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Protective Effects of Melatonin Against 2,4-Dichlorophenoxyacetic Acid Induced Altered Haematological Variables in Mice: An *In Vivo* and *In Silico* Approach

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Abstract

2,4-Dichlorophenoxyacetic acid (2,4-D) is a systemic phenoxy herbicide that induces oxidative stress. In contrast, melatonin is a secretory product of the pineal gland with antioxidant properties. In the present study, the ameliorative potential of melatonin (10 mg/kg body weight) was investigated against 2,4-D (low, mid, and high dose-16.5, 33.0, and 66.0 mg/kg body weight) induced altered haematological variables using *in vivo* and *in silico* models. Doses of 2,4-D and melatonin were administered orally for 28 days. The evaluated haematological indices in the present study were Haemoglobin (Hb), Red Blood Corpuscles (RBC), Haematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), White Blood Corpuscles (WBC), Lymphocytes, Monocytes, Granulocytes, Platelet Count (PT), Mean Platelet Volume (MPV), Plateletcrit (PCT), and Erythrocyte Sedimentation Rate (ESR). The statistical significant value was considered at $p<0.05$. Molecular docking study was performed for interaction of 2,4-D and melatonin with haemoglobin. *In vivo* results revealed that 2,4-D treatment showed a significant dose-dependent alteration in above all studied haematological indices. No significant auto reversal effects were observed in the withdrawal study, on the contrarily, the altered haematological indices were normalized and comparable to control when melatonin was given alone and in combination with 2,4-D. *In silico* results also demonstrated that 2,4-D and melatonin showed competitive bindings with haemoglobin. In nutshell, these *in vivo* and *in silico* findings depicted those haematological indices were altered by 2,4-D toxicity and can be abridged by melatonin attributed to its ameliorative potential as also evidenced by molecular docking.

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1. Introduction

Pesticides are extensively used in modern agriculture and are an effective and economical way to enhance the yield quality and quantity, thus ensuring food security for the ever-growing population around the globe. Approximately, 2 million tonnes of pesticides are utilized annually worldwide with increased usage of 3.5 million tonnes by the year 2020[1]. The largest of these pesticide categories are herbicides which compose 50% of the pesticides worldwide. They are a broad class of pesticides that are commonly used by farmers to remove nuisance plants, such as grasses and weeds, that may compromise the growth and yield of desired crops that are in close proximity[2]. 2,4-Dichlorophenoxyacetic acid (2,4-D) has become a widely-used herbicide in agricultural and urban areas around the world due to its efficiency, selectivity, low cost, and broad-spectrum in pest control[3]. At recommended doses, 2,4-D is able to act with the properties of a natural auxin hormone. The recommended application doses level of 2,4-D in agro practices are between 0.5 to 2 L ha^{-1} (equivalent to 5 to 10 mM) depending on the crop[4]. 2,4-D has a Maximum Acceptable Concentration (MAC) of 0.10 mg/L (100 µg/L) for drinking water[5] and has maximum residue limits ranging between 0.01 to 5 ppm for a variety of food products (including fruits, vegetables, and animal tissues/organs)[6]. However, regardless of the decent results of using 2,4-D in agriculture, their high concentrations or frequent exposure and its persistence involve the risk of its retention in crops and soils. Moreover, this compound may also pass from surface to groundwater. The chemical through surface runoff may reach unrestricted areas like ponds and rivers and alters the physicochemical properties of water. In this way, 2,4-D directly or indirectly pollutes crops, air, water, soil, and the overall ecosystem causing a serious health hazard for living beings[7]. Blood is an important component for studying the effects of toxicants as it is highly susceptible to environmental fluctuations[8]. Many studies have directed on changes in blood parameters resulting from exposures to 2,4-D conditions and/or the presence of contaminants[9–14]. 2,4-D increases the level of methemoglobin and changes of oxygen affinity of haemoglobin[9]. Apart from haemoglobin, 2,4-D also alters the balance of erythrocytes and leucocytes by revealing the general immune suppressive effect[10–14]. The endogenous antioxidant defenses may be inadequate to offer protection against 2,4-D damage. Several therapeutic and dietary antioxidants have been reported as immune boosters, i.e., melatonin, vitamin C, and curcumin. Out of them, melatonin is an indoleamine derived from the pineal gland, which modulates circadian rhythms and participates in a wide range of physiological processes[15,16]. In mammals, melatonin is produced by the pineal gland in the vertebrate brain and numerous other organs[17]. Melatonin ameliorates the toxicity of many drugs used to treat haematological malignancies, including myelotoxicity and toxicity on non-haematological tissues[18]. It is also known to influence haematological parameters and various immune parameters[19]. Previously, exogenous melatonin could effectively reduce 2,4-D toxicity in kidney tissue of Swiss albino mice[20,21]. However, to the best of our knowledge, there is no information available on the combined effects of 2,4-D and melatonin on haematological parameters. Therefore, the hypothesis of present work was designed to study the efficiency of melatonin against 2,4-D induced haematological parameter disorders using *in vivo* and *in silico* models.

