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EVALUATION OF HEPATOTOXICITY IN MICE BY 2,4- DICHLOROPHENOXY ACETIC ACID (2,4-D): A PROTECTIVE EFFECT OF MELATONIN

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ABSTRACT: As a selective herbicide, 2,4- dichlorophenoxy acetic (2,4-D) acid is worldwide used in agricultural field. It has been associated with a variety of toxicities in rodents including man.The present study was performed to evaluate alterations in biochemical and antioxidant biomarkers of liver in adult male mice(*Musmusculus*) exposed to it at different concentrations (30, 60, 90 mg/kgbw) for 45 days daily and its mitigation by melatonin (10 mg/kgbw). Liver dysfunctions were confirmed by decrements in metabolic indices i.ephosphatases, adenosine triphosphatase, succinate dehydrogenase and total proteins. Similarly, various oxidative stress indices like glutathione, total ascorbic acid, total –SH, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione –s-transferase levels remainedaltered by 2,4-D feeding. Contrarily increased activities of glycogen and lipid peroxidation (LPO) levels were confirmed by administration of toxicant in a dose dependent manner.These consequences were supported by hepato-histopathological study. However, melatonin administration to 2,4-D treated mice mitigated these metabolic, oxidativestress as well as histopathological effects and it is hence concluded that melatonin ameliorates 2,4-D induced toxicity in this vital organdue to its antioxidant properties.

Key Words: Herbicide, Hepatotoxicity, Biochemical and Antioxidant indices, Histopathology, Liver, Mice.

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INTRODUCTION

The 2,4-dichlorophenoxy acetic (2,4-D) acid is one of the most selective herbicide used worldwide in agriculture (Marouani *et al.*,2017) .In India, it is one of the most successful and commonly used types of modern agriculture to control the growth of broad-leaved weeds. It is present everywhere like air, water, soil and food (Jamakala and Rani, 2015) .Through food chain, it enters the healthy individual's physique and induces carcinogenesis, embryotoxicity, immunotoxicity, teratogenicity, and endocrine effects (Venkov et al., 2000; Tuschl and Schwab,2003; Maurouani et al., 2017).Wafa et al.(2012) and Nakbi et al. (2010) suggested that 2,4-D exposure can lead to oxidative stress through the unregulated generation of (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radical and singlet oxygen in varieties of animal species.In the healthy individuals, the generation of reactive oxygen species (ROS) is well balanced by the counter balancing act of antioxidant defenses.Recently, this herbicide exerted reproductive effects in rodents leading to loss of reproductive functions (Joshi et al., 2012 and Mehta et al., 2016). Studies in our laboratory documented these toxicant induced effects in kidney, testes and liver of the adult mice (Patel et al., 2016; Upadhaya et al., 2017; Rao and Satapathy, 2017).

Various antioxidants including vitamins and herbal products are well known to ameliorate toxicant generated physiological and genetic effects in *in vivo* and *in vitro* conditions (Rao and Bhatt, 2012; Rao and Narenchania, 2016). Melatonin is a direct scavenger of OH, O_2^-, and NO and can easily cross the cell membranes and the blood-brain barrier (Rao and Satapathy, 2017). Besides its function, it has been referred to as a terminal (or suicidal) antioxidant (Tan et al., 2000). Very few studies have been conducted on its mitigation against 2,4-D toxicity*in vivo* and *in vitro*(Bongivanni et al.,2007;Patel et al., 2016; Upadhaya et al., 2017; Mehta et al., 2016). Hence, we supplemented melatonin in our study to mitigate the effects of 2,4-D in the vital organ of mice.

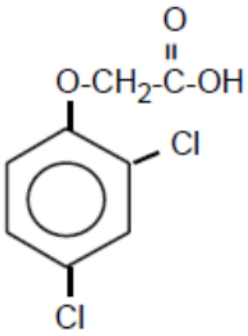

Fig.1:Chemical Structure of 2,4-D

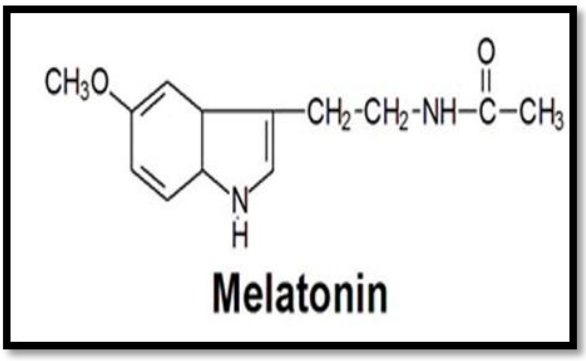

Fig. 2: Chemical Structure of Melatonin

MATERIALS AND METHODS

Chemicals

The compound 2, 4-Dichlorophenoxyacetic acid (2,4-D) was obtained from Sigma Chemicals (USA), whereas melatonin (MEL)was from Hi media laboratories(Mumbai, India). All the other chemicals used in different assays were procured from Sigma Chemicals (USA) and Merck and Hi Media (Mumbai).

