrate was taken in stopper glass tube (5 stoppered glass tubes for each set, i.e. for C, D, DA, and A)and 0.50 ml of Griess reagent was added into it. Griess reagent was prepared by mixing 1:1 ratio of sulphanilamide (1 % w/v in 3N HCl) and 0.1 % w/v N-naphthyl ethylenediamine dihydrochloride. The absorbace of the solutions were measered at 540 nm, 10 min after the addtion of Griess reagent against a blank solution containing 5.00 ml of distilled water and 0.50 ml of Griess reagent. The concentrations of NO were calculated from the standard curve.

A standard curve was prepared by using Sodium nitrite solution.A series of standard solution were prepared by mixing 0.50 ml of freshly prepared Griess reagent with several aliquots of standard Sodium nitrate solution in graduated tubes. The volume of the tubes was then made up to 10.00 ml with phosphate buffer and the absorbance of the solutions was measured at 530 nm. The blank solution was prepared by mixing buffer and Griess reagent. Obsersed absorbances were plotted against concentrations to obtain the best-fit equation i.e. y=0.109x, where y=absorbance, x=concentration. The value of R^2=0.997 & standard error mean (sem)=0.019.Shimadzu UV-1800 Double Beam Spectrophotometer was used to measure the absorbance.

Estimation of GSH level from the tissue homogenate samples

Estimation of GSH was done per Ellman's methods [29]. The level of GSH in the samples was determined at 2 h and 4 h of incubation. After each specified hour of incubation 1.00 ml of an incubated mixture was transferred into the centrifuge tubes (5 centrifuge tubes for each set, i.e. for C, D, DA, and A) and one ml of 5 % w/v TCA solution in 1 mM EDTA was added to each tube. Then the mixture was centrifuged at 3000 r.p.m for 10 min and filtered to collect the supernatant. 1 ml of the filtrate was mixed with 5.00 ml of 0.10 M phosphate buffer (pH 8.0), and 0.40 ml of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB 0.01 % w/v in phosphate buffer) was added to it and the absorbance of the solutions was estimated at 412 nm.The blank solution was prepared from 6.00 ml of phosphate buffer and 0.40 ml of DTNB. The concentration of the test solutions was determined using standared curve, constructed as follows.

Different aliquots of the standard GSH solution were taken in 10.00 ml volumetric flasks. 0.40 ml of DTNB solution was added to each flask, and the volume was made upto the mark

with freshly prepared phosphate buffer (pH 8.0) solution. The absorbance was recorded at 412 nm, against a blank. The blank solution was prepared by using 9.60 ml of phosphate buffer and 0.40 ml DTNB solution. Observed absorbances were plotted against concentrations to obtain the best-fit equation i.e. y=0.0005x, where y=absorbance, x=concentration. The value of R^2=0.995 & standard error mean (sem)=0.011.Shimadzu UV-1800 Double Beam Spectrophotometer was used to measure the absorbance.

RESULTS & DISCUSSIONS

The lipid peroxidation induction capacity of Mahasudarshan churna was reported as average percentage (%) change in MDA (Table 1), NO (Table 2) and GSH (Table 3) concentration with respect to control group

Table 1: Average % change in mda concentration with respect to control group

After 4 h of incubation period					After 2 h of incubation period				
Sets	**D**	**DA**	**A**	**ANOVA**	**Sets**	**D**	**DA**	**A**	**ANOVA**
1	-3.73[a]	-23.06[a]	-6.45[a]	F1=2.007	1	-6.72[a]	9.13[a]	-12.22[a]	F1=7.138
2	-3.68[a]	-14.29[a]	-4.49[a]	(df=6,12)	2	-10.20[a]	6.82[a]	-11.73[a]	(df=6,12)
3	-5.79[a]	-11.96[a]	-3.47[a]	F2= 35.424	3	-6.93[a]	9.51[a]	-10.54[a]	F2= 27.695
4	-7.36[a]	-14.70[a]	-9.56[a]	(df=2,12)	4	-6.52[a]	6.82[a]	-7.66[a]	(df=2,12)
5	-6.47[a]	-21.29[a]	-12.54[a]	Critical	5	-11.03[a]	6.72[a]	-12.11[a]	Critical
6	-8.07[a]	-23.17[a]	-11.94[a]	difference:	6	-5.79[a]	14.99[a]	-7.03[a]	difference:
7	-16.61[a]	-24.72[a]	-18.92[a]	(p = 0.05)#	7	-20.71[a]	9.13[a]	-12.22[a]	(p = 0.05)#
Mean	-7.39	-19.03	-9.63	Ranked mean**	Mean	-9.70	-21.98	-10.09	Ranked mean**
±sem	±0.39	±0.51	±0.46	(D,DA,A)	±sem	±0.93	±1.06	±1.35	(D,DA,A)

