Full Length Article

PROTECTIVE EFFECTS OF RAW AND NANO FORMS OF RESVERATROL AND SILIBININ IN DIETHYL NITROSAMINE INDUCED HEPATIC DAMAGE IN WISTAR RATS

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ABSTRACT

Oxidative stress is a common mechanism contributing to initiation and progression of hepatic damage in a variety of liver disorders. Hence, there is a great demand for the development of agents with potent antioxidant effect. The aim of the present investigation is to evaluate the efficacy of raw and nanoforms of resveratrol and silibinin as a hepatoprotective and an antioxidant against diethylnitrosamine induced hepatocellular damage. 0.01% diethylnitrosamine administration to rats resulted in significantly elevated levels of lipid peroxidation (LPO) and decreased levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSH) and total protein (TP) in the liver tissue. Post-treatment with the nano resveratrol (50 mg/kg), nano silibinin (50 mg/kg), raw resveratrol (50 mg/kg), raw silibinin (50 mg/kg) orally for 30 days significantly reversed the diethylnitrosamine induced alterations in the liver tissue and offered almost complete protection. The results from the present study indicate that both nano forms exhibit good hepatoprotective and antioxidant potential against diethylnitrosamine induced hepatocellular damage in rats as compared to raw forms.

Keywords: Resveratrol, silibinin, diethyl nitrosamine, antioxidants, solid lipid nanoparticles

Received : 25.10.2022 Revised : 27.02.2023 Accepted : 28.02.2023

INTRODUCTION

According to the World Health Organization (WHO), around 150 million people worldwide suffer from various types of chronic liver damage including hepatocellular carcinoma (Rawla *et al*., 2018). A sizeable portion of research has demonstrated that

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oxidative stress is the root cause of chronic liver disorders in both humans and laboratory animals (Li *et al*., 2015).

It has been documented that the generation of oxygen-derived free radicals and reactive oxygen species (ROS) resulting from lipid peroxidation during hepatocellular membrane damage cause the advancement of inflammatory responses, that result in necrosis, steatosis, fibrosis, and apoptosis (Madkour, 2020). Resveratrol (3,5,4′-trans-trihydroxystilbene), a phytoalexin, is a polyphenol that is mostly present in red grapes, red wine, peanuts, and berries (Mates *et al*., 2011). Resveratrol has effects on lipid metabolism, and it also has antioxidant, anti-inflammatory, anticarcinogenic, and antifibrogenic properties (Chavez *et al*., 2008). Resveratrol is metabolized to resveratrol sulfate, and in its low concentrations, it is converted into resveratrol glucuronide by enzymes glucuronosyltransferase (UGT) or sulfotransferase (ST) (Yu *et al*., 2002).

The medicinal plant *Silybum marianum* (Asteraceae), also known as "milk thistle," produces silibinin, a flavonolignan, hepatoprotective agent and active substance which makes up about 40% to 50% of the main extract, silymarin (Chhabra *et al*., 2013). Silibinin has hepatoprotective, antioxidant, free radical scavenging, and membrane stabilizing properties against liver damage caused by various drugs and chemicals in animal models (Vargas *et al*., 2014).

Solid lipid nanoparticles (SLNs) are lipid nanocarriers with a solid core that can carry both hydrophilic and hydrophobic medicines. Plant-based compounds like silibinin and resveratrol have been loaded into SLNs because they have the potential to pass the blood-brain barrier, resulting in increased concentration in deeper tissues (Sarangi and Padhi, 2016). In the current study, we investigated the alleviating properties of raw and nano forms of resveratrol and silibinin against DEN-induced disruption in the homeostasis of the antioxidant defense parameters.

MATERIALS AND METHODS

Animals

Wistar albino male rats weighing 150 ± 10 g purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, were used in this study. They were housed in polypropylene cages with 12 hrs light and dark cycle. Animals were fed standard pellet feed and water *ad libitum*. All animal experiments were performed in accordance with the strict guidelines prescribed by the Institutional Animal Ethical Committee (IAEC) and after getting necessary approval (Approval Lr. No. 370/DFBS/IAEC/2021, dated: 16.08.2021).