2. Materials and Methods

2.1 Chemicals

The herbicide, 2,4-D (CAS No. 94-75-7; Purity: ≥ 98.0%) was obtained from Loba Chemie, Mumbai, India. Melatonin (CAS No. 73-31-4; Purity: ≥ 99.0%) was obtained from HiMedia Laboratories, Mumbai, India. All the other chemicals (AR grade) utilized in different assays were procured from Sigma Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), and HiMedia Laboratories (Mumbai, India).

2.2 Experimental Animals

Healthy, adult male albino mice of the Swiss strain (*Mus musculus*) obtained from Zydus Cadila Pharmaceutical, Ahmedabad, Gujarat, India were used for all experiments. Mice were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 22 ± 3°C, 12 hours light/dark cycle, and relative humidity 50-55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and water *ad libitum*. All the experimental protocols were approved by the Committee for the Purpose of Control

Table 1. Experimental groups

Groups	Treatment and Dose	Duration (days)	Day of Autopsy
I	Control (CN)	28 days	29th day
II	Melatonin (MT) alone (10 mg/kg body weight)	28 days	29th day
III	2,4-D Low Dose (LD) (16.5 mg/kg body weight)	28 days	29th day
IV	2,4-D Mid Dose (MD) (33 mg/kg body weight)	28 days	29th day
V	2,4-D High Dose (HD) (66 mg/kg body weight)	28 days	29th day
VI	2,4-D High Dose (HD) (66 mg/kg body weight) + Withdrawal (WD)	28 days + 28 days	57th day
VII	2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight)	28 days	29th day

and Supervision of Experiments on Animals (Reg-GU/IAEC/02/2017/CPCSEA), New Delhi, India. Mice were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India[22].

2.3 Dose Selection

Different doses of 2,4-D were selected based on its LD_{50} value (330 mg/kg body weight)[23]. Mice of the 2,4-D treated groups received three different doses of 2,4-D which are 1/20, 1/10, and 1/5 of LD_{50} value (low, mid, and high dose-16.5, 33.0, and 66.0 mg/kg body weight) respectively. For the amelioration of 2,4-D induced toxicity dosage of melatonin (10 mg/kg body weight) was selected based on the study of Upadhyaya and co-workers[20,21].

2.4 Treatment

As shown in Table 1, forty-two mice were randomly divided into seven groups with six mice in each group. Group I served as control and received an equivalent amount of sterile distilled water used as a vehicle; Group II (antidote control) was given administration of melatonin alone (10 mg/kg body weight), mice of groups III, IV, and V received three different doses of 2,4-D (low, mid, and high dose-16.5, 33.0, and 66.0 mg/kg body weight), however, mice of Group VI received a high dose of 2,4-D (66 mg/kg body weight) followed by 28-day withdrawal (auto-recovery) period. Group VII mice were administered a 2,4-D high dose (66 mg/kg body weight) along with melatonin (10 mg/kg body weight). The mode of administration in all the groups was oral by force-feeding. The volume of administration (0.2 ml/mice/day) in all the groups was adjusted throughout the experiment. Group I-V and Group VII mice received 28-day treatment and were sacrificed on the 29th day whereas in Group VI mice received treatment for 28 days and then remained untreated for the next 28 days and were sacrificed on the 57th day. For sacrification of the mice, the cervical dislocation method was opted. Blood samples were collected by cardiac puncture in 0.5 M EDTA tubes and used for the analysis of the haematological profile.

2.5 Haematological Indices

Haemoglobin (Hb), Red Blood Corpuscles (RBC), Haematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), White Blood Corpuscles (WBC), Lymphocytes, Monocytes, Granulocytes, Platelet Count (PT), Mean Platelet Volume (MPV), and Plateletcrit (PCT) were analysed by automated method on Mindray - Auto

Haematology Analyzer (Model: BC-2800 Vet). Moreover, Erythrocyte Sedimentation Rate (ESR) was also analysed by automated method on Alcor Scientific - Fully Automated ESR Analyzer (Model: miniiSED).

2.6 Percentage Amelioration

Percentage amelioration in all studied parameters was calculated by using the following formula:

$$\text{Percentage Amelioration (\%)} = \frac{\text{Prooxidant Group-Respective Antioxidant Group}}{\text{Prooxidant Group-Control}} \times 100$$

2.7 Statistical Analysis

All the data were expressed as mean±standard error (SE). Statistical analysis was performed using GraphPad Prism software version 7.04. The data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was accepted at $p<0.05$.

2.8 Molecular Docking

To identify the plausible mechanism of 2,4-D and melatonin, blind molecular docking was performed using Autodock Vina[24] version 1.1.2. This analysis provided the interaction and structural insight to 2,4-D and melatonin compounds. The chemical structures of 2,4-D (CID: 1486) and melatonin (CID: 896) were downloaded from NCBI PubChem database[25] using compound identifiers respectively. The three-dimensional structure of haemoglobin (PDB code: 2HHB)[26] having resolution 1.74 Å was retrieved from the RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank[27]. The binding affinity and the mode were analyzed using Discovery Studio Visualizer[28] version 1 6.1.0.15350.

The molecular docking experiment resulted in the top 10 conformations. Amongst them, the best binding structures were taken into the consideration according to most negative Gibbs free energy (-ΔG) of the protein-ligand complex and type of interaction. Moreover, the interaction profiles have been evaluated to understand the mechanism of better binding affinity based on van der Waals, conventional hydrogen bond, alkyl, Pi-alkyl, unfavourable donor–donor, unfavourable acceptor–acceptor, Pi–Pi T-shaped, carbon-hydrogen bond, Pi–anion, and Pi– Sigma interactions to anticipate the interacting atoms with the respective selected receptors.