* Percent changes of D, DA and A with respect to controls of corresponding hours are shown in the Table. sem= Standard Error of Means of four sets (n=7). Significance of 't' values of the changes of MDA content (df = 2) are shown as: a > 99%. #Critical values of F at p = 0.05 level, F1 = 2.99 [df = (6, 12)], F2 = 3.88 [df = (2, 12)] at p=0.05. F1 and F2 corresponding to variance ratio between groups and within groups, respectively. **Two means are included within the same parenthesis, hence are not statistically significantly different at p = 0.05 level.

Table 2: Average % change in no concentration with respect to control group

After 4 h of incubation period					After 2 h of incubation period				
Sets	**D**	**DA**	**A**	**ANOVA**	**Sets**	**D**	**DA**	**A**	**ANOVA**
1	25.12[a]	51.76[a]	17.54[a]	F1=3.607	1	26.03[a]	53.73[a]	18.60[a]	F1=4.682
2	4.14[a]	41.01[a]	13.38[a]	(df=6,12)	2	29.74[a]	63.52[a]	17.12[a]	(df=6,12)
3	13.81[a]	51.87[a]	8.44[a]	F2= 131.342	3	22.60[a]	50.62[a]	17.55[a]	F2= 140.96
4	29.04[a]	45.15[a]	25.49[a]	(df=2,12)	4	24.77[a]	49.37[a]	10.92[a]	(df=2,12)
5	20.46[a]	59.20[a]	10.68[a]	Critical	5	29.95[a]	56.77[a]	10.82[a]	Critical
6	16.64[a]	47.07[a]	14.06[a]	difference:	6	12.35[a]	56.62[a]	6.81[a]	difference:
7	14.42[a]	48.67[a]	11.05[a]	(p = 0.05)#	7	16.49[a]	37.45[a]	2.95[a]	(p = 0.05)#
Mean	20.09	48.67	14.38	Ranked mean**	Mean	23.14	52.59	12.11	Ranked mean**
±sem	±1.06	±1.43	±0.99	(D,DA,A)	±sem	±1.15	±1.21	±1.23	(D,DA,A)

* Percent changes of D, DA and A with respect to controls of corresponding hours are shown in the Table. sem= Standard Error of Means of four sets (n=7). Significance of 't' values of the changes of NO content (df = 2) are shown as: a > 99%. #Critical values of F at p = 0.05 level, F1 = 2.99 [df = (6, 12)], F2 = 3.88 [df = (2, 12)] at p=0.05. F1 and F2 corresponding to variance ratio between groups and within groups, respectively. **Two means are included within the same parenthesis, hence are not statistically significantly different at p = 0.05 level.

Table 3: Average % change in GSH concentration with respect to control group

After 4 h of incubation period					After 2 h of incubation period				
Sets	**D**	**DA**	**A**	**ANOVA**	**Sets**	**D**	**DA**	**A**	**ANOVA**
1	21.95[a]	50.85[a]	26.40[a]	F1=3.253	1	25.12[a]	50.45[a]	27.34[a]	F1=44.153
2	23.23[a]	54.94[a]	33.62[a]	(df=6,12)	2	37.82[a]	69.72[a]	48.76[a]	(df=6,12)
3	18.74[a]	46.29[a]	30.86[a]	F2= 168.30	3	15.31[a]	41.30[a]	24.40[a]	F2=320.98
4	23.98[a]	51.97[a]	32.38[a]	(df=2,12)	4	20.28[a]	47.32[a]	26.93[a]	(df=2,12)
5	17.83[a]	45.42[a]	30.93[a]	Critical	5	19.85[a]	46.37[a]	28.50[a]	Critical
6	13.61[a]	50.15[a]	18.41[a]	difference:	6	16.82[a]	48.25[a]	21.15[a]	difference:
7	21.75[a]	55.00[a]	28.40[a]	(p = 0.05)#	7	20.73[a]	50.66[a]	29.94[a]	(p = 0.05)#
Mean ±sem	20.16 ±0.42	50.66 ±0.80	28.72 ±0.54	Ranked mean** (D,DA,A)	Mean ±sem	22.24 ±0.81	50.59 ±0.92	29.58 ±0.66	Ranked mean** (D,DA,A)