Animals

Three months old Swiss strain male albino mice (*Musmusculus*) weighing between 30-50 gm/kg body weight were obtained from Cadila Pharmaceutical, Ahmedabad, Gujarat, India, under the Animal Maintenance Registration No. 167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Govt. of India and were kept in stainless steel mesh cages, housed under standard laboratory conditions (26±2º C, 30-70% relative humidity, 12 hour light-dark cycle) with standard animal chow (Pranav Agro Industries, Baroda, India) and drinking water *ad libitum*. The mice were acclimatized to the laboratory conditions for 10 days.

Dose and Duration

The dosage of 2,4-D (Fig. 1) was used based on LD_{50}370 mg/kg in mice followed by Gervais et al.(2008)**.** The dose of melatonin (Fig. 2) was decided based on earlier work of Rao and Satapathy (2017).The treatments were given orally (2,4-D) or intraperitoneally (Melatonin) to experimental animals with gavages and syringe respectively. The control animals were provided the only standard diet with distilled water throughout the study. The duration of treatment was for 45 days.

The animals were divided into eight (I to VIII) groups. The eight groups were mainly: Control (untreated), Melatonin alone (10mg/kg), 2,4-D treated (low dose;30mg/kg),2,4-D treated (mid dose;60mg/kg), 2,4-D treated (high dose; 90mg/kg), 2,4-D treated + Melatonin (LD+MEL), 2,4-D treated + Melatonin (MD+MEL), 2,4-D treated + Melatonin (HD+MEL).

Isolation of tissues

The control and treated animals were sacrificed by cervical dislocation at the 46th days of each treatment. The liver was excised carefully, blotted free of blood before weighing and used for carrying out gravimetric, metabolic andoxidative stress parameters followed by the histopathological study.

Metabolic Indices

The metabolic parameters evaluated were acid phosphatase and alkaline phosphatase (ACPase: EC 3. 1. 3. 2 and ALPase: EC 3. 1. 3. 1; Bessey et al., 1946), adenosine triphosphatase (ATPase:EC 3. 6. 1. 3; Quinn and White, 1968), succinate dehydrogenase (SDH:EC 1. 3. 5. 1; Beatty et al., 1966), total protein (TP; Lowery et al.,1951) and glycogen (GLG; Stalmans et al.,1974) using standard techniques.

Oxidative Stress Profile

The non-enzymatic antioxidant parameters in liver of all groups included glutathione (GSH; Ellman,1959), total ascorbic acid (TAA; Roe and Ku¨ether, 1943), total sulfhydryl (Total-SH; Sedlak and Lindsay,1968) and lipid peroxidation (LPO;Ohkawa et al., 1979) followed by the enzymatic antioxidant enzymes such as superoxide dismutase (SOD: EC 3. 15. 1. 1;Kakkar et al., 1984), catalase(CAT: EC 3. 11. 1. ; Luck, 1963), glutathione peroxidase (GPx:EC 1. 11. 1. 9; Rotruck et al.,1973), glutathione reductase (GR: EC 1. 8. 1. 7; Carlberg and Mannervik, 1985) and glutathione –s-transferase (GST: EC 2. 5. 1. 18; Habig et al.,1974) respectively.

Statistical Analysis: The data were subjected to statistical analysis such as mean, standard deviation (SD), and analysis of variance (ANOVA), using standard statistical software, the statistical package for social sciences (SPSS; version 16). All values are expressed as mean ± SD of 10 individual samples. A value of $P < 0.05$ was considered significant.

RESULTS

Body weight was significantly ($P<0.01$, $P<0.001$) reduced by 2,4 –D feeding with dose increments (Gr. I Vs.VI and V).The same pattern was noticed with liver (Gr. I Vs. VI and V). As compared with high dose, mid dose and low dosesalso had significant ($P<0.05$, $P<0.01$) changes. Melatoninsupplementation at high dose level of 2,4-D exhibited partial recovery. But a significant ($P<0.05$) mitigation was observed when compared to other doses (Gr. VIIIVs. VII & VI) (Table 1).