3. Results

3.1 Effects of 2,4-D and/or Melatonin on Hb, RBC, and HCT of Mice

The administration of 2,4-D (Group III-V) caused a significant dose-dependent decrease in the levels of Hb (low dose-$p<0.05$; mid dose-$p<0.001$; high dose-$p<0.0001$), RBC (low dose-$p<0.01$; mid dose-$p<0.001$; high dose-$p<0.0001$), and HCT (low dose-$p<0.05$; mid dose-$p<0.01$; high dose-$p<0.0001$) whereas melatonin alone treatment (Group II) presented no significant change in mice in all three parameters as compared to control (Group I). No significant reversal effects were observed in the withdrawal study (Group VI) of the above three parameters on the contrarily, significant ameliorative effects ($p<0.0001$) in all three parameters with percentage amelioration (Hb-84.52%; RBC-85.73%; HCT-82.61%) were noted in mice when melatonin was co-administered with a high dose of 2,4-D (Group VII) as compared to alone 2,4-D high dose induced mice (Group V) (Figure 1).

3.2 Effects of 2,4-D and/or Melatonin on MCV, MCH, and MCHC of Mice

No significant change was observed in MCH and MCHC values of 2,4-D low dose exerted mice (Group III), as well as antidote control (Group II) in all three parameters whereas significant 2,4-D dose-dependent decrement (Group III-V) was observed in MCV (low dose-$p<0.01$; mid dose-$p<0.001$; high dose-$p<0.0001$), MCH (mid dose-$p<0.01$; high dose-$p<0.0001$), and MCHC (mid dose-$p<0.01$; high dose-$p<0.001$) values as compared to control (Group I). No significant auto-recovery was noted in the withdrawal study (Group VI) of the above three parameters, on the other hand, melatonin supplemented with a high dose of 2,4-D (Group VII) showed significant recovery (MCV-$p<0.0001$; MCH-$p<0.001$; MCHC-$p<0.0001$) with percentage amelioration (MCV-92.64%; MCH-73.49%; MCHC-85.56%) as compared to the alone 2,4-D high dose administered mice (Group V) (Figure 2).

3.3 Effects of 2,4-D and/or Melatonin on WBC, Lymphocytes, Monocytes, and Granulocytes of Mice

Low dose of 2,4-D treatment (Group III) led no significant change in granulocytes value as well as antidote control (Group II) in all four parameters whereas significant 2,4-D dose-dependent increment (Group III-V) was noted in WBC (low dose-p<0.05; mid dose-p<0.01; high dose-p<0.0001), lymphocytes (low dose-p<0.05; mid dose-p<0.001; high dose-p<0.0001), monocytes (low dose-p<0.05; mid dose-p<0.01; high dose-p<0.0001), and granulocytes (mid dose-p<0.05; high dose-p<0.001) values as compared to control (Group I). Mice showed no significant auto-recovery in withdrawal investigation (Group VI) of the above four parameters whereas significant mitigative effects (WBC-p<0.0001; lymphocytes-p<0.0001; monocytes-p<0.0001; granulocytes-p<0.001) with percentage amelioration (WBC-84.73%; lymphocytes-89.80%; monocytes-85.25%; granulocytes-92.62%) were observed in mice when melatonin was fed along with a high dose of 2,4-D (Group VII) as compared to alone 2,4-D high dose administered mice (Group V) (Figure 3).

3.4 Effects of 2,4-D and/or Melatonin on PT, MPV, PCT, and ESR of Mice

No significant alteration was observed in the PCT value of 2,4-D low dose administered mice (Group III), as well as antidote control (Group II) in all four parameters whereas significant 2,4-D dose-dependent decrement (Group III-V) was observed in PT (low dose-p<0.01; mid dose-p<0.001; high dose-p<0.0001) and PCT (mid dose-p<0.01; high dose-p<0.0001) values as compared to control (Group I). Moreover, 2,4-D treatment

