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RESEARCH ARTICLE

Pharmacological evaluation of *Dactylorhiza Hatagirea* against cyclophosphamide-induced reproductive toxicity in male wistar rats

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Abstract

D. hatagirea (DH) is a species belonging to the Orchidaceae family predominantly found in higher altitude regions such as the Himalayas,, which is reported to possess several biological properties such as anti-oxidant, anti-viral and anti-inflammatory potential. Cyclophosphamide (CP) is a commonly used chemotherapeutic drug that is reported to cause severe reproductive toxicity due to oxidative stress induction. The current study aims at evaluating the effect of DH root extract on mitigating reproductive toxicity induced via CP administration using male wistar rats owing to its previously reported antioxidant potential. Briefly, sequential extraction of DH root was performed using petroleum ether, chloroform and ethanol, followed by its phytochemical evaluation. Biochemical evaluation of the ethanolic DH extract showed optimum antioxidant potential compared to other extracts. Further, *in vivo* evaluation of the effect of DH extract on CP-induced reproductive toxicity was conducted using varying dosages of 100 mg/kg and 200 mg/kg for 4 weeks and 8 weeks time periods. Our results indicate the efficacy of DH extract by the restoration of tissue in CP-induced damage post 8 weeks of administration with 200 mg/kg DH treatment. Moreover, an elevated level of testosterone with respect to vehicle control in 4 and 8 weeks of 200 mg/kg DH treatment was also observed, which suggests the ability of DH root extract to elevate reproductive capacity in animals. An increase of 163.37 % and 116.25 % w.r.t. control was observed for testosterone and follicle-stimulating hormone (FSH) was obtained. An up-regulation of antioxidant enzyme catalase (CAT) and down-regulation of malondialdehyde (MDA) levels suggest the possible mode of action of DH extract towards mitigating the toxic effects of CP administration in the test groups. Thus, our study proposes DH root extract as a potential therapeutic agent to mitigate CP-induced reproductive damage.

Keywords: anti-oxidant; cyclophosphamide; *Dactylorhiza hatagirea*; medicinal orchids; reproductive toxicity

Abbreviations: DH - *Dactylorhiza hatagirea*; CP - Cyclophosphamide; FSH - Follicle stimulating hormone; CAT - Catalase; MDA - Malondialdehyde; LH - Luteinizing hormone; HCl - Hydrochloric acid; DMSO - Dimethyl sulfoxide; NBT - Nitro blue tetrazolium; NEDA - Naphthalene diamine dihydrochloride; DPPH - 2,2-diphenyl 2-picrylhydrazyl hydrate; CPK - Serum creatine phosphokinase; LDH - Lactate dehydrogenase; SGOT - Serum glutamic oxaloacetic transaminase; SGPT - Serum glutamate pyruvate transaminase

Introduction

Reproductive process in males is a complex system involving coordinated regulation by Sertoli cells, spermatogenic epithelium and the production of androgen by Leydig cells. This system is regulated via the production of various hormones, including gonadotropin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Chemotherapeutic drugs such as cyclophosphamide (CP), cisplatin and doxorubicin are xenobiotic reproductive toxicants that cause functional damage to the reproductive system (1-3). Exposure to Lead acetate, tartrazine, ethanol and bisphenol has also been reported to induce significant reproductive toxicity, either directly or indirectly, through various mechanisms (4-7).

CP is an alkylating agent used in the treatment of leukaemia, myeloma, lymphoma and other immune-related diseases (8). Clinical anti-neoplastic effect of CP is associated with phosphoramide mustard, which is an active metabolite of CP, whereas the toxic effect of CP is imparted via acrolein, which is the second active metabolite produced from CP. Latter causes severe adverse side-effects, including alteration of human fertility (8). CP treatment reduces sperm counts and causes alterations in the spermatogenic cycles of testicular tissue (9). Although the exact mechanism underlying reproductive toxicity remains unclear, disruption of redox balance leading to elevation in oxidative stress is considered a potential mode of action (8). Based on previous reports, compounds with potential antioxidant activity may be considered as a potential therapeutic agent against CP-induced reproductive toxicity (10).

Plant-derived compounds are well-known for their antioxidant potential owing to the presence of a broad spectrum of phenolics, terpenoids, saponins and tannins (11). DH is a species belonging to the Orchidaceae family predominantly found in higher altitude regions such as the Himalayas (12). DH is previously reported to contain several secondary metabolites, including flavonoids, phenolics, alkaloids, terpenoids and saponins, with anti-inflammatory, antiviral, diuretic, neuroprotective and antioxidant properties (12). DH is previously reported to possess the presence of resveratrol and trans-stilbenes, which often exert antioxidant, anti-inflammatory and antimicrobial effects. The species is believed to enhance the bodys' antioxidant defence, thereby reducing oxidative stress and potentially protecting against chronic diseases such as cancer and cardiovascular conditions (12, 13). CP-induced reproductive toxicity is due to elevated levels of oxidative stress generated due to acrolein, which is a secondary metabolite of CP. Ameliorative effect of DH on CP-induced reproductive toxicity has not yet been reported, to per best of our knowledge. Thus, in our current study, owing to the antioxidant potential of DH, we aimed at pharmacological evaluation of DH extract against reproductive toxicity induced via CP induction using male albino wistar rat model.

Materials and Methods

Plant materials and other chemicals

The bulbous roots of DH were collected from local shops of Lahaul, Himachal Pradesh and identified morphologically. The roots were washed in water, dried in the shade and then ground into a fine powder using a grinding machine. LDH assay kit was purchased from Sigma Aldrich (MAK066). CPK assay kit (E-BC-K558-S), SGOT assay kit (E-BC-K236-M) and CAT assay kit (E-BC-K031-S) were purchased from Elabscience. Rat FSH (RTF100783) and testosterone (QSES050) ELISA kit was purchased from AssayGenie. MDA assay kit was purchased from MedChem Express (HY-K0319). All other chemicals and reagents used in this study were of analytical grade and obtained from Sigma-Aldrich.