The data in levels of phosphatases, SDHand total protein in the liver showed a significant ($P<0.01$, $P<0.001$) reduction, whereascontrarily a significant ($P<0.01$, $P<0.001$) increase in glycogen were recorded in high doses fedmice exhibiting dose dependency. The ATPase and SDH levels were ($p<0.05$) significantly altered in melatonin supplementation with 2,4-D high dose treated mice whereas there were no changes observed in ACPase, ALPase, total protein and glycogen levels. A significant ($p<0.05$) mitigation was seen when compared with the high dose of 2,4-D treated melatonin supplementation group versus low dose of 2,4-D treated melatonin supplementation group in these levels.No substantial results were recorded in other groups(Table 2).

The mice exposed to 2,4-D resulted in significant ($P<0.01$, $P<0.001$) decline in GSH, TAA and -SH followed by significant ($P<0.01$, $P<0.001$) rise in LPO levels at mid dose and high dose levels. The high dose treated mice showed a significant ($P<0.05$, $P<0.01$) alterations in GSH, TAA, -SH and LPO level in comparison to other groups. In contrast, except TAA, melatonin marginally mitigated high dose of 2,4-D toxicity. However, significant ($P<0.05$) mitigation was observed in GSH, -SH and LPO levels by comparing with high dose of 2,4-D treated with melatonin supplementation to other groups(Table 3).

Further, our results revealed that SOD, CAT,GR and GST activities were decreased markedly($P<0.05$, $P<0.001$) in all mice exposed to toxicant gradually.When we compared high dose with other doses, significant changes ($P<0.05$, $P<0.01$) were observed with respect to above indices in all the groups. In SOD, CAT and GST activities, melatonin was able to alleviate 2,4-D toxicity at high dose level. Similarly there were no significant changes observed comparing with low and mid doses. A significant ($P<0.05$) mitigation was noted in GPx and GR enzyme activitiesby comparing with control as well as 2,4-D treated melatonin supplementation groups with other doses of 2,4-D treated melatonin supplementation groups. But mitigation was partial ($P< 0.05$) with high dose by melatonin supplementation (Table 4).

Histopathology: The hepatic parenchyma consists of cords of hepatocytes which are separated from other hepatic cords by vascular sinusoids in control mice (Fig. 1). Treatment of mice with 2,4-D for 45 days produced mild vascular and hepatocellular lesions with necrotic changes and focal areas of necrosis in the liver (Fig. 2). Supplementation of melatonin (MEL) (group VIII), few hepatocytes exhibited resumption of the anatomical features similar to that of control mouse (Fig. 3).

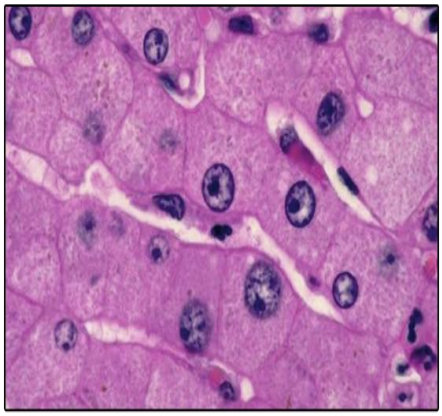

Fig. 1: Normal mouse liver (Gr. 1) section appearances a network of hexagonal shaped cells known as hepatocytes(H), blood vessels BV) followed by sinusoid cells (S). X840.

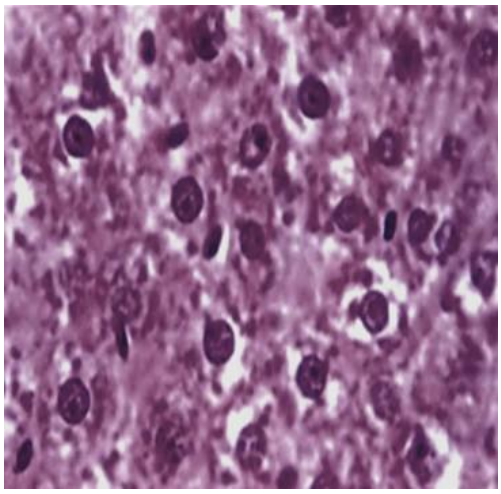

Fig. 2: This figure revealing necrosis (N) followed by vacuolated hepatocytes (VH) in treated mice (Gr. V)with HD. X840.