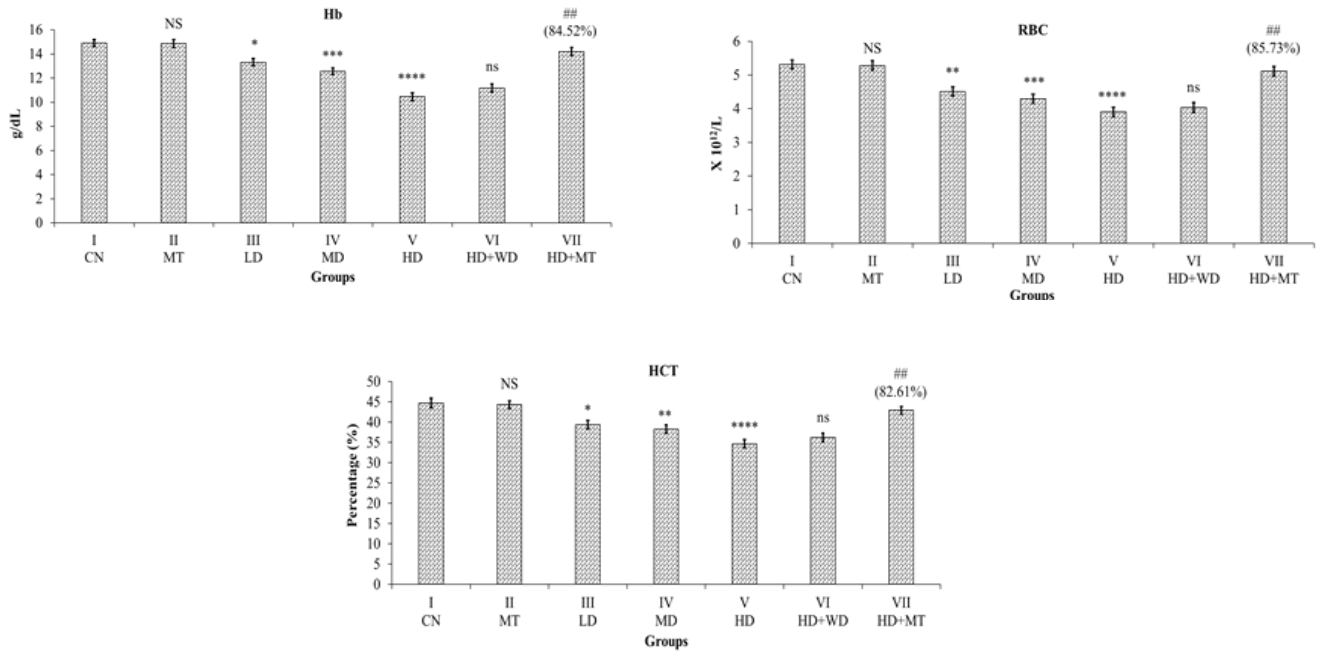


Figure 1. Effects of 2,4-D and/or melatonin on Hb, RBC, and HCT of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose.

NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I);

ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

Haemoglobin (Hb); Red Blood Corpuscles (RBC); Haematocrit (HCT).

caused a significant dose-dependent increase (Group III-V) in MPV (low dose-p<0.05; mid dose-p<0.001; high dose-p<0.0001) and ESR (low dose-p<0.05; mid dose-p<0.01; high dose-p<0.0001) values when compared with control (Group I). No significant auto-recovery was noted in the withdrawal study (Group VI) of the above four parameters in contrast melatonin supplemented with a high dose of 2,4-D (Group VII) showed significant recovery (PT-p<0.0001; MPV-p<0.0001; PCT-p<0.001; ESR-p<0.0001) with percentage amelioration (PT-95.43%; MPV-84.73%; PCT-61.70%, ESR-91.46%) as compared to the alone 2,4-D high dose administered mice (Group V) (Figure 4).

3.5 Molecular Docking of 2,4-D and Melatonin with Haemoglobin Protein (2HHB)

The binding affinities of ligands 2,4-D and melatonin towards the receptor protein 2HHB is investigated in detail. The binding energy, hydrogen bonds, and contacting receptor residues of the interactions are listed in Table 2 and shown in Figures 5 and 6.

The docking complex of 2,4-D and 2HHB showed a binding affinity of -5.4 kcal mol^{-1}. This complex involves 2 hydrogen bonds with Phe 103 and His 92 (one hydrogen bond per residue) and other weak interactions. These interactions include 9 van der Waals interactions and 3 alkyl bonds.