Resveratrol-SLNPs and Silibinin-SLNPs were prepared by single emulsification-solvent evaporation method (Pooja *et al.,* 2016) and administered to rats. Rats were divided into nine groups with six animals in each group viz. Control group (Sham control); Blank Solid Lipid Nanoparticles (BSLNPs) group (1.5 ml thrice in a week / P.O); DEN group (0.01% in drinking water every day for

17 weeks); raw resveratrol and raw silibinin groups (0.01% in drinking water every day for 17 weeks + 50 mg/ kg b. wt. thrice a week P. O); nano-resveratrol and nano silibinin groups (0.01% in drinking water every day for 17 weeks + 50 mg/ kg b. wt. thrice a week P. O); Nano combination group (0.01% DEN in drinking water every day for 17 weeks +50 mg/ kg b. wt. of nano silibinin thrice a week P. O +50 mg/ kg b. wt. of nano resveratrol thrice a week P.O) and Sorafenib group (0.01% DEN in drinking water every day for 17 weeks + 7.5 mg/ kg b. wt. thrice a week P. O) respectively . At every 30, 60, 90, 120 days interval, rats were anaesthetized by using isoflurane and immediately after sacrificing the animals by cervical dislocation, the liver tissue was excised and washed in ice cold normal saline, blotted dry and stored at -20°C for further analysis.

The collected liver tissue was crushed in tissue homogenizer and 10% w/v liver homogenate was prepared with 0.05M PBS (pH 7.4) was used for the estimation of lipid peroxidation [LPO] and reduced glutathione [GSH]. The remaining homogenate was centrifuged at 4500 rpm for 15 min at 4°C and the supernatant was used for the estimation of superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidise [GPx]. The lipid peroxidation is estimated by the formation of thiobarbituric acid reactive substances (TBARS) which were estimated spectrophotometrically at 548 nm by the method of Ohkawa *et al*. (1979). The enzymatic antioxidant of superoxide dismutase (SOD) levels were estimated by the method of Marklund and Marklund (1974), glutathione peroxidase (GPx) by the method of Rotruck *et al*. (1973), and catalase (CAT) by the method of Caliborne and Greenwald (1985). The non-enzymatic antioxidant reduced glutathione (GSH) levels were estimated by the method of Moron *et al*. (1979). The total protein values were estimated by the method of Lowry *et al.* (1951).

Statistical analysis

The data generated from different parameters of the experimental study was subjected to one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test by using IBM SPSS software version 20 for windows. The differences among the groups were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

The mean (±SE) lipid peroxidation and antioxidant values are presented in Table 1-4. The mean value of total LPO revealed significant ($P<0.05$) between the DEN and sham control/ BSLNPs (Blank solid lipid nanoparticles) groups. There was a significant increase in the liver lipid peroxidation (Fig.1) in the DEN group alone as compared to treated groups. Treated groups did not show any significant difference in LPO value as compared to sham control group. There was no significant difference in the LPO of the sham control and BSLNPs groups.

Significant difference in the non-enzymatic (GSH; Fig.2) and enzymatic (CAT, GPX and SOD) antioxidant values

Table 1. Mean (±SE) Liver antioxidant status in hepatic cancer induced and treated rats- 30 days