Plant identification

The plant material collected was identified as DH based on the morphological characteristics. The plant material collected was further certified as DH by the Botany Department of Sri Venkateswara University, Tirupati. A herbarium specimen of the same was prepared and deposited to TBGT bearing the collection number 107818; voucher specimen no: TBGT107818. The herbarium was verified by Dr A. Nazarudeen, Senior Scientist and HoD, Plant Systematics and Evolutionary Science division, JNTBGRI, Kerala, India.

Extraction of DH

Soxhlet extraction was performed to extract the components using petroleum ether (68 °C), chloroform (60 °C) and ethanol (78 °C) as solvent media at or near the solvents' boiling point. The heating mantle is set to the particular boiling point of each solvent to ensure continuous vaporisation and cycling through the apparatus. Finely powdered root of DH (100 g) was packed in a thimble and loaded into the main chamber of the Soxhlet extractor. The extraction process was carried out using 1000 mL of selected solvents for 72 h each by employing the continuous heat method. Upon completion n, the solvent was removed from the extract using a rotary evaporator. Dried samples were stored at 4 °C till further use.

Qualitative phytochemical analysis

Confirmatory qualitative phytochemical screening of the freshly prepared crude extract of DH roots was performed to determine the major classes of phytochemical compounds (phenols, tannins, saponins, flavonoids, alkaloids, glycosides, steroids, quinone, fatty acid and terpenoids) present in the DH extract from petroleum ether, chloroform and ethanol following standard protocols (14, 15).

Quantitative Phytochemical analysis of ethanolic extract

Phytochemical constituents such as phenol, tannin, flavonoid, alkaloid and saponin tested highly positive (+++) in qualitative analysis for ethanolic extract of DH and were subsequently analysed quantitatively. For all quantitative assays, a standard graph of R^2 value above 0.99 was used and a minimum of 3 technical replicates for each standard concentration.

Determination of phenolics and tannins (Folin and Ciocalteu method)

The test sample extract (1mg/mL) was treated with 0.5 mL of Folin Ciocalteu reagent mixture and 2 mL of 20 % sodium carbonate. The tubes were incubated for one minute in a boiling water bath, then cooled and the absorbance was measured at 750 nm in a spectrophotometer against a reagent blank. Gallic acid dilutions were also treated as above and the results were expressed in terms of Gallic acid equivalents (16, 17).

The presence of tannins was measured based on the formation of a blue colour by the reduction of phosphotungsto-molybdic acid by tannin compounds at alkaline pH. To the prepared DH root extract (1 mg/mL), 0.5mL of Folin-Ciocalteu reagent and 35 % 1mL sodium carbonate solution was added and the final volume was made up to 10mL with distilled water. The absorbance was measured at 700nm with tannic acid dilutions as standard (mg/mL tannic acid equivalents) (18, 19).

Determination of flavonoids (Aluminium chloride method)

1 mg/mL of DH extract was prepared and transferred to a 10ml volumetric flask. To this, 3mL of distilled water and 0.30 mL of sodium nitrite (5 %) were added and incubated for 5 min. After the incubation, 0.3 mL of 10 % aluminium chloride was added and the mixture was incubated for an additional 5 min. To this solution, 2 mL of 1 M Sodium hydroxide was added and the volume was made up to 10 mL with distilled water. The absorbance was read at 510 nm using a Thermo Scientific, Orion Aquamate 8000 UV/Visible spectrophotometer (18, 20, 21). A standard curve of quercetin was prepared to estimate the quantity of flavonoids present in DH extract (mg quercetin/ g dried extract).

Determination of saponins (Vanillin-sulphuric acid method)

The root sample extract (1 mg/mL) was treated with 0.25 mL of 0.8 % (w/v) vanillin in ethanol and 2.5 mL of 72 % (v/v) sulphuric acid in water for 15 min at 60 °C in a shaking water bath. The mixture was cooled to room temperature and the absorbance was measured at 544 nm. Various concentrations of diosgenin were treated as described in the above protocol to get a standard graph and were used for assessing the saponin content in the DH extract (22).

Determination of alkaloids

The DH root extract (1 mg/mL in dimethyl sulfoxide) was treated with 1 mL of 2N hydrochloric acid (HCl). The solution was filtered and transferred to a separating funnel, then 5 mL of bromocresol green and phosphate buffer were added and the mixture was vigorously

shaken with an increasing volume of chloroform and collected in a 10 mL volumetric flask. A set of reference standard solutions of atropine was prepared in the same manner as described earlier. The absorbance for test and standard solutions was determined against the reagent blank at 470 nm with a UV/Visible spectrophotometer (23).

In vitro antioxidant activity of the ethanolic extract of DH

Superoxide scavenging activity

The standard method was used to detect the superoxide anion scavenging activity of DH ethanolic extract (24). 0.1 mL Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 mL EDTA (0.1M EDTA), 0.05 mL riboflavin (0.12 mM) and 2.55 mL of phosphate buffer (0.067 M phosphate buffer) were added to the sample. Dimethyl sulfoxide (DMSO) was used as a negative control and ascorbic acid as the reference compound. The reaction mixture was illuminated for 30 min and the absorbance was read at 560 nm. All the tests were performed in triplicate and the results were averaged. The percentage inhibition was calculated by comparing the results of the control and test samples.

