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RESEARCH ARTICLE

Chemoprotective action of *Dactylorhiza hatagirea* (D. Don) Soo ethanol extract against cyclophosphamide induced reproductive toxicity and oxidative stress on TM3 cells

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Abstract

Cyclophosphamide (CP) is one of the common therapeutic agents which possesses anti-cancer potential, however it causes neuronal and reproductive toxicity due to rise in oxidative stress which is a major limitation to its therapeutic use. Despite of this, several research findings suggest that phytocompounds possess antioxidant potential and thus can be utilized as potential therapeutic agent for reactive oxygen species (ROS) induced damage to the cells. So as to explore the effect of root of *Dactylorhiza hatagirea* (D. Don) Soo belonging to the family Orchidaceae towards reproductive toxicity induced by cyclophosphamide the current study was conducted towards TM3:mouse leyding cells. In this study, ethanolic extraction roots was performed by using soxhlet and compound identification was done through GC-MS. Further, cytotoxicity and protection against reproductive toxicity effect of ethanolic root extract was analysed by using MTT assay followed by evaluation of ROS scavenging effect of extract post-treatment with CP in TM3 cells. In order to elucidate the molecular mechanism behind the protective effect of the extract, qRT-PCR was performed to analyse the expression of antioxidant and steroidogenic gene markers. The results from GC-MS analysis showed presence of two major compounds namely Benzenemethanol, 3fluoro (C1) and 2-Mercaptophenol (C2). It was also identified that the extract was non-toxic to TM3 cells and pre-treatment with enhanced cell viability in CP-treated TM3 cells. Apart from this, reduction in ROS generation was observed in pre-treated *D. hatagirea* CP induced cells. Additionally, the extract has enhanced the antioxidant response (via *CAT, GPx1, SOD1* and *SOD2*) to combat the oxidative stress caused by CP, which could help in reducing the side effects of the treatment. Moreover, the extract seems to modulate the steroidogenic gene response induced by cyclophosphamide via upregulation of AR, 3βHSD, Cyp11a1, Cyp19 and StAR. These findings suggest that DH has potential therapeutic value in mitigating the adverse effects of CP through antioxidant and steroidogenic pathways.

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

Introduction

Reproductive toxicity mediated male infertility is increasing each year (1). Elevated levels of oxidative stress due to factors such as endocrine disruption, environmental pollution, side-effects of pharmaceutical drugs such as CP and doxorubicin, by-products of industrial processes and genetic factors are major influencers in this increase in rate of infertility in male (1, 2).

CP is a DNA alkylating agent which is widely administered drug for tumor therapy and organ transplantation (prevent rejection of transplants) but still has many adverse side-effects including impairment of reproductive function (1, 3). CP gets converted to phosphoramide mustard and acrolein *in-vivo*. Phosphoramide mustard is the product responsible for its anti-cancer activity whereas the byproduct acrolein is the major contributor to its toxicity towards reproductive system (4). Previous reports suggest that CP can cause severe damage to testis and increase the risk of oligospermia or azoospermia eventually leading to infertility (1, 5). Apart from this, organs such as heart, lungs, ovary and liver were also adversely affected by CP administration (3). Toxicity to male reproductive system is caused due to oxidative stress and whereas apoptosis induction is due to CP administration which further leads to decrease in testosterone synthesis (3, 4, 6, 7). Acrolein causes damage to sertoli cells, further stimulates apoptosis in germ cells and impairs testosterone levels in Leydig cells (4). Testosterone is the vital hormone responsible for secondary sex characteristics of male (1).

Previous reports suggests that phenolic compounds and other natural compounds can act as adjuvant drugs and protect male reproductive system from CP induced toxicity (3, 8). *Dactylorhiza hatagirea* is naturally found in alpine and sub-alpine regions (9). Traditional ayurvedic knowledge shows that it has several medicinal effects in treatment of disorders related to nervous, skeletal, respiratory and reproductive system (9). It is also used as a dietary supplement to improve testosterone levels (9). Scientific evidence for the afore-mentioned properties of this species is minimal (10, 11) and its effect on protection of Leydig cells against damage induced due to CP treatment is not yet explored. The current study to evaluate the protective effect of pre-treatment on reproductive toxicity induced to TM3 cells will provide scientific evidence to this traditional knowledge. This may also provide appropriate insights into its therapeutic potential as a supplement to cancer patients undergoing chemotherapy treatment to ameliorate the side-effects of CP.