* Percent changes of D, DA and A with respect to controls of corresponding hours are shown in the Table. sem= Standard Error of Means of four sets (n=7). Significance of 't' values of the changes of GSH content (df = 2) are shown as: a > 99%. [#]Critical values of F at p = 0.05 level, F1 = 2.99 [df = (6, 12)], F2 = 3.88 [df = (2, 12)] at p=0.05. F1 and F2 corresponding to variance ratio between groups and within groups, respectively. **Two means are included within the same parenthesis, hence are not statistically significantly different at p = 0.05 level.

Interpretation of the results was supported by Student "t" test. Analysis of variance (ANOVA) was performed on the % changes data of samples D, DA and A with respect to control group at 2 h and 4 h of incubation period [30-31]. Multiple comparison analysis and ANOVA was performed on the % changes in MDA, NO and GSH content to compare the means of the samples. There were no significant differences found among various groups of D, DA and A, statistically they are not different from each other.

Lipid peroxidation is a common phenomenon occurs invariably in almost all the cells of living organisms. It serves as an instance of cells involving in free radical reaction by generating reactive oxygen species (ROS). Drug-induced toxicity was linked to peroxidation effects. In this work change of MDA, NO, and GSH content in goat liver samples was measured to quantify the lipid peroxidation induction potency of chemotherapeutic agents. The change in MDA, NO, and GSH content were observed after the specified time intervals and the results were verified by statistical analysis methods. The % of change in MDA, NO, and GSH level were calculated with respect to the corresponding control considered as the indicator of the extent of lipid peroxidation. It was observed that when the goat liver sample was treated with Mahasudarshan churna the MDA content decreased. This indicated the lipid peroxidation reduction capacity of the drug as MDA is a byproduct of lipid peroxidation. Groups where the sample was treated with Mahasudarshan churna and ascorbic acid both, the MDA content decreased in comparison with drug treated group. It suggested the synergistic effect of drug with ascorbic acid. Groups which were treated with only ascorbic acid there was a decrease in MDA content compared to drug treated group. This was might be due to the fact that ascorbic acid can reduce the making of hydroxyl radicals and other reactive oxygen species through Fenton's reaction.

Nitric oxide (NO) is one of the most important biogenic molecules participates in many vital physiological processes within the mammalian body. It has both beneficial and harmful effects. The body should the appropriate levels of NO to protect the organs such as the liver from ischemic damage. Decrease in NO production results in tissue toxicity which may contribute to the vascular collapse associated with septic shock leads to inflammatory conditions including ulcerative colitis, multiple sclerosis, arthritis and juvenile diabetes. The results clearly shown that the level of NO increased in all the three samples, whereas the drug and antioxidant treated group showed the maximum increase in NO content. Again this was might synergistic effects of Mahasudarshan churna and ascorbic acid. This suggested that the drug is having lipidn peroxidation lowering capacity.

The average % changes in reduced glutathione (GSH) level of seven samples were showed that the level of GSH increased in all the three samples, whereas the drug and antioxidant treated group showed the maximum increase in GSH content. Again this was might synergistic effects of Mahasudarshan churna and ascorbic acid. This suggested that the drug is having lipidn peroxidation lowering capacity.

Histograms of average % change in MDA, NO and GSH along with standard error mean were shown in Fig. 1, 2,3,4,5 and 6 respectively.