Nitric oxide scavenging activity

The Garrat method was used to analyse the nitric oxide scavenging activity of the DH extract (25). The test sample was treated with 2 mL of sodium nitroprusside (10 mM) and 0.5 mL phosphate buffer saline (1M) and the reaction volume was made upto3ml. The tubes were incubated at 25 ºC for 2.5 h. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1mL of sulphanilic acid reagent (0.33 %) and allowed to stand for 5 min to complete diazotisation. 1 mL of naphthalene diamine dihydrochloride (1 % NEDA) was then added, mixed and allowed to stand for 30 min. Sodium nitroprusside readily releases nitric oxide in slightly alkaline aqueous solutions. The liberated nitric oxide can subsequently react with oxygen to form nitrite ions, which can be quantitatively estimated using the Griess-Illosvay reaction, typically measured at 540 nm.

DPPH radical scavenging assay

The antioxidant activity was evaluated using 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay, following the method described with slight modifications. DPPH is a stable free radical that reacts with antioxidant compounds capable of donating hydrogen atoms, leading to its reduction (26). Upon reduction, the DPPH reagent changes colour from deep violet to light yellow and this change can be quantitatively measured at 515 nm using a UV-visible spectrophotometer.

Hydroxyl free radical scavenging activity

The assay was carried out by using a standard method with minor modifications. Various concentrations of the sample extracts were treated with 0.1 mL deoxyribose (2.8 mM), 0.1 mL EDTA (0.1 mM), 0.1 mL hydrogen peroxide (1 mM), 0.1 mL ascorbic acid (0.1 mM), 0.1 mL Potassium dihydrogen phosphate-Potassium hydroxide buffer, pH 7.4 (20 mM) in a final volume of 1 mL. The reaction mixture was incubated at 37 ˚C for 60 min. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated (27).

Experimental animals

Healthy Male Wistar Albino rats weighing about 150-200 g were used for the study. The entire study was approved by the Institutional Animal Ethical Committee (IAEC) of Cape Bio Lab and Research Centre with approval number CBLRC/14/01 - 2020.

The animals were kept in clean and dry polycarbonate cages and maintained in a well-ventilated animal house with a 12 h light – 12 h dark cycle. The animals were fed with a standard pellet diet and water was given ad libitum. For experimental purposes, the animals were kept fasting overnight but allowed free access to water.

36 albino wistar male rats were used for the present study. The animals were randomly divided into six groups of six animals each and were treated as follows. Group I consists of 6 animals administered with vehicle control orally for 4 weeks. Group II consists of 6 animals administered with 6.5 mg/kg CP for 4 weeks orally, followed by vehicle control administration for 4 weeks. Group III consists of 6 animals administered with 6.5 mg/kg CP orally for 4 weeks, followed by vehicle control administration for 8 weeks. Group IV consists of 6 animals administered with 6.5 mg/kg CP orally for 4 weeks, followed by DH extract (100 mg/kg) treatment orally for 4 weeks. Group V consists of 6 animals administered with 6.5 mg/kg CP orally for 4 weeks, followed by DH extract (200 mg/kg) treatment orally for 8 weeks. Group VI consists of 6 animals administered with 6.5 mg/kg CP orally for 4 weeks, followed by DH extract (200 mg/kg) treatment for 8 weeks. Dosages were chosen based on previous literature studies using DH (28). The animals were euthanised using isoflurane anaesthesia at the end of the study and blood was collected by cardiac puncture immediately after euthanasia. Sections were studied microscopically for changes in histoarchitecture or morphology.

Biochemical studies

Blood serum was subjected to Biochemical analysis. Biochemical parameters include are serum concentration of FSH, measurement of CPK, LDH and SGOT. Measurement of serum testosterone, MDA and CAT concentrations in the testicular tissue was done.

Histopathology assessment

Tissues were fixed with neutral formalin 10 %, embedded in paraffin and then manually sectioned with a microtome to obtain 3-5 µm thick paraffin sections. Dewaxed sections are then stained with hematoxylin and eosin (H and E)

Statistical analysis

All experiments were carried out in triplicate and data are presented as mean ± standard deviation (SD) and analysed using Students' t-test. A value of $p<0.05$ is considered statistically significant.

Results and Discussion

Extraction of DH

Among the different solvents used, the maximum weight and yield percentage were obtained for ethanol, followed by chloroform and petroleum ether, with 7.33 g, 0.511 g and 0.087 g yield and 7.33 %, 0.511 % and 0.087 % yield percentage, respectively, from 100g of samples. Petroleum ether extract showed a semi-solid sticky consistency, whereas chloroform and ethanol extract showed sticky resinous and thick paste consistency, respectively.

Qualitative phytochemical analysis

The three root extracts of DH showed significant phytochemical activity in qualitative terms, as evidenced by notable colour changes. Preliminary screening confirmed the presence of phenols, tannins, flavonoids, alkaloids and saponins in all three extracts. Among the different solvents used, the ethanolic root extract showed the highest abundance of secondary metabolites, indicated by a strong

degree of precipitation (+++), whereas petroleum ether and chloroform extracts revealed a lower diversity of these compounds. Terpenoid, glycoside, quinine and fatty acid were present only in trace amounts (+) and steroids were absent in all three extracts.

Quantitative phytochemical analysis

The pharmacological effects of extracts are due to the presence of bioactive chemical constituents. The spectrophotometric analysis for total alkaloid content in the ethanolic root extract (EEDH) revealed a high alkaloid content in the root powder (66.21 mg/g extract). Ethanolic extract of root parts demonstrated higher concentration of total phenolics (98.27 mg/g extract), flavonoid (82.66 mg/g extract), total alkaloid and tannin content (53.93 mg/g extract) (Fig. 1). All the values were obtained from triplicate experiments and are represented as mean ± SD. Plant-derived secondary metabolites such as safranal, sinapic acid, hesperidin, crocin and chlorogenic acid, belonging to terpenoid aldehyde, phenols, flavanone glycoside, carotenoid and polyphenols, respectively, have previously been reported to possess the potential to alleviate reproductive toxicity induced due to CP treatment (29-33). Owing to the high content of phenols, alkaloids and flavonoids in DH extract, further studies have been carried out to explore its antioxidant potential *in vitro* and CP induced reproductive toxicity reduction *in vivo*.