Considering the above facts, the present study was performed on its ethanolic extract and analysed for its potential towards protecting leydig cells from toxicity induced by CP. However, cytotoxic effect of the extract was initially analysed and its effect on ROS scavenging produced due to CP treatment was also performed. Finally, effect of DH pre-treatment on CP treated leydig cells were analyzed for gene expression levels of oxidative stress, steroidogenesis and apoptotic markers.

Materials and Methods

Dacytlorhiza hatagirea has been selected for the study. Dulbeccos modified eagle medium (DMEM), Fetal bovine serum (FBS) was purchased from HiMedia, India. Antibiotic-Antimycotic (100x) solution was purchased from Gibco, India. cDNA synthesis kit was obtained from Bio-Rad, India. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma-aldrich. All other chemicals and reagents used were analytical grade.

Collection and processing of *D. hatagirea*

Root samples were collected from Lahaul, Himachal Pradesh and it was confirmed through Sanger sequencing. The collected samples were shade dried and powdered prior to extraction.

50 g dried sample was powdered to obtain a coarse powder and placed inside a thimble, which is loaded into the main chamber of the Soxhlet extractor. The extraction of weighted root powder with respective volume of ethanol was carried out with its boiling point. The Soxhlet extractor is positioned into a flask containing the extracting solvent. The Soxhlet is then fitted with a condenser. The solvent is heated to reflux. The solvent vapor travels up a distillation tube and condensed into the extractor housing the thimble holding the solid. The chamber containing the material slowly fills with warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the extracting flask. This cycle may be allowed to repeat many times, over hours or days, until the solvent gets colourless in extracting chamber. After many cycles the desired compound is concentrated in the distillation flask. After extraction the solvent is removed typically by means of a rotary evaporator yielding the extracted compound. Dried samples were then stored at 4 °C until for further use.

GC-MS analysis

GC-MS analysis was performed to elucidate the volatile components present in the extract. GC Clarus 500GC-MS instrument (Perkin Elmer) was used for compound separation and the obtained mass spectrum was interpreted by using NIST11 and RTLPEST3 databases. Hexane was utilized as volatile solvent for GC-MS analysis.

Cell culture and maintenance

TM3 (Mouse Leydig cell line) was purchased from NCCS, Pune, India. Cells were maintained in Ham's F12 medium supplemented with 10 % FBS and 1X antibiotic-antimycotic solution in 5 % CO_2 incubator at 37 °C. Medium was replenished every alternate day.

Cell Viability of TM3 cells

In order to evaluate the potential cytotoxic effect of DH extract on TM3 cell lines, MTT assay was performed. TM3 cells were cultured on a 96 well plate and upon reaching 80 % confluency, cells were treated with varying concentrations of DH extract (6.25-100 µg/mL) for 24 hr. After incubation, MTT assay was performed to evaluate the cell viability. After completion of incubation period, medium was removed and then the cells were washed once with 1X PBS and incubated with 100 µL of MTT solution (0.5 mg/mL) for 3 hr. The solution was then removed and formazan crystals were solubilized by using DMSO and absorbance was measured at 540 nm.

Protective effect of *D. hatagirea* extract against CP induced toxicity

Protective effect of the extract on CP induced toxicity was evaluated by using MTT assay. TM3 cells were seeded on to 96 well plates and upon reaching 80 % confluency, cells were treated with its extract in varying concentrations (6.25-100 µg/mL) for 4 hr and then treated with CP (5 µg/mL). Later, the treated cells were incubated for 24 hr in CO_2 incubator at 5 % CO_2 and 37 °C. Then the untreated cells were kept as negative control and CP treated cells alone were kept as positive control. After 24 hr, MTT assay was performed as mentioned earlier.