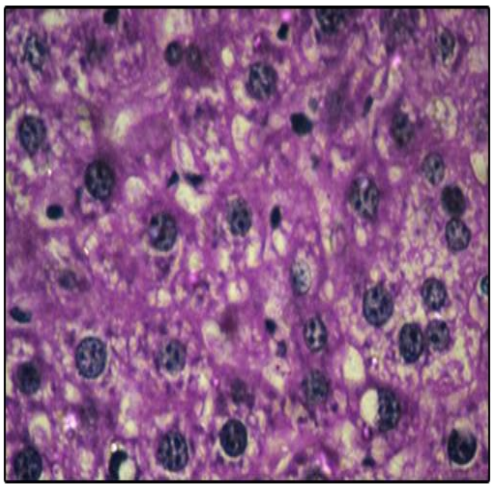

Fig. 3: Normal features of hepatic tissue supplemented with melatonin to treated mice (Gr. VIII). X840.

Table 1: Body and organ weights of control and experimental groups

PARAMETERS	CON (G.I)	MEL (G.II)	TREATED(2,4-D)			TREATED+MEL		
			LD (G.III)	MD (G.IV)	HD (G.V)	LD+ MEL (G.VI)	MD+ MEL (G.VII)	HD +MEL (VIII)
Total Body Weight gm)	42.63±1.21	41.9±1.55^{NS}	38.21± 1.11^{NS,b}	32.13± 1.6^{**,a}	29.54± 1.43^{+}	39.5± 1.43^{NS,d}	38.14± 1.12^{NS}	35.14± 1.13*
Liver (mg)	2.78±0.28	2.68±0.28^{NS}	1.89± 0.19^{NS,b}	2.49± 0.16^{**,a}	.98± 0.14^{+}	2.69± 0.15^{NS,d}	2.67± 0.01^{NS}	2.08± 0.50*

Values are Mean ± S.E, NS= Non Significant, (Gr. II, III,VI,VII Vs. Gr. I), (G.VIII Vs G.VII).*=P<0.05, **=P<0.01, +=P<0.001 (Gr. II to VIII verses Gr. I), a=P<0.05, b=P<0.01 (G.V Vs G.IV & G.III), d=P<0.05 (G.VIII Vs G.VI).

Table 2: Biochemical indices in liver of control and experimental groups.

PARAMETERS	CON (G.I)	MEL (G.II)	TREATED(2,4-D)			TREATED+MEL		
			LD (G.III)	MD (G.IV)	HD (G.V)	LD +MEL (G.VI)	MD+ MEL (G.VII)	HD+MEL (G.VIII)
Acid phosphatase[m]	3.29 ±0.04	3.19 ±0.04^{NS}	3.01± 0.02^{NS,b}	2.01± 0.14^{**,a}	1.67± 0.31^{+}	2.99± 0.18^{NS,d}	2.88± 0.24^{NS}	2.69± 0.14^{NS}
Alkaline phosphatase[n]	2.09 ±0.01	1.69 ±0.12^{NS}	1.84± 0.11^{NS,b}	1.56± 0.07^{**,a}	0.98± 0.13^{+}	1.83± 0.11^{NS,d}	1.80± 0.18^{NS}	1.71± 0.17^{NS}
Adenosine triphosphatase[o]	2.19 ±0.11	2.14 ±0.01^{NS}	2.03± 0.01^{NS,b}	1.41± 0.01^{**,a}	1.09± 0.10^{+}	2.14± 0.10^{NS,d}	1.95± 0.28^{NS}	1.57± 0.06*
Succinate dehydrogenase [p]	46.18 ±1.03	42.09 ±0.01 ^{NS}	42.88± 0.51^{NS,b}	35.54± 1.21^{**,a}	28.54± 0.11$^{+,}$	41.98± 0.21^{NS,d}	40.78± 0.01 ^{NS}	39.74± 0.01*
Total proteins [q]	16.76±. 81	15.63± 0.11^{NS}	13.18± 0.24^{NS,b}	11.58± 0.31^{**,a}	9.58± 0.31^{+}	14.28± 0.11^{NS,d}	14.16± 0.22^{NS}	13.38± 0.23^{NS}
Glycogen [r]	1406 ±43.03	1457± 34.21^{NS}	1647± 20.51^{NS,b}	2051± 67.91^{**,a}	2401± 51.1^{+}	1602± 21.41^{NS,d}	1621± 32.31^{NS}	1648± 51.11^{NS}