Nitric oxide scavenging activity

The nitric oxide radical was found to be reduced by ethanolic DH extract in a concentration-dependent manner, which is illustrated in Table 1. The test sample was found to be most effective in scavenging nitric oxide radical activity compared to the standard ascorbate significantly. The IC50 of the ethanolic extract of DH and ascorbate were found to be 853 µg/mL and 442 µg/mL, respectively.

Superoxide scavenging activity (NBT dye reduction method)

The percentage scavenging of superoxide anion examined at different concentrations of ethanolic extracts of DH (100, 200, 400, 800 µg/mL) is presented in Table 1. The IC_{50} values of ethanolic DH extract were found to have strong superoxide radical scavenging activity, where the IC_{50} of the ethanolic extract was found to be 818 µg/mL.

Hydroxyl free radical scavenging activity

Hydroxyl radicals (OH) are highly reactive and damaging free radicals produced in the body that can oxidise various cellular components, leading to cell damage and contributing to various diseases. The hydroxyl radical scavenging assay helps in understanding the antioxidant potential of compounds and thereby evaluating the effectiveness of potential therapeutic agents. Scavenging of hydroxyl radical is an important antioxidant activity because of the very high reactivity of the OH radical, enabling it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides (34). Thus, removing OH is very important for the protection of living systems. The EEDH extract showed increasing hydroxyl radical scavenging activity with an increase in the concentration of extract. The IC_{50} value for hydroxyl radical scavenging activity observed was 818 µg/mL (Table 1). Phenolic compounds have high antioxidant activity and they are effective in the prevention of oxidative stress (34). Our results are consistent with the data published previously (35). Here, we assume that the antioxidant activity and reducing power capacity of the extracts were likely due to the presence of polyphenols, which can act as free radical scavengers by donating an electron or hydrogen. Flavonoids are plant secondary metabolites that possess a broad spectrum of chemical and biological activities, including radical scavenging properties. This study suggests that the ethanolic extract of DH has a potent antioxidant activity; as a result, the root extracts of DH may serve as a possible source of natural antioxidants.

Table 1. Effect of ethanolic extract DH (EEDH) of the plant on nitric oxide, superoxide and hydroxyl free radical scavenging activity

Concentration (µg/mL)	% of activity (mean ± std dev)					
	Nitric Oxide scavenging		Superoxide scavenging		Hydroxyl free radical scavenging	
	EEDH	Standard (Ascorbate)	EEDH	Standard (Ascorbate)	EEDH	Standard (Ascorbate)
100	11.50 ± 0.30	28.80 ± 0.30	11.50 ± 0.30	28.80 ± 0.30	16.20 ± 0.48	45.39 ± 0.22
200	17.31 ± 0.71	33.26 ± 0.50	17.31 ± 0.71	33.26 ± 0.50	20.30 ± 0.96	49.55 ± 0.47
400	26.52 ± 0.90	45.84 ± 0.45	26.52 ± 0.90	45.84 ± 0.45	29.22 ± 0.20	58.51 ± 0.58
800	47.35 ± 0.77	74.36 ± 0.28	47.35 ± 0.77	74.36 ± 0.28	43.70 ± 0.85	75.50 ± 0.78
IC50	853.68 µg/mL	442.19 µg/mL	853.68 µg/mL	442.19 µg/mL	951.25 µg/mL	207.24 µg/mL

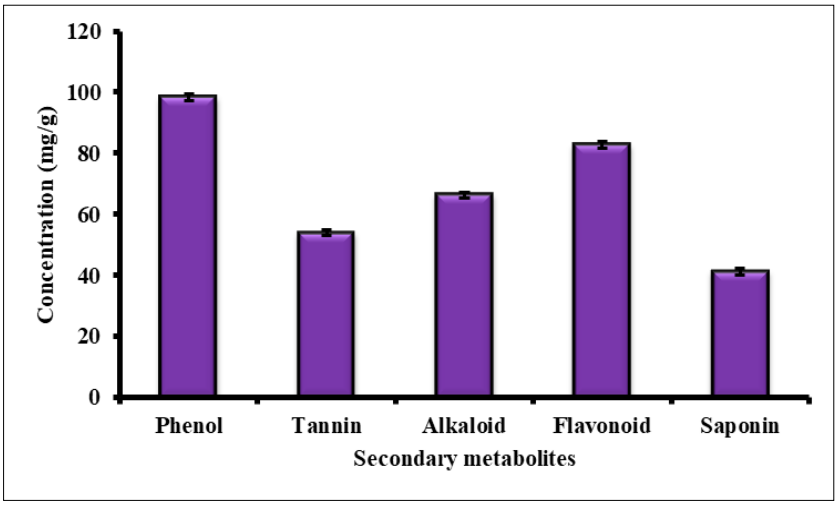

Fig.1 Quantitative analysis: Quantitative estimation of phenol, tannin, alkaloid, flavanoid and saponin in ethanolic root extract of DH represented in mg/g. Experiments were done in triplicates and values are provided as mean _+SD.