Group (30d)	LPO	GSH	GPX	SOD	CAT	TP
Control	107.96^{c}±0.92	13.09^{d}±0	68.56^{d}±0.11	18.43^{e}±0.39	91.82^{c}±8.72	7.52^{d}±0.04
BSLNPs	107.77^{c}±1.00	13.09^{d}±0.1	68.67^{d}±0.11	18.04^{de}±0.39	83.1^{bc}±8.72	7.47^{d}±0.04
DEN	185.25^{a}±2.72	7.52^{a}±0.03	50.41^{a}±0.07	10.68^{a}±0.31	56.20^{a}±0.64	4.44^{a}±0.06
Raw resveratrol	102.15^{b}±0.42	11.45^{b}±0.06	65.74^{b}±0.29	16.78^{bc}±0.23	76.22^{b}±2.05	6.37^{b}±0.09
Raw silibinin	104.78^{bc}±2.75	11.48^{b}±0.04	66.03^{b}±0.29	16.51^{b}±0.07	74.15^{b}±0.04	6.30^{b}±0.03
Nano resveratrol	104.18^{bc}±1.53	12.36^{c}±0.32	67.24^{c}±0.74	17.3^{cd}±0.10	79.88^{bc}±0.49	6.94^{c}±0.12
Nano silibinin	105.93^{bc}±0.20	12.62^{c}±0.22	67.91^{cd}±0.07	17.48^{cd}±0.09	79.83^{bc}±0.48	7.08^{c}±0.02
Nano combination	104.37^{bc}±1.64	12.51^{cd}±0.32	67.17^{c}±0.71	17.40^{cd}±0.15	80.17^{bc}±0.25	6.94^{c}±0.12
Sorafenib	102.59^{bc}±0.94	11.68^{b}±0.17	65.47^{b}±0.16	17.07^{bc}±0.09	79.47^{bc}±0.69	6.78^{c}±0.16

Means with different superscripts differ significantly ($p<0.05\%$) from each other with in the same column

Table 2. Mean (±SE) Liver antioxidant status in hepatic cancer induced and treated rats- 60 days

Group (60d)	LPO	GSH	GPX	SOD	CAT	TP
Control	113.32^{d}±1.56	6.31^{e}±0.06	52.95^{e}±0.28	22.32^{d}±0.28	84.84^{d}±0.56	9.92^{d}±0.06
BSLNPs	112.24^{d}±1.54	6.28^{e}±0.03	53.91^{e}±0.72	22.36^{d}±0.27	84.88^{d}±0.54	9.88^{d}±0.05
DEN	331.74^{a}±0.38	3.22^{a}±0.06	34.13^{a}±0.37	10.22^{a}±0.06	43.65^{a}±0.22	5.35^{a}±0.04
Raw resveratrol	160.11^{b}±0.16	5.88^{b}±0.06	47.94^{bc}±0.25	20.62^{b}±0.29	76.56^{b}±0.53	9.28^{bc}±0.13
Raw silibinin	160.37^{b}±0.39	5.90^{bc}±0.04	47.33^{b}±0.73	20.30^{b}±0.07	77.36^{b}±1.28	9.11^{b}±0.04
Nano resveratrol	172.10^{c}±1.25	6.08^{d}±0.03	52.07^{de}±0.20	21.37^{c}±0.10	82.74^{cd}±0.34	9.56^{c}±0.06
Nano silibinin	170.72^{c}±0.29	6.07^{cd}±0.02	51.85^{de}±0.18	21.32^{c}±0.11	83.06^{cd}±0.36	9.52^{c}±0.07
Nano combination	170.58^{c}±0.39	6.06^{cd}±0.02	51.67^{de}±0.32	21.35^{c}±0.10	82.92^{cd}±0.31	9.52^{c}±0.08
Sorafenib	168.7^{c}±3.97	6.02^{bcd}±0.09	49.97^{cd}±2.02	20.93^{bc}±0.34	81.92^{c}±1.05	9.35^{bc}±0.15

Means with different superscripts differ significantly ($p<0.05\%$) from each other with in the same column

Table 3. Mean (±SE) Liver antioxidant status in hepatic cancer induced and treated rats- 90 days