DCFH-DA staining

TM3 cells were cultured on 35 mm culture dish and upon reaching 60 % confluency, cells were treated with 25 and 50 µg/mL of extract and after 4 hr cells were treated with CP (5 µg/mL). Thereafter, untreated cells were kept as negative control and CP treated cells alone were kept as positive control. After 24 hr, DCFH-DA staining was performed according to previous reported protocol. Cells were washed twice with 1X PBS and incubated with DCFH-DA solution for 30 min in dark. After incubation, staining solution was removed, washed twice and observed under fluorescence microscope.

RT-qPCR

TM3 cells were cultured in 6 well plates and upon reaching 80 % confluency, cells were treated with extract 25 and 50 µg/mL for 4 hr and CP (5 µg/mL) treatment was provided. Untreated cells were kept as negative control and CP treated cells alone were kept as positive control. After 24 hr, RNA isolation was performed by using TRI-zol method and cDNA synthesis was performed through 2x RT Easy Mix by G Bioscience. RT-qPCR was then performed by using SYBR green qPCR Mix (GBiosciences) for antioxidant and steroidogenic marker genes (Table S1). Amplification reaction was performed in a CFX Connect Real-Time system (Bio rad). In each PCR analysis, template and RT controls were included to account for contamination.

Results

DH roots were dried and extraction was performed using ethanol as solvent using Soxhlet apparatus. A final yield of 5 % was obtained and component analysis was performed using GC-MS analysis (Fig. 1).

GC-MS analysis

From the GC-MS analysis of crude ethanolic extract of DH, 2 compounds were detected namely Benzenemethanol, 3fluoro (C1) and 2-Mercaptophenol (C2) (Supplementary Fig. 1). GC-MS analysis of ethanolic extract of *Dactylorhiza hatagirea* root revealed presence of Fluorobenzyl alcohol and 2-Mercaptophenol (Table S2, Fig. 2).

Cell viability of TM3 cells

Mouse Leydig cells were treated with different concentrations of root extract for 24 hr and evaluated its cytotoxicity towards the cells (Fig. 3). The cell images revealed the addition of root extract has not altered the cell morphology which was further validated by using MTT assay. A very slight reduction in cell viability was observed with the highest concentration treatment suggesting its possible toxicity at concentrations above 100 µg/mL. Thus, concentrations below 100 µg/mL were utilized for further analysis.

Protective effect of the extract against CP induced toxicity

Inhibitory effect of CP on TM3 cells were evaluated by using MTT assay and the results showed that 5 µg/mL of CP treatment for 24 hr has caused a decrease in cell viability of TM3 to 52.69 % (Fig. 4). Pre-treatment with its extract was found to be beneficial in improving the cell viability in a dose-dependent manner and highest efficiency was showcased by 100 µg/mL of DH pre-treatment. However, no significant improvement in cell viability was observed between 50 µg/mL and 100 µg/mL treated groups. This might be due to the slight toxic effect of DH extract in higher concentrations. Thus, the pretreatment of concentrations less than 50 µg/mL was utilized for further evaluations.