Biochemical studies

Phytochemical and biochemical screening of DH showed a strong presence of secondary metabolites and antioxidant potential. Thus, we further evaluated the pharmacological activity of the DH extract in ameliorating reproductive toxicity in male Wistar rats due to CP treatment. SGOT and SGPT enzymes are biomarker indicators for liver function (36). Elevated levels of these enzymes in serum indicate possible damage to liver tissue or any alterations thereof. Chemotherapeutic drugs are known for their side effects on hepatic cells, leading to elevated levels of these enzymes in the blood circulation (37). In our study, elevated SGOT levels were observed in CP-treated group II and group III compared to the control, which suggests potential damage to liver functions. Additionally, an increase in SGOT levels in group III compared to group II points towards further deterioration of liver health if left untreated, suggesting the requirement for therapeutic intervention to ensure a better quality of life for cancer patients post-chemotherapy treatment. All DH-treated groups showed a decrease in SGOT levels compared to both CP-treated groups, supporting the positive effect of DH extract on improving liver health. However, no dose or duration-dependent alteration was observed in SGOT levels between groups IV, V and VI (Fig. 2a). The finding suggests that a low dosage for 4 weeks post-CP induction is sufficient to improve liver damage. A similar study using gallic acid, which is a phenolic compound, as a preventive and therapeutic model for CP-induced cellular liver damage, proved that pre- and co-treatment with gallic acid significantly reduced serum SGOT and SGPT levels compared to CP-treated groups (38). The results were also in concordance with this previous finding and this might be due to the presence of phenolic compounds in the ethanolic DH extract.

LDH levels are another commonly used indicator for assessing cellular damage due to xenobiotic treatment. In this study, we performed an LDH assay to evaluate the effect of DH treatment in improving the cellular conditions of CP-treated wistar rats. The results suggest that LDH levels were increased in CP-treated groups compared to controls, suggesting that cellular damage occurred due to CP treatment. However, no significant alteration in LDH levels between group II and group III indicates that no further deterioration or improvement in cellular condition occurred with no treatment provided. No significant reduction in LDH levels of group IV compared to group II and III suggests that a low dosage for 4 weeks is not sufficient to improve the condition of CP-induced damaged cells (Fig. 2b). On the contrary, a high-dose treatment for 4 weeks or 8 weeks may play a significant role in improving cellular damage. Group VI showed LDH levels comparable to the control group, suggesting the damage repair potential of high-dose treated groups after 8 weeks.

CP treatment has resulted in elevated levels of CPK in blood, indicating potential damage to muscles and the absence of any therapeutic intervention has resulted in further elevation of CPK levels in group III compared to group II (Fig. 2c). Even though our results suggests that treatment with DH extract has negligible role to play in improvement of muscle damage, treatment with DH might help in prevention of further damages post treatment with CP. However, a previous study using *Achillea millefolium* aqueous extract treatment at a dosage of 1.2 g/kg for 4 weeks to CP-induced male wistar rats has shown a significant decrease in levels of CPK (39). In the current study, researchers have utilised a significantly lower dosage of 200 mg/kg, which might be considered as a potential reason for no significant alteration in CPK levels observed in DH-treated groups. Thus, the negligible effect of DH treatment on CPK levels is a potential drawback of the current study and future evaluations may be conducted with a higher dosage or longer time period of DH treatment to evaluate its potential in lowering CPK levels.

Induction of oxidative stress is the mode of action of CP towards imparting cell damage, which is known to cause lipid peroxidation, leading to elevated levels of MDA. Our findings suggest