Group (90d)	LPO	GSH	GPX	SOD	CAT	TP
Control	518.1^{d}±39.48	25.43^{d}±0.22	100.78^{d}±0.45	39.10^{d}±0.65	80.85^{c}±1.21	6.86^{e}±0.04
BSLNPs	473.61^{cd}±30.88	25.21^{d}±0.22	100.33^{cd}±0.44	38.45^{d}±0.65	79.64^{c}±1.21	6.82^{de}±0.04
DEN	715.33^{a}±6.66	5.94^{a}±0.14	44.39^{a}±0.58	5.36^{a}±0.06	32.97^{a}±0.13	4.89^{a}±0.20
Raw resveratrol	386.02^{b}±3.75	21.89^{bc}±1.46	96.20^{b}±0.77	33.41^{b}±0.04	76.33^{b}±0.33	6.07^{b}±0.03
Raw silibinin	382.27^{b}±3.75	23.35^{cd}±1.46	96.98^{b}±0.77	33.37^{b}±0.04	76.00^{b}±0.33	6.04^{b}±0.02
Nano resveratrol	450.99^{c}±0.33	24.52^{d}±0.22	100.58^{d}±0.04	35.95^{c}±0.40	80.51^{c}±0.03	6.67^{cde}±0.0
Nano silibinin	435.91^{bc}±10.04	20.86^{b}±0.41	100.31^{cd}±0.34	36.26^{c}±0.19	80.73^{c}±0.37	6.51^{c}±0.04
Nano combination	436.37^{bc}±3.81	21.56^{bc}±0.4	99.59^{cd}±0.70	36.62^{c}±0.03	80.11^{c}±0.44	6.58^{cd}±0.01
Sorafenib	418.21^{bc}±7.17	21.27^{bc}±0.34	98.81^{c}±0.03	34.14^{b}±1.26	79.88^{c}±0.11	6.66^{cde}±0.03

Means with different superscripts differ significantly (p<0.05%) from each other with in the same column

Table4. Mean (±SE) Liver antioxidant status in hepatic cancer induced and treated rats- 120 days

Group (120d)	LPO	GSH	GPX	SOD	CAT	TP
Control	753.80^{e}±1.77	27.08^{d}±1.78	87.28^{d}±3.51	49.85^{d}±0.26	101.29^{d}±1.37	6.44^{d}±0.05
BSLNPs	752.02^{e}±1.77	25.30^{cd}±1.78	83.77^{bcd}±3.51	49.58^{d}±0.26	99.92^{d}±1.37	6.39^{d}±0.05
DEN	922.89^{a}±0.23	6.21^{a}±0.05	47.45^{a}±0.05	4.85^{a}±0.29	34.92^{a}±2.70	2.59^{a}±0.18
Raw resveratrol	666.57^{d}±1.98	18.13^{b}±0.02	78.48^{b}±0.32	43.29^{b}±0.36	79.33^{b}±0.33	3.70^{b}±0.07
Raw silibinin	664.59^{d}±1.98	18.10^{b}±0.02	78.8^{bc}±0.32	42.92^{b}±0.36	79^{b}±0.33	3.63^{b}±0.07
Nano resveratrol	654.35^{cd}±42.11	21.82^{bc}±0.18	84.28^{bcd}±1.14	46.03^{c}±0.36	80.5^{bc}±10.03	4.29^{bc}±0.59
Nano silibinin	606.68^{c}±16.74	25.23^{cd}±2.31	86.18^{d}±1.84	46.94^{c}±0.81	80.36^{bc}±0.37	4.23^{bc}±0.33
Nano combination	556.88^{b}±14.31	18.69^{b}±0.44	84.94^{cd}±1.60	47.47^{c}±0.92	80.55^{bc}±0.44	4.0^{bc}±0.34
Sorafenib	727.57^{e}±15.55	19.28^{b}±1.18	82.70^{bcd}±0.21	47.59^{c}±0.96	84.10^{c}±1.78	4.74^{c}±0.073

Means with different superscripts differ significantly (p<0.05%) from each other with in the same column