m,n=µ moles of p-nitro phenol released / mg protein, o= µ moles of inorganic phosphate released / 30 min. / mg protein, p= µg formazan formed/ 15 min/ mg protein, q=mg/100 mg tissue weight, r=µ moles/ 100 mg tissue weight, Values are Mean ± S.E, NS= Non Significant, (Gr. II, III,VI,VII Vs. Gr. I), (G.VIII Vs G.VII).*=P<0.05, **=P<0.01, +=P<0.001 (Gr. II to VIII verses Gr. I), a=P<0.05, b=P<0.01 (G.V Vs G.IV & G.III), d=P<0.05 (G.VIII Vs G.VI).

Table 3: Non-enzymatic antioxidants in liver of control and experimental groups

PARAMETERS	CON (G.I)	MEL (MEL) (G.II)	TREATED(2,4-D)			TREATED+MEL		
			LD (G.III)	MD (G.IV)	HD (G.V)	LD +MEL (G.VI)	MD+ MEL (G.VII)	HD +MEL (G.VIII)
Glutathione [m]	70.25± 2.04	69.24± 2.13^{NS}	64.21± 1.12^{NS,b}	46.87± 2.13^{**,a}	37.17± 1.41^{+}	64.01± 0.92^{NS,d}	60.31± 0.92^{NS}	52.11± 1.12*
Total ascorbic acid [n]	3.19± 0.01	2.89± 0.11^{NS}	2.74± 0.21^{NS,b}	2.26± 0.07^{**,a}	1.98± 0.13^{+}	2.83± 0.01^{NS,d}	2.79± 0.18^{NS}	2.75± 0.17^{NS}
Total sulfhydryl [o]	2.19± 0.11	2.13± 0.01^{NS}	2.13± 0.11^{NS,b}	1.32± 0.11^{**,a}	2.08± 0.10^{+}	2.13± 0.10^{NS,d}	1.92± 0.28^{NS}	1.73± 0.16*
Lipid peroxidation [p]	29.76± 0.11	31.86± 0.32^{NS}	42.16±0 .23^{NS,b}	43.58± 0.31^{**,a}	47.43± 0.31^{+}	32.11± 0.23^{NS,d}	33.31± 0.13^{NS}	40.21± 0.23*

m=µ moles/ 100 mg tissue weight, n= mg/ gm tissue weight, o= mg/ 100 mg tissue weight, p= n moles of MDA formed/100mg tissue weight. Values are Mean ± S.E, NS= Non Significant, (Gr. II, III,VI,VII Vs. Gr. I), (G.VIIIVs G.VII).*=P<0.05, **=P<0.01, +=P<0.001 (Gr. II to VIII verses Gr. I), a=P<0.05, b=P<0.01 (G.V Vs G.IV & G.III), d=P<0.05 (G.VIII Vs G.VI).

Table 4: Enzymatic antioxidant in liver of control and experimental groups.

PARAMETERS	CON (G.I)	MEL (G.II)	TREATED(2,4-D)			TREATED+MEL		
			LD (G.III)	MD (G.IV)	HD (G.V)	LD +MEL (G.VI)	MD+ MEL (G.VII)	HD +MEL (G.VIII)
Superoxide dismutase [m]	2.15± 0.04	2.02± 0.01^{NS}	2.01± 0.05^{NS,b}	1.57± 0.03^{**,a}	1.19± 0.01^{+}	2.07± 0.11^{NS,d}	2.09± 0.03^{NS}	2.08± 0.03^{NS}
Catalase [n]	30.19± 0.19	28.81± 0.15^{NS}	28.74± 0.07^{NS,b}	25.23± 0.13^{**,a}	19.58± 0.21^{+}	29.84± 0.05^{NS,d}	29.93± 0.11^{NS}	29.81± 0.17^{NS}
Glutathione peroxidase [o]	29.27± 0.11	24.71± 0.31^{NS}	25.12± 0.01^{NS,b}	21.41± 0.11^{*,a}	19.17± 0.19**	25.61± 0.31^{NS,d}	25.22± 0.04^{NS}	20.26± 0.21*
Glutathione reductase[p]	34.06± 1.03	32.13± 1.20^{NS}	27.77± 0.91^{NS,b}	25.41± 1.01^{**,a}	21.01± 1.01^{+}	31.43± 1.01^{NS,d}	31.43± 0.81^{NS}	27.40± 1.01*
Glutathine –s-transferase[q]	0.029±. 001	0.027±. 001^{NS}	0.025± .001^{NS,b}	0.017± .003^{**,a}	0.012± 0.02^{+}	0.025± .002^{NS,d}	0.024± .002^{NS}	0.023± .001^{NS}