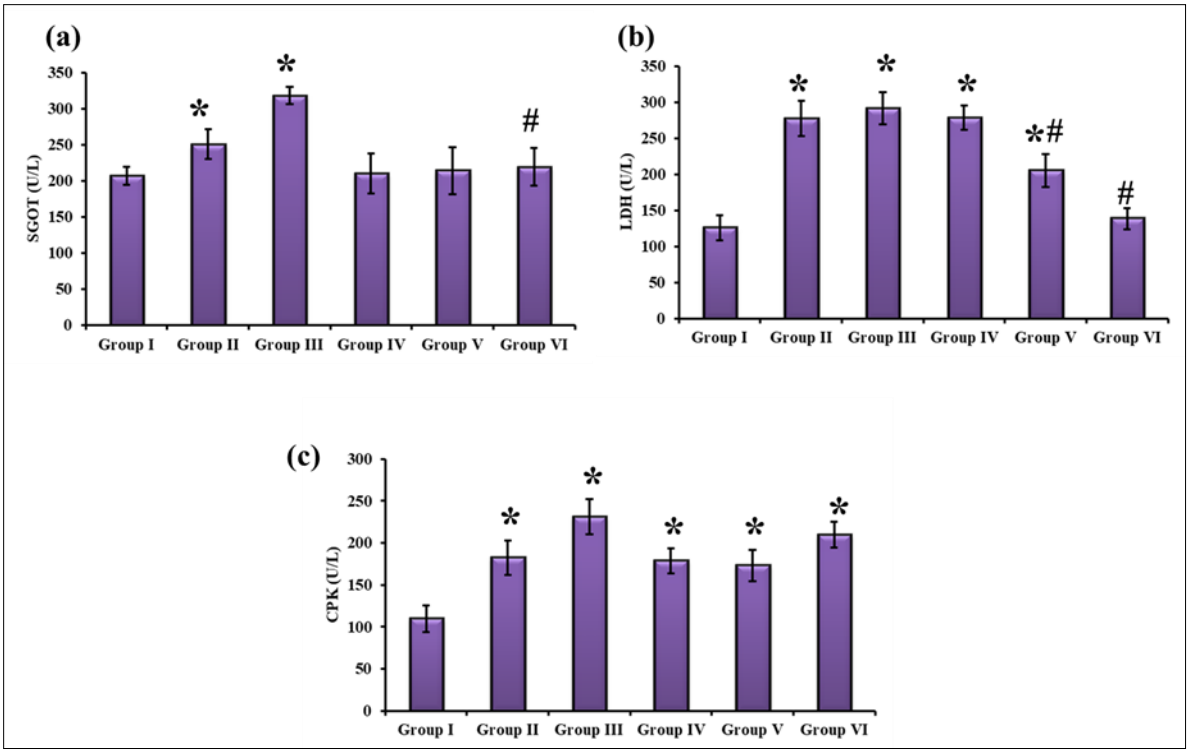

Fig. 2. DH treatment in CP induced male wistar rats showed restoration of SGOT and LDH levels comparable to control. Effect of DH extract treatment on **a.** SGOT, **b.** LDH and **c.** CPK levels in test animals; Sample size n=6 (*$p<0.05$ significantly different w.r.t. control, #$p<0.05$ significantly different w.r.t. respective CP control).

that CP treatment for 4 weeks followed by 4 weeks vehicle treatment resulted in elevated MDA levels with respect to control, whereas after 8 weeks of vehicle control treatment has further reduced the levels of MDA (Fig. 3a). Thus, our results were in concordance with previous findings of CP-induced elevated levels of MDA and also suggest that oxidative stress levels may be mitigated once the CP treatment is stopped. But persistent ROS can lead to significant cell membrane damage. Thus, treatment with DH extract will be beneficial in reducing oxidative stress in lesser duration of time, which eventually reduces the risk of potential further damage to the cells post-treatment with CP. A dose and duration-dependent effect was not observed, suggesting that a low dosage for a shorter duration of time itself is sufficient for protecting the cells against further damage due to ROS. A reduction in MDA levels is associated with decreased levels of oxidative stress. Multiple previous findings using CP-treated *in vivo* models have successfully established that a reduction in levels of MDA in sample-treated groups when compared to CP-alone-treated groups is a key parameter indicating its therapeutic potential in treating reproductive toxicity (40-42). Our findings were in correlation with these previous reports and suggest its potential application in mitigating CP-induced reproductive toxicity.

Oxidative stress response involves elevated levels of antioxidant enzymes to ameliorate the effect of ROS, leading to cellular damage. CAT activity is tampered with when treated with CP, suggesting that the natural mitigating mechanism is non-functional in CP-treated groups, followed by vehicle treatment. CAT levels were found to be reduced in CP-treated group II compared to the control, contrary to group III showing an increase in CAT activity, pointing towards the possibility of a gradual increase in the antioxidant enzymes post-treatment with CP without any external intervention (Fig. 3b). DH extract treatment may reduce the time required for an increase in CAT activity, which might provide a protective effect to cells from any further damage occurring due to persistent ROS levels. These results were in concordance with MDA levels, where an increase in MDA levels resulted in a decrease in CAT levels in corresponding groups. Aqueous extract of *Phyllanthus fraternus Webster* treatment for 35 days at varying dosage levels for 35 days with a treatment frequency of once a week, has significantly elevated activity of antioxidant enzymes such as CAT (39). Similarly, treatment with extracts of *Ginkgo biloba*, *Trigonella foenum-graecum* and pure compounds such as Gallic acid and Rutin has also shown a similar effect of elevated levels of CAT enzyme activity in CP-induced animals and thereby promoting the mechanism of mitigating reproductive toxicity induced (43-46).

Overall improvement in the aforementioned parameters suggests the potential of DH in ameliorating damage induced due to CP treatment to various organs. CP-induced damages are known to be an after effect of ROS generated due to acrolein, a byproduct formed due to CP metabolism. Elevated levels of anti-oxidant enzymes such as CAT suggest the mode of action of DH extract in improving overall toxicity is via reducing oxidative stress induced by acrolein. Reduction in FSH due to CP treatment is known to negatively affect testosterone synthesis by Leydig cells (47). CP treatment caused FSH levels to decrease with respect to control, suggesting reproductive toxicity induced due to drug treatment. Vehicle control-treated groups for both 4 and 8 weeks showed no significant improvement in levels of FSH, suggesting the need for external intervention for ameliorating the toxicity induced via CP treatment. DH treatment for 4 weeks, low dosage (group IV) showed a significant improvement in levels of FSH, which was further found to be elevated in group V and group VI, suggesting the ability of DH extract in improving reproductive health in both dose and time-dependent manner (Fig. 4a). DH-treated group VI showed an increase of 116.25 % in FSH levels compared to Control was observed. A significant decrease in testosterone levels in CP-treated groups confirms the reproductive toxicity induced in animals due to CP treatment since testosterone is a key steroid sex hormone regulating fertility in males. DH-treated groups showed a significant increase in testosterone levels compared to CP-treated groups and the control group (Fig. 4b). DH-treated group VI showed an increase of 163.37 % in testosterone levels compared to the Control was observed. This result suggests that the effect of DH extract is not limited to improving testosterone levels to normal levels, but it can also elevate them beyond normal levels. A reduction in levels of testosterone due to multiple reasons is considered to be the major factor for the increase in the incidence of male infertility. Thus, DH extract may be considered as a potential therapeutic agent to mitigate male infertility arising due to a defect in testosterone synthesis. *Crataegus monogyna* extract treatment at a lower dosage of 20 mg/kg to CP-injured male Wistar rats has shown a similar result of elevated levels of testosterone and FSH post-treatment (48). FSH levels are the regulating factor for testosterone synthesis. Our study result also shows that any alteration in FSH levels is reflected correspondingly in testosterone levels. CP-treated mice were previously reported to have reduced FSH levels and post-treatment with *Cynomorium songaricum Rupr.* The extract was shown to upregulate levels of FSH w.r.t. blank and CP-treated groups and the aforementioned effect was proposed to be due to the presence of phenolic compounds with antioxidant potential present in the crude