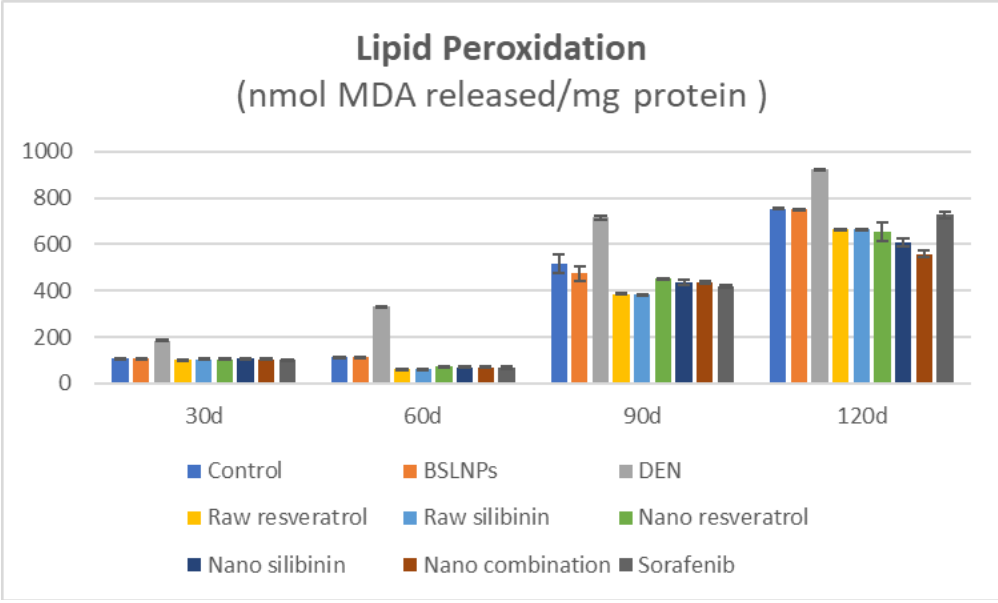

Fig 1: Effect of DEN toxicity and treatment on LPO in liver tissue of rats

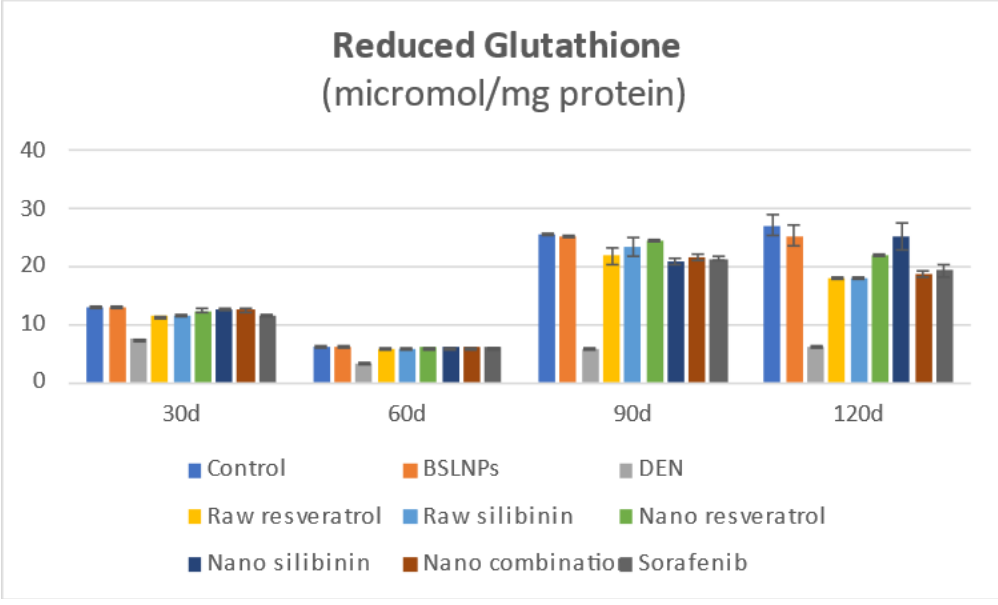

Fig 2: Effect of DEN toxicity and treatment on GSH in liver tissue of rats

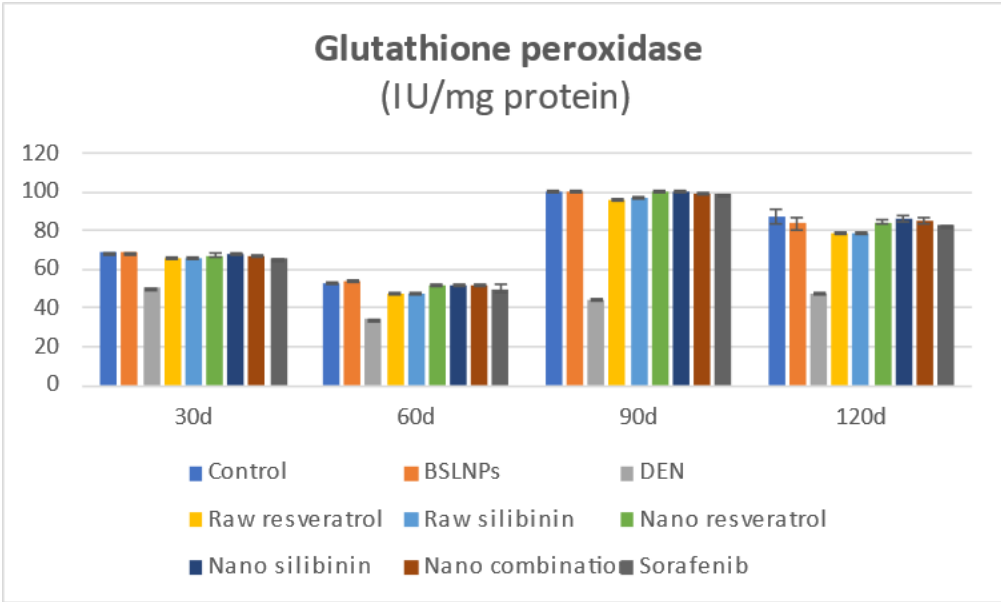

Fig 3: Effect of DEN toxicity and treatment on GPX in liver tissue of rats

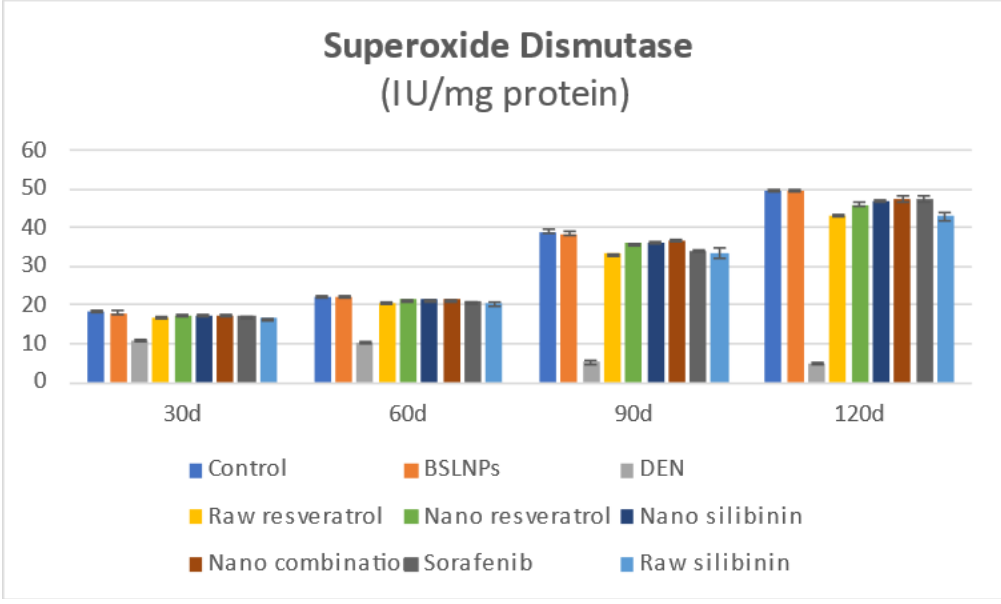

Fig 4: Effect of DEN toxicity and treatment on SOD in liver tissue of rats