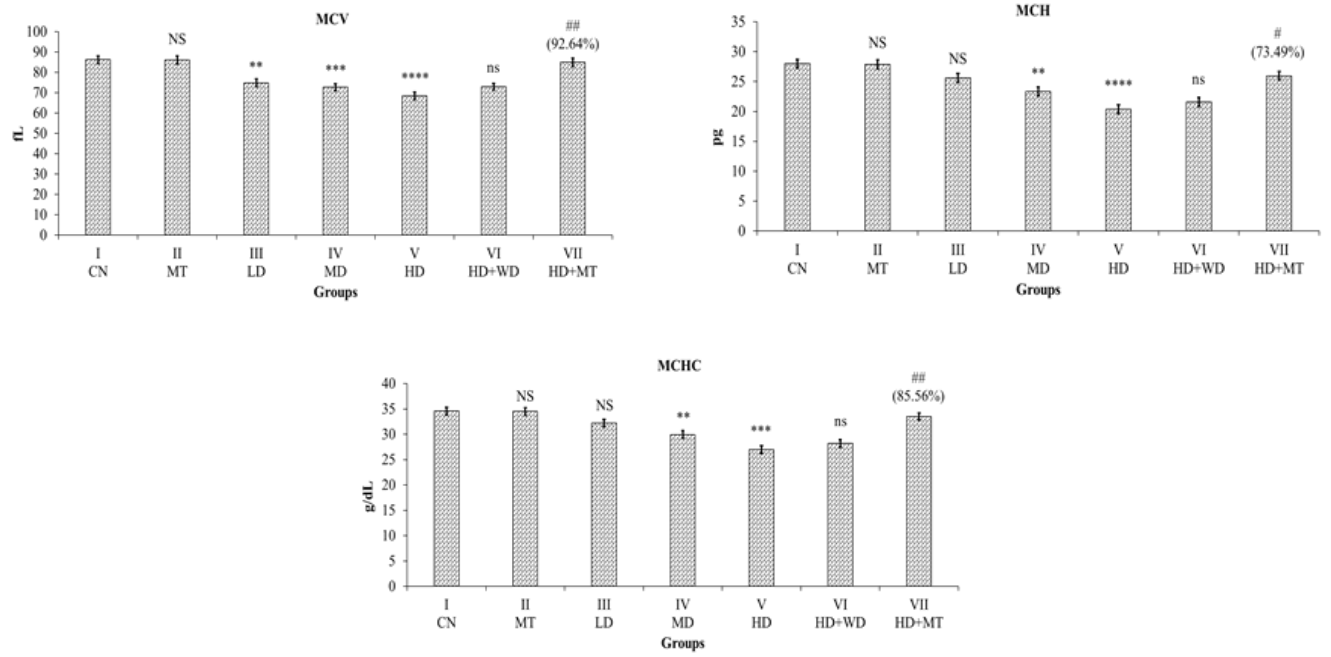


Figure 2. Effects of 2,4-D and/or melatonin on MCV, MCH, and MCHC of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose.

NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I);

ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

Mean Corpuscular Volume (MCV); Mean Corpuscular Haemoglobin (MCH); Mean Corpuscular Haemoglobin Concentration (MCHC).

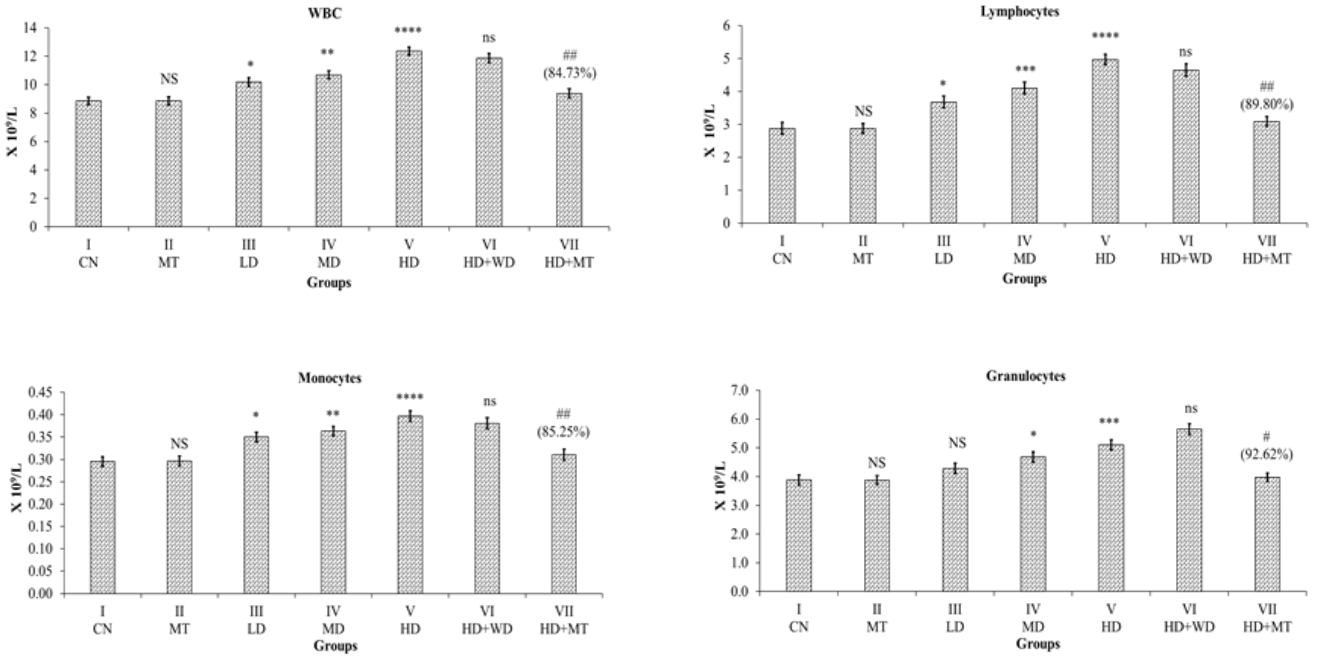


Figure 3. Effects of 2,4-D and/or melatonin on WBC, Lymphocytes, Monocytes, and Granulocytes of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose.

NS = Non Significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$ when groups II to V were compared with control (group I);

ns = non significant; # = $p<0.001$; ## = $p<0.0001$; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

White Blood Corpuscles (WBC).

Table 2. Molecular docking result analysis: binding energy, hydrogen bond, and contacting receptor residues

Protein	Ligands	Binding Energy (kcal mol^{-1})	Hydrogen Bond	Contacting Receptor Residues
Haemoglobin (2HHB)	2,4-D	-5.4	Phe 103, His 92	Phe 103, His 92, Leu 88, Phe 71, Phe 85, Val 134, Val 137, Arg 104, Ala 138, Gly 107, Leu 106, Val 67, Leu 141, Ala 70
	Melatonin (MT)	-7.3	His 63, Phe 103	His 63, Phe 103, Ala 70, Leu 88, Val 67, His 92, Phe 41, Phe 42, Val 98, Asn 102, Leu 141, Leu 106, Val 137, Phe 71

The total number of contacting receptor residues are 14, out of them, Phe 103 and His 92 play a vital role in interaction with 2,4-D (Table 2; Figure 5).