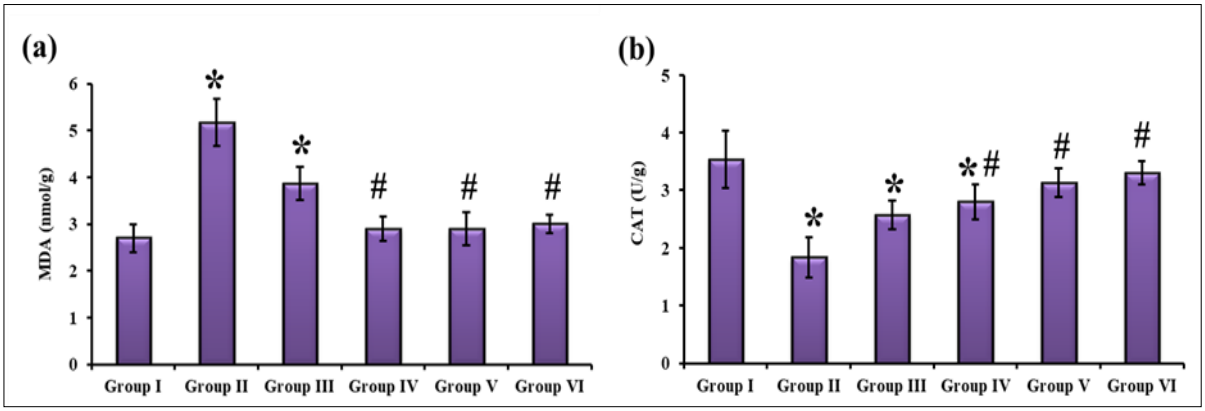

Fig. 3. DH treatment in CP induced male wistar rats showed restoration of MDA and CAT levels comparable to control. Effect of DH extract treatment on **a.** MDA and **b.** CAT levels in test animals; Sample size n=6 (*$p<0.05$ significantly different w.r.t. control, #$p<0.05$ significantly different w.r.t. respective CP control).

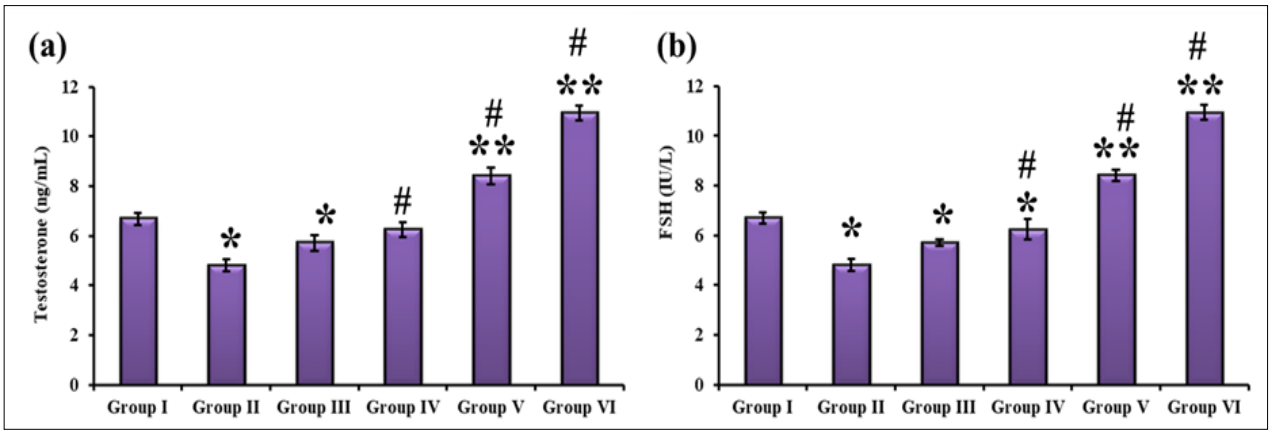

Fig. 4. Elevated levels of testosterone and FSH with respect to control were observed in the DH root extract-treated male wistar rat. Effect of DH extract treatment on **a.** Testosterone and **b.** FSH levels in test animals; Sample size n = 6 (*$p<0.05$ significantly downregulated w.r.t. control, **$p<0.05$ significantly upregulated w.r.t. control, #$p<0.05$ significantly upregulated w.r.t. respective CP control.

extract. Our quantitative and qualitative results showed the presence of phenolic compounds in the DH crude extract, which might be the potential reason behind enhanced FSH levels in DH-treated groups w.r.t CP-treated groups (47).

The overall biochemical studies show that treatment with DH extract in dosages of 100 mg/kg and 200 mg/kg for 4 and 8 weeks has elevated the levels of antioxidant enzymes such as CAT and downregulated MDA. It suggests a potential decrease in oxidative stress induced via CP treatment. This reduction in oxidative stress might be the potential reason for the corresponding improvement in cellular damage assessed using SGPT and LDH levels. Our group has already reported the effect of DH extract on improving the expression of antioxidant enzymes in mouse Leydig cells (TM3) induced with CP damage. This previous finding also points towards the elevation of antioxidant enzymes due to DH treatment as a potential mechanism of action of DH in providing reproductive toxicity protection (49). The current study showed a CP-induced toxicity protection with 200 mg/kg DH treatment for 8 weeks in male wistar rats, whereas previous studies with *American ginseng* and *Withania somnifera* showed a similar protective effect with much higher dosage levels of 500 mg/kg, making DH extract a potentially better candidate for the aforementioned activity (50, 51).