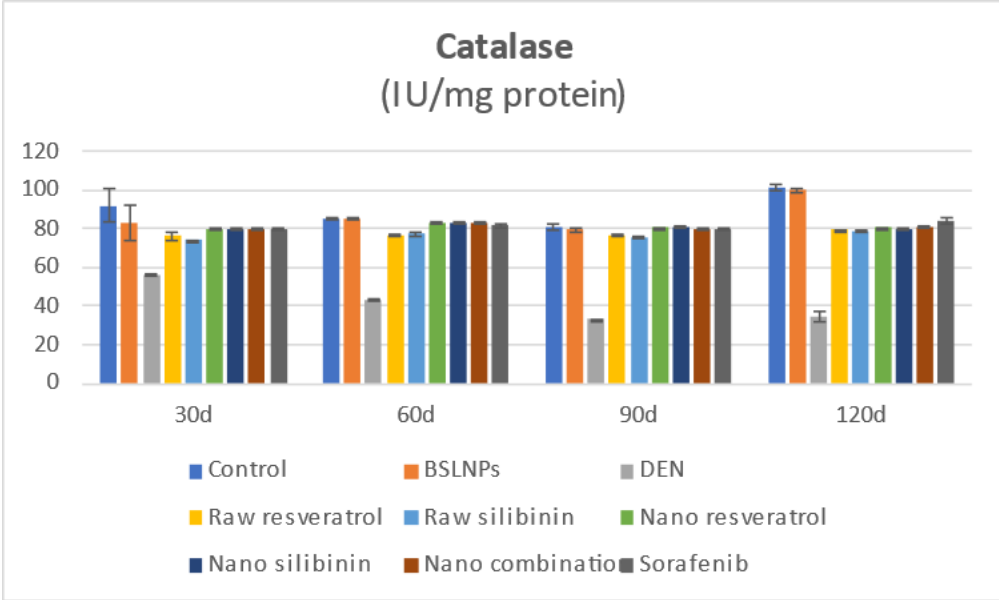

Fig 5: Effect of DEN toxicity and treatment on CAT in liver tissue of rats

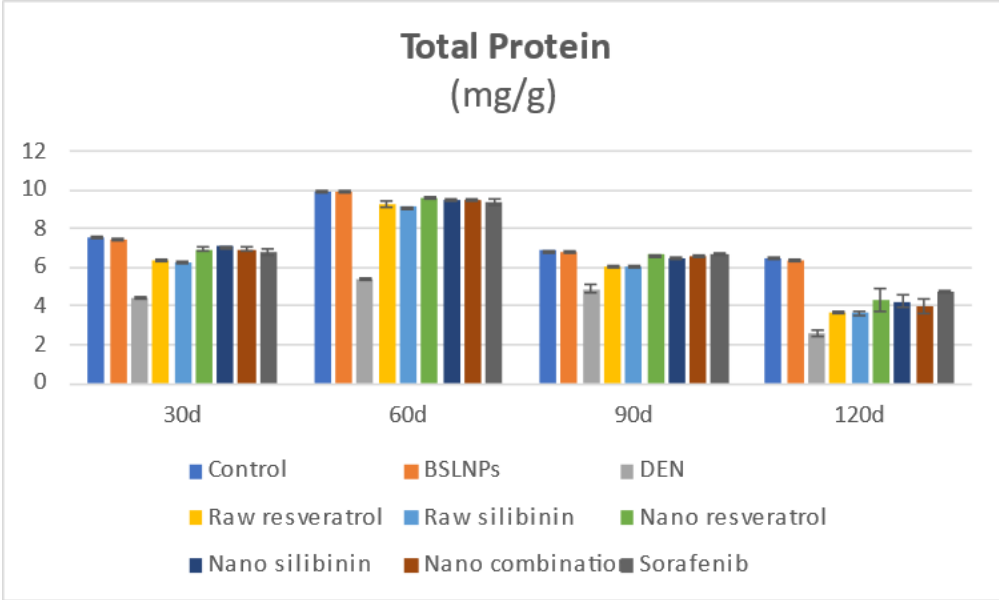

Fig 6: Effect of DEN toxicity and treatment on TP in liver tissue of rats

were observed between the DEN and the sham control/ BSLNPs groups. There was a significant ($p<0.05$) reduction in the GPX (Fig.3), SOD (Fig 4.), catalase (Fig.5) values and GSH values in the DEN treated groups as compared to the sham control/ BSLNPs groups. SLNPs showed no influence on the antioxidant status. Sorafenib group showed significant ($p<0.05$) decrease in the GPX, GSH, catalase and SOD values as compared to sham/ BSLNPs groups. Total protein (Fig.6) values did not differ significantly among the various treatment groups. There was a significant difference in the antioxidant status with time. As the time of exposure to the carcinogen increased, there is more reduction in the antioxidant status of the liver tissue.