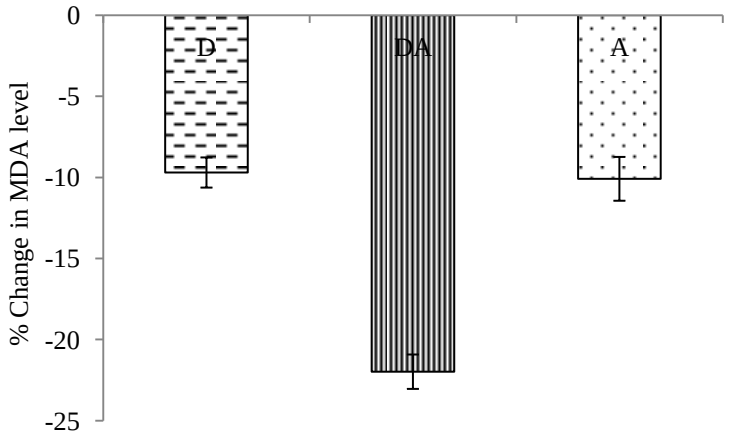

Fig. 1: Average % change in MDA content after 2 h of incubation period

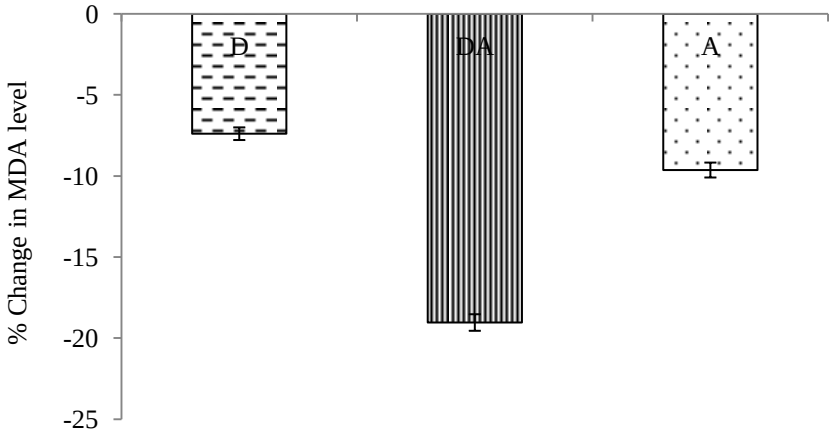

Fig. 2: Average % change in MDA content after 4 h of incubation period

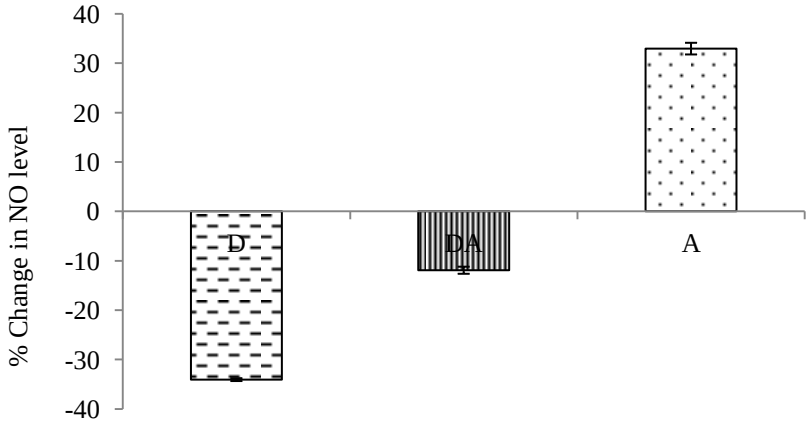

Fig. 3: Average % change in NO content after 2 h of incubation period

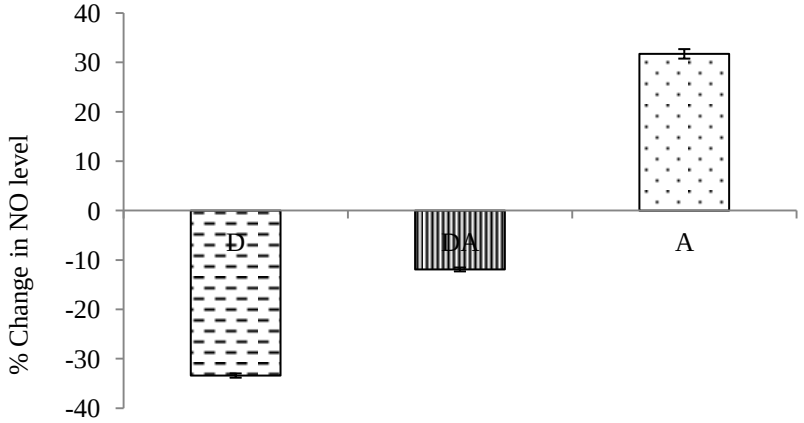

Fig. 4: Average % change in NO content after 4 h of incubation period

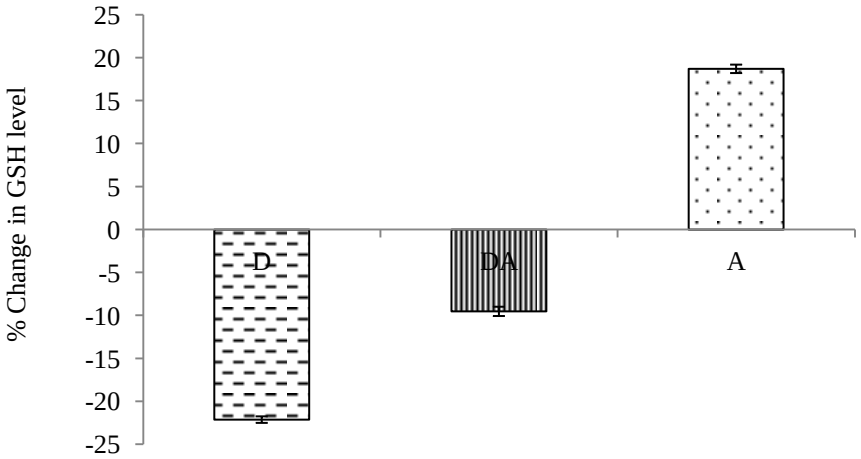

Fig. 5: Average % change in GSH content after 2 h of incubation period

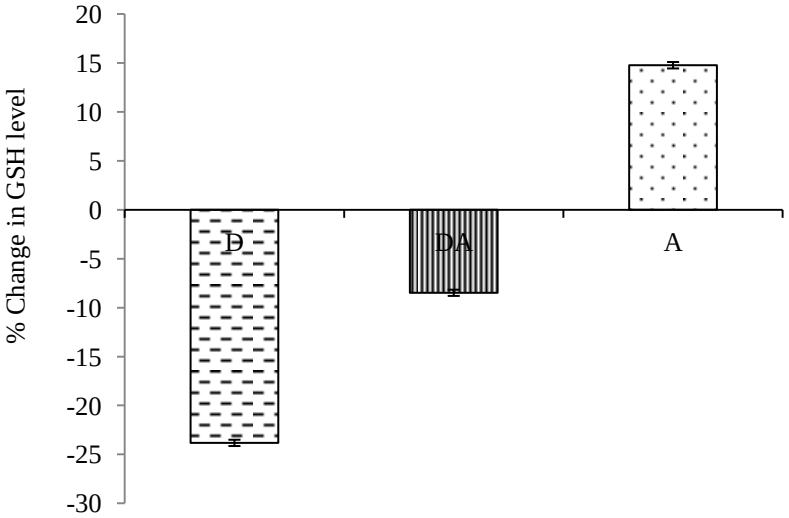

Fig. 6: Average % change in GSH content after 4 h of incubation period

CONCLUSION

The central theme of this working hypothesis is laid behind the fact that the detectable changes of lipid in presence of drug in the body, changes in lipid constituents might be an important contribution to the therapeutic and toxic effect of the concern drug. The work was deigned to find out the lipid peroxidation induction capacity of Mahasudarshan churna and found that

lipid peroxidation is not the cause of toxicities produced by Mahasudarshan churna. The study revealed that there might be a different mechanism through which Mahasudarshan churna showed toxicity. Lipid peroxidation is not the contributing factor of its toxicity.

FINANCIAL ASSISTANCE
Nil

CONFLICT OF INTEREST
The authors declare no conflict of interest

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