Another docking complex of melatonin and 2HHB showed a binding affinity of -7.3 kcal mol^{-1} which is much lower than the 2,4-D-2HHB complex. This complex

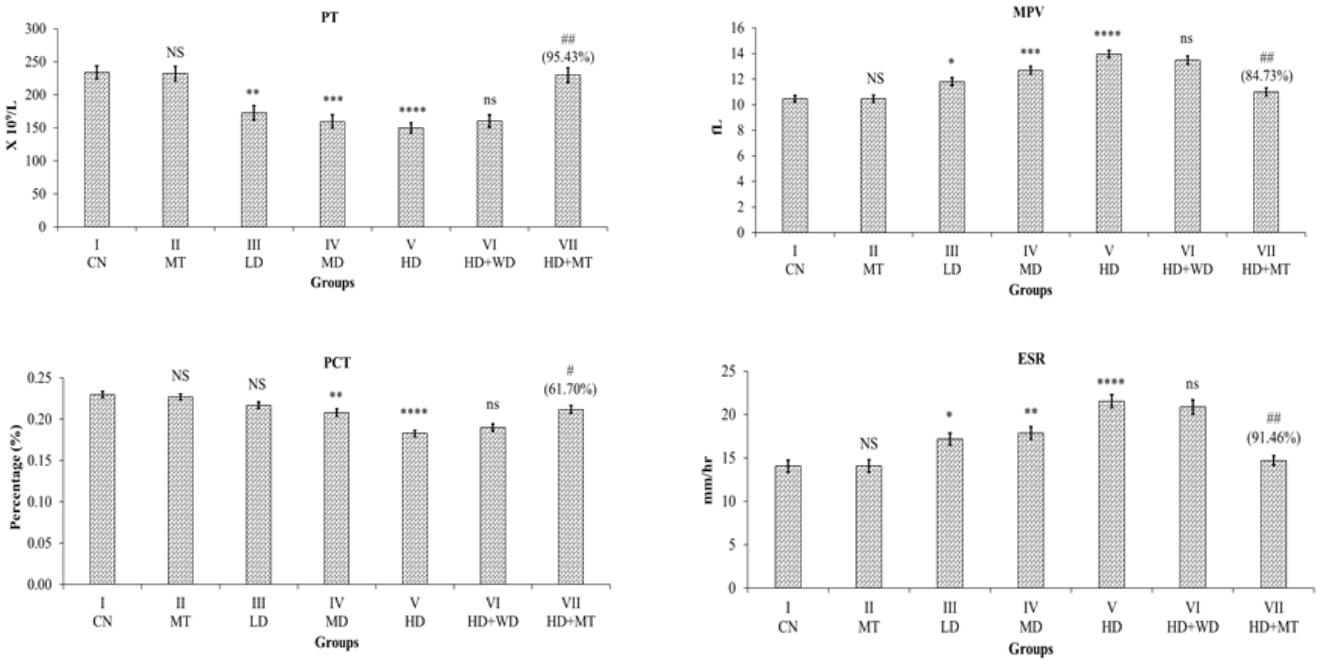


Figure 4. Effects of 2,4-D and/or melatonin on PT, MPV, PCT, and ESR of mice.

Values are Mean ± S.E.; Group-I = Control (CN); Group II = Melatonin (MT) (10 mg/kg body weight); Group III = 2,4-D Low Dose (LD) (16.5 mg/kg body weight); Group IV = 2,4-D Mid Dose (MD) (33 mg/kg body weight); Group V = 2,4-D High Dose (HD) (66 mg/kg body weight); Group VI = 2,4-D High Dose (HD) (66 mg/kg body weight) – 28 Days Exposure + 28 Days Withdrawal (WD); Group VII = 2,4-D High Dose (HD) (66 mg/kg body weight) + Melatonin (MT) (10 mg/kg body weight).

Values in parenthesis are percent amelioration after melatonin administration along with 2,4-D high dose.

NS = Non Significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 when groups II to V were compared with control (group I);

ns = non significant; # = p<0.001; ## = p<0.0001; when groups VI and VII were compared with high dose of 2,4-D treatment (group V).

Platelet Count (PT); Mean Platelet Volume (MPV); Plateletcrit (PCT); Erythrocyte Sedimentation Rate (ESR).

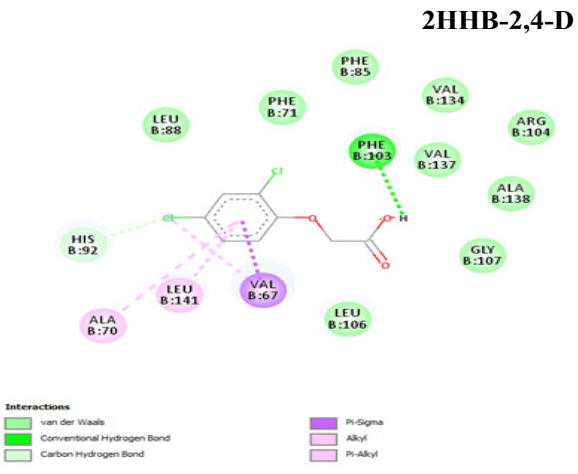


Figure 5. Binding modes of 2,4-D with the crystal structure of haemoglobin protein (2HHB). Receptor-ligand interactions with 2D representation of 2,4-D.