The oxidative stress is measured by measuring liver lipid peroxidation and antioxidant status. The present study reports significant increase in the liver LPO in DEN groups as compared to treatment and control groups. Treatment group livers showed a significant difference in LPO value as compared to the DEN group. Nano groups showed better protection as compared to raw treated groups. This finding indicated the protective effect of nano resveratrol, nano silibinin, nano combination on DEN induced cell membrane damage. Restoration in the levels of lipid peroxidation after administration of resveratrol and silibinin could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane lipids. This is in accordance with findings of Kasdallah *et al*. (2006).

The present study showed a significant decrease in GPX, SOD, CAT and GSH liver antioxidants in the DEN treated group when compared to the control. Nano groups showed comparatively better protection than raw treatment groups. There was a significant difference between treatment and carcinogen group. These findings were in accordance with Anjaladevi (2020) who found that GPX, CAT and SOD activities very significantly decreased in the DEN treated groups as compared to control. The above antioxidant findings is in agreement with Rajasekaran *et al*. (2011) who also reported significant decrease in the GSH, GPX, SOD and CAT in the DEN treated rats and significant increase in the antioxidant status in the DEN+ resveratrol group and reported a protective effect of resveratrol in DEN induced oxidative stress. SOD is a sensitive index in hepatocellular damage and it scavenges the superoxide anion to form hydrogen peroxide, diminishing the toxic effect caused by the free radical (Mondal *et al*., 2020). The present study reports reduction in total protein in the DEN treated rats that might be due to inhibition of protein synthesis and hepatic lesions from hepatoxicity which lead to hepatocellular damage and defective protein biosynthesis in liver.

Important player in resveratrol's antioxidant activity is suggested to be run by the OH groups and blocking OH group methylation showed that resveratrol and trimethylated resveratrol provide some degree of protection, but the latter one has a better protective effect (Rivera *et al*., 2008). Another hepatoprotection mechanism of

resveratrol comes from its ability to activate genes related to antioxidant system or from its ability to inhibit enzymes. Zhu *et al*. (2011) shown that, in mice, administration of resveratrol increased the antioxidant system (SOD, GPx, and GSH). It has been proposed that resveratrol indirectly activates peroxisome-proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α), which can regulate mitochondrial energetics and induce the expression of many ROS-detoxifying enzymes including SOD, CAT, and GPX. To explain our findings, we hypothesize that GSH may be maintained in hepatocytes after pre-treatment with resveratrol, as suggested by the concentration of GSH in liver in our results. The observations of the present study were in accordance with Arirudran and Saraswathy (2014).

Treatment with nano resveratrol and nano silibinin for 30 days after DEN administration restored the activities of SOD and CAT in the liver tissue to that of near normalcy. The decrease in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the metabolism of diethylnitrosamine. This is further substantiated by an elevation in the levels of lipid peroxidation (Yassin *et al*., 2022). Similar reports have shown an elevation in the status of lipid peroxidation in the liver during diethylnitrosamine treatment and our results are in accordance with these reports. The decreased levels of GSH observed during diethylnitrosamine administration might be due to the excessive utilization of GSH in scavenging the free radicals formed during the metabolism of diethylnitrosamine. Silibinin treatment effectively restored the depleted levels of these nonenzymic antioxidants caused by diethylnitrosamine.

CONCLUSION

The results of the present study show silibinin and resveratrol to be effective in scavenging the free radicals released during the metabolism of diethylnitrosamine. It also showed that the silibinin and resveratrol exhibits good hepatoprotective property in both nanoforms and raw forms by restoring the hepatic antioxidant property by reversing the oxidant–antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats.

ACKNOWLEDGEMENT

The author thank Tamil Nadu Veterinary and Animal Sciences University for all the support extended.

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