Histopathology assessment

Negative control group (Fig. 5a) showed intact basal membrane, seminiferous tubules and loose connective tissue between the tubules. CP-treated groups in Fig. 5b-5c (Group II and Group III) showed a decrease in the thickness of the basal layer, suggesting damage to the cells. Less compact spermatogenic cells separated by vacuoles and cytoplasmic vacuolization in spermatogonia were also observed in these groups. Animals treated with 100 mg/Kg for 4 weeks (Group IV) showed the presence of degenerating spermatogonia as well as regenerating spermatogonia, suggesting a potential damage repair occurring due to DH treatment in damaged tissues (Fig. 5e). A further increase in dosage and duration of treatment in Group V and Group VI has resulted in the appearance of Seminiferous tubules with compact spermatogenic cells and nearly normal Leydig cells (Fig. 5d-5f). Pathological alterations in the testis are considered a biomarker for reproductive function (47). CP treatment is reported to cause thinning of spermatogenic epithelium and reduction in epithelial layers and our results were in concordance with these previous findings (47). Ginger and Pumpkin seed extract treatment to CP-induced male wistar rats has shown both elevated levels of antioxidant enzymes and an increase in the count of spermatogonia, suggesting the positive effect of treatment with compounds with antioxidant potential in protecting cells

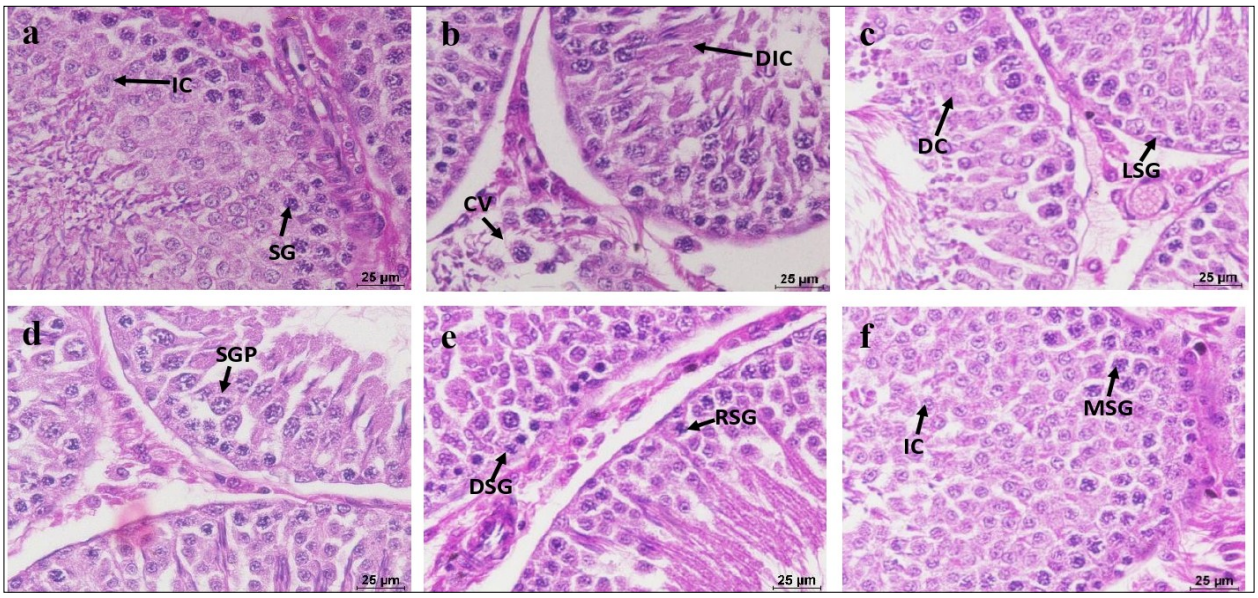

Fig. 5. Histopathological evaluation of testis tissue: **a.-f.** Group I- Group VI male wistar rats. **CV** – Cytoplasmic vacoulization, **DC** – Degenerating cells, **DIC** – Degenerating interstitial cells, **DSG** – Degenerating spermatogonia, **IC** – Interstitial cells, **LSG** – Less number of spermatogonia, **MSG** – Maturing spermatogonia, **RSG** – Regenerating spermatogonia, **SG** – Spermatogonia, **SGP** – Spermatogonia becomes more prominent

against reproductive toxicity (52). Our results were also in correlation with this finding, where elevated levels of antioxidant enzymes and improvement in spermatogenic cells were observed. An improvement in histomorphology of reproductive tissues was observed in similar studies, which utilised extracts of *Cucurbita pepo var. styriaca* and *Trigonella foenum graecum* as therapeutic agents CP induced toxicity (44, 53).

Conclusion

In the current study, we evaluated the pharmacological effects of DH extract in protecting CP-induced reproductive toxicity in male wistar rats. A comparative study among phytochemical constituents present in different solvents used for extraction revealed that the ethanolic DH extract showed the highest bioactivity. Subsequent *in vivo* evaluation revealed that DH treatment post CP induction in male wistar rats has a significant effect in repairing cellular damage, as evidenced by the restoration of LDH and SGOT levels. Additionally, DH extract treatment significantly increased testosterone levels in the study animals. These findings suggest a potential therapeutic application of DH extract in ameliorating CP-induced reproductive damage. Moreover, its ability to increase the testosterone levels compared to the negative control further underscores a future direction to be explored for its potential applications in a wide spectrum of conditions where reduced levels of testosterone synthesis are causative for diminished reproductive health. Given its bioactivity and potential to modulate hormone levels, DH extract may offer a promising therapeutic option for a wide range of reproductive health issues, particularly those associated with oxidative stress and chemotherapeutic-induced damage.

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Authors' contributions

RG conceived of the presented idea, developed the theory and performed the experiments. SKB conceived of the presented idea. RS and DS verified the analytical methods. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final version of manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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