m= n moles of MDA formed/100mg tissue weight, n= units/ mg protein, o= µ moles of H_2O_2 consumed/ min/ mg protein, p= µ moles of GSH consumed/ min/ mg protein, q= n moles of NADPH oxidized/ min/ mg protein. Values are Mean ± S.E, NS= Non Significant, (Gr. II, III,VI,VII Vs Gr. I) (G. VIII Vs G.VII).*=P<0.05, **=P<0.01, +=P<0.001 (Gr. II to VIII verses Gr. I), a=P<0.05, b=P<0.01 (G.V Vs G.IV & G.III), d=P<0.05 (G. VIII Vs G.VI).

DISCUSSION

Feedingto serial doses of 2,4-Dto mice for 45 days significantly decreased body and organ weight due to loss of food intake in a dose dependent manner where the high dose was maximum. This can also be explained by loss of appetite and improper metabolic status followed by protein synthesis inhibition. Similar reports were reported by other researchers working with varieties of tissue in treated rodents (Upadhyaya et al., 2017; Patel et al., 2016; Joshi et al., 2012)

Similarly, the decline in protein levels in our studies bytreatment with 2,4-D was also noticed indicating the loss of body andliver functions in fed mice. Reduction in SDH and ATPase activities in this tissue hence resulted in a loss of energy status inintoxicant mice. Studies of Rao and Satapathy (2017) also support these results, where metabolic enzymes were reduced in this vital organof fed mice. Further, it is evident that glycogen levels are increased markedly in it altering the level of blood glucose levels affecting carbohydrate metabolism. Moreover, the altered phosphatase levels in treated liver of mice substantiated pathogenic effects of this tissue in our cohort to support liver damage as reported(Nakbi et al., 2010). Same results were evident in other tissues like kidney and testes by this treatment in mice and rats(Patel et al.,2016;Upadhayaya et al.,2017; Joshi et al., 2012).

In the present study, there is a decrement in the level of GSH and total-SH contents. Their depletions to about 20% to 30% levelsimpair cell defenses against toxic action.This might lead to cell injury and death. The reductionof ascorbate also supported the stress condition imposed bythis herbicide. Thus, 2,4-D led to a reduction in non- enzymatic indicators to reflect on imposition of oxidative stress (OS) in this organ .This was still noticed by an increase inlipid peroxidation levels in the liver of intoxicated mice .Hence, increased lipid peroxides, reduced non enzymatic factors, this oxidative stress can affect the activities of other protective antioxidant enzymatic machionary which are sensitive indicators of exaggerated stress. Therefore, a marked decline in an antioxidant enzymes such as SOD, CAT, GPx and GR in the liver were observed. .Same data werealso obtained by Patel et al.(2016) and Upadhayaya et al.(2017)in testes and kidney of mice .These consequences of oxidative stress were correlated with liver pathological symptoms as noticed in our cohort.These findings were also in agreement with Nakbiet al. (2010) who studied intoxicated rats.Anusuya andHemlatha(2014) also reported oxidative stress inducted by 2,4-D pesticide /herbicide in fish and found significant reductions in SOD and catalase levels in agreement to our results.Kawashima et al (1984) also documented increased peroxisomal enzymes in treated rat liver mitochondria supporting our data andcould be related to toxicant accumulation in it (Pitchard et al., 1982).

However, the 2,4-D induced effects could be effectively mitigated by supplementation of melatonin as noticed in our study. Melatonin thus mitigates numerous toxic effects exerted by various toxicants due to its free radicals scavenging cascade (Tan et al., 2000, 2002). The role of melatonin in alleviation of herbicide induced toxicity is well demonstrated*in vitro* conditions in rat cerebral granule cells (Bongivanni et al., 2007). Similar way*in vivo*studies in our earlier data also (Patel et al., 2016; Upadhayaya et al., 2017) supported these observations.

CONCLUSION

The effect of 2, 4-D toxicant exerted hepatotoxicity is evidenced by induction of oxidative stress affecting mouse liver structure and function .It is further mitigatedby melatonin supplementation, due to its antioxidant properties to certain extent.

Conflict of interest statement

There are no conflicts of interest.

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