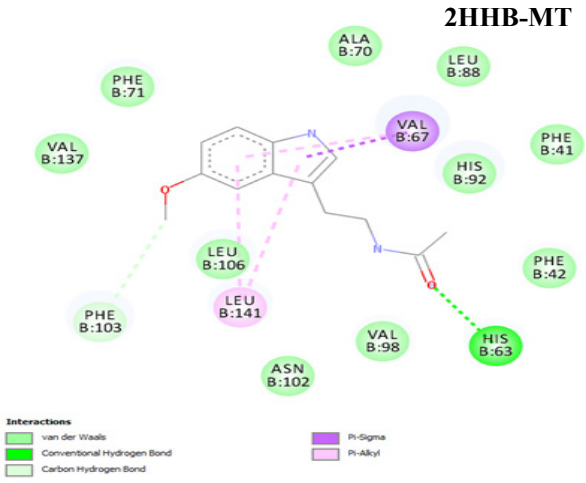


Figure 6. Binding modes of melatonin (MT) with the crystal structure of haemoglobin protein (2HHB). Receptor-ligand interactions with 2D representation of melatonin.

involves 2 hydrogen bonds with His 63 and Phe 103 (one hydrogen bond per residue) and other interactions. These interactions include 10 van der Waals interactions, 2 alkyl bonds, and 1 sigma bond. The total number of contacting receptor residues are 14, amongst them, His 63 and Phe 103 play a vital role in interaction with melatonin (Table 2; Figure 6).

In both interactions, Phe 103 is a common residue with which both ligands are interacting. Moreover, melatonin interacts with more negative free energy. Hence, it can be predicted that melatonin showed the best binding affinity.

4. Discussion

Living organisms are exposed to 2,4-D in many ways[7]. While acute exposure is easily detected and diagnosed, the effects resulting from long term exposure of 2,4-D are often difficult to assess. Haematological examination is an important tool for monitoring as the small amount of blood collected provides extensive information about the physiological status of an organism i.e. oxygen transport capacity, immune functions, stress, and various metabolic functions[8]. In recent years, haematological parameters have been also evaluated with innovative automatic methods. The present study revealed that 2,4-D induced changes in haematological indices of mice supported by some literature on other species and organisms[9-14]. These altered values depend on the species, chemical, dose, and duration of the exposure. Amongst all the studied haematological indices, Hb level, which reveals the status of an organism oxygen level and is responsible for supplying oxygen to the major organs in response to higher metabolic demands[29], showed a dose-dependent decline in 2,4-D treated mice in the present study. Our results are in accordance with the earlier studies, which showed a reduction in Hb level in fish after 2,4-D[13] as well as other herbicides[30–33] intoxication, whereas Kubrak and co-workers reported no change in Hb level in goldfish after the 96 hours of 2,4-D exposure[14]. Reduction in the value of Hb is usually caused by the effect of pesticide on the blood by diminishing its oxygen-carrying capacity, which also implies anaemia or validates the toxic effect of pesticide[34]. Along with the Hb, it is well known that herbicide exposure promotes the reticence of cells, causes mortality of red blood cells, and inhibits haemoglobin formation resulting in anaemia and impaired erythropoiesis[35]. 2,4-D treatment in mice caused dose-dependent decrement in RBC, HCT, MCV, MCH, and MCHC values in the present study suggesting erythrocytopenia. Similar results were also obtained by other authors including fish in 2,4-D[13] and other herbicides[30–33] treatment in support of our study whereas no change in HCT value was reported by Kubrak and co-workers in 2,4-D treated fish[14]. The reduction in RBC, HCT, MCV, MCH, and MCHC values may result from declined Hb level accompanied by inhibition of RBC, haem synthesis, osmoregulatory dysfunction, and destruction of RBC in haematopoietic organs as mentioned in an earlier report[34].

WBC and differential counts (lymphocytes, monocytes, and granulocytes) are recognized as sensitive indicators of environmental stress. WBC play a major role in defense mechanisms, lymphocytes produce antibodies, and monocytes and granulocytes act as phagocytes to rescue debris from injured tissue. These all components are involved in the regulation of immunological function and their alterations could be connected with the immunotoxic potential of substances[36]. Some studies reported the dose-dependent reduction of WBC, lymphocytes, monocytes, and granulocytes values in fish after 2,4-D[14] as well as other herbicides[30–32] treatment. In our experiment, these parameters were dose-dependently increased in the 2,4-D treated mice suggesting leukocytosis. Previous findings also showed similar changes in 2,4-D[13] and another herbicide[33] intoxicated fish which support the results observed in the present study. These increased levels could be due to the stimulation of the immune system causing an increase in lymphocytes by an injury or tissue damage attributed to herbicide treatment[36].

Platelets, besides their specialized role in haemostasis and atherothrombosis, actively modulate innate and adaptive immune responses with crucial roles in immune surveillance, inflammation, and host defense during infection[37]. In our study, PT and PCT values showed dose-dependent decrement whereas increased MPV value was observed signifying the effect of 2,4-D on platelet production. Soni and co-workers had noted a similar decrement in these values in fish after 2,4-D treatment[13] which supports the results observed in the present study. This alteration suggests thrombocytopenia and could be explained by thrombostatin content which can be regulated through the homeostasis process[38].

ESR indicate and monitor inflammatory activity within the body caused by one or more conditions such as autoimmune disease, infections or tumours[39]. The present study showed an elevated ESR value in a dose-dependent manner after 2,4-D treatment in mice. Our results are in line with the previous study that indicated similar findings in pesticide intoxicated fish[40]. The increased level of ESR could be due to stress, anaemia, and inflammatory conditions as well as the high proportion of fibrinogen occurred by pesticide administration[41].

All the haematological indices of the withdrawal study were unchanged compared to a high dose of 2,4-D suggesting an accumulation of the chemical into the physiological system[42,43] and hence, confirming a strong need of a mitigating agent. Oxidative stress in the blood is portrayed by altered antioxidant status[44]. Melatonin is known to be a powerful antioxidant[45]. It exhibits direct antioxidant properties, acts as a scavenger of free radicals, indirectly enhances the activity of anti-oxidative enzymes, and can stabilize their concentrations[46]. Melatonin is a pivotal component of the body's internal time-keeping system that is associated with human health[47]. Numerous studies had shown that melatonin inhibits carcinogenesis in both humans and animals[48,49]. Hence to evaluate the possible role of melatonin against 2,4-D induced haematological alteration, melatonin was used as an antidote in the present study. The RBC profile (Hb, RBC, HCT, MCV, MCH, MCHC, and ESR) were found to be normalized significantly in the present study evident by melatonin co-administration with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment. The RBCs can extract melatonin from circulation and its accumulation promotes the activity of glutathione peroxidase[50,51]. RBC is the only cell that probably synthesizes melatonin without mitochondria. This ability further enhances the melatonin concentration in RBC[52]. Melatonin is considered a redeemer to preserve structurally and functionally compromised RBCs from oxidative stress[53]. Furthermore, Melatonin chelates free iron formed during RBC apoptosis and causes forceful destruction under stressed conditions to inhibit Fenton reaction and subsequent ROS generation. Melatonin is also applied in RBC storage to eliminate the detrimental effects of oxidative stress[54].

The altered values of WBC and differential counts were significantly ameliorated when melatonin was co-administered along with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment. Melatonin is also a modulator of immune cell function and haematopoiesis[15,18,55]. Physiologically, melatonin is associated with T-helper 1 (Th1) cytokines and induces activation of Th1. In both normal mice and those with acute mid-stage erythroleukemia, melatonin administration results in a quantitative and functional enhancement of natural killer (NK) cells, which mediate endogenous defenses against cancer cells. Melatonin regulates cell dynamics of host defense, including the proliferative and maturational stages of haematopoietic and immune cells (NK cells, T and B lymphocytes, granulocytes, and monocytes), thereby improving their normal physiological function. Notably, in mice bearing mid-stage leukaemia, daily administration of melatonin results in a survival index of 30–40% (more than 3 months) compared with 0% in untreated mice[55].

The platelets profile (PT, MPV, and PCT) had shown significant recovery when melatonin was given along with a high dose of 2,4-D as compared to 2,4-D high dose alone treatment. Melatonin, a metabolite of serotonin (5-hydroxytryptamine; 5-HT) is a growth factor for haematopoietic stem/progenitor cells. 5HT may enhance megakaryopoiesis and proplatelet formation via the extracellular signal-regulated kinase 1/2 (Erk1/2) pathway and cytoskeleton reorganization. 5-HT also promoted the megakaryocytes proliferation and reduced the cell apoptosis via the activation of 5-hydroxytryptamine receptor 2B (5-HT2BR) and protein kinase B (Akt) pathway. In this way, melatonin exerts a protective effect on megakaryocyte and platelet recovery in the mouse model evident by its proven role in platelet formation and actin filaments (F-actin) reorganization[56].

In silico data of the present study showed that melatonin was bound to haemoglobin with better affinity compared to 2,4-D in terms of docking score, hydrogen bond, and contacting receptor residues. Previously, neuroprotective effects of melatonin against ethanol-induced neurodegeneration were observed in the computational approach attributed to the antioxidant efficacy of melatonin[57]. However, to the best of our knowledge, this *in silico* study is the first evidence signifying the interaction of 2,4-D and melatonin with haemoglobin. Along with that, this *in vivo* study also

retained novelty related to the combined effects of 2,4-D and melatonin on haematological variables.

5. Conclusion

Taken together, our data proved that 2,4-D generates low, moderate, and high toxicity in the blood of mice in a dose-dependent manner confirming that the high concentration or long term exposure of low concentration promotes alteration in haematological variables. Melatonin can play an important role in the protection of cells from oxidative damage caused by 2,4-D. Moreover, *in silico* data confirmed that melatonin showed better binding affinity towards haemoglobin compare to 2,4-D. Thus, melatonin can be an effective agent and can prevent and mitigate 2,4-D induced haematological alteration by encouraging immunomodulation both in *in vivo* and *in silico* manner.

6. Conflict of Interest

The authors declare that there is no conflict of interest.

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