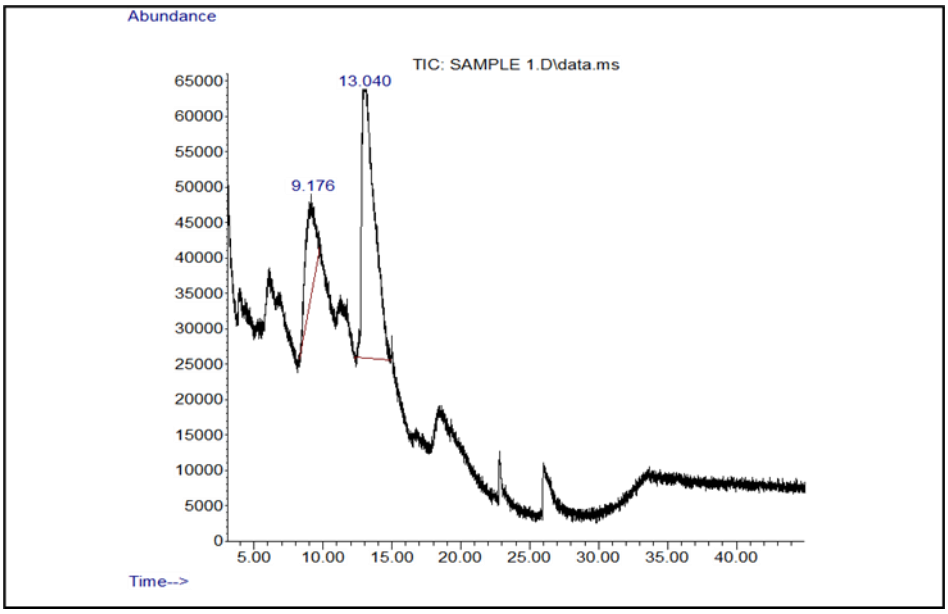

Fig. 1. GC-spectrum of *Dactylorhiza hatagirea* root ethanolic extract.

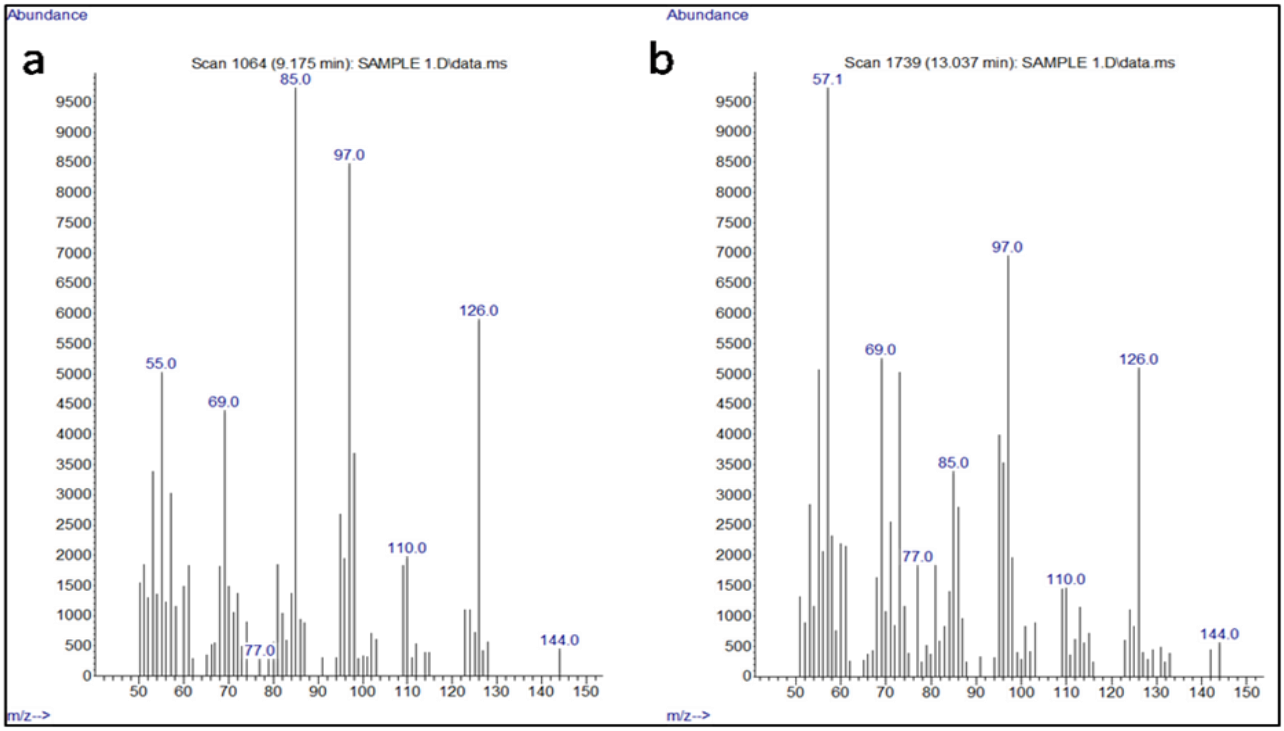

Fig. 2. a) Mass spectrum of peak 1 at 9.175 RT, b) Mass spectrum of peak 2 at 13.037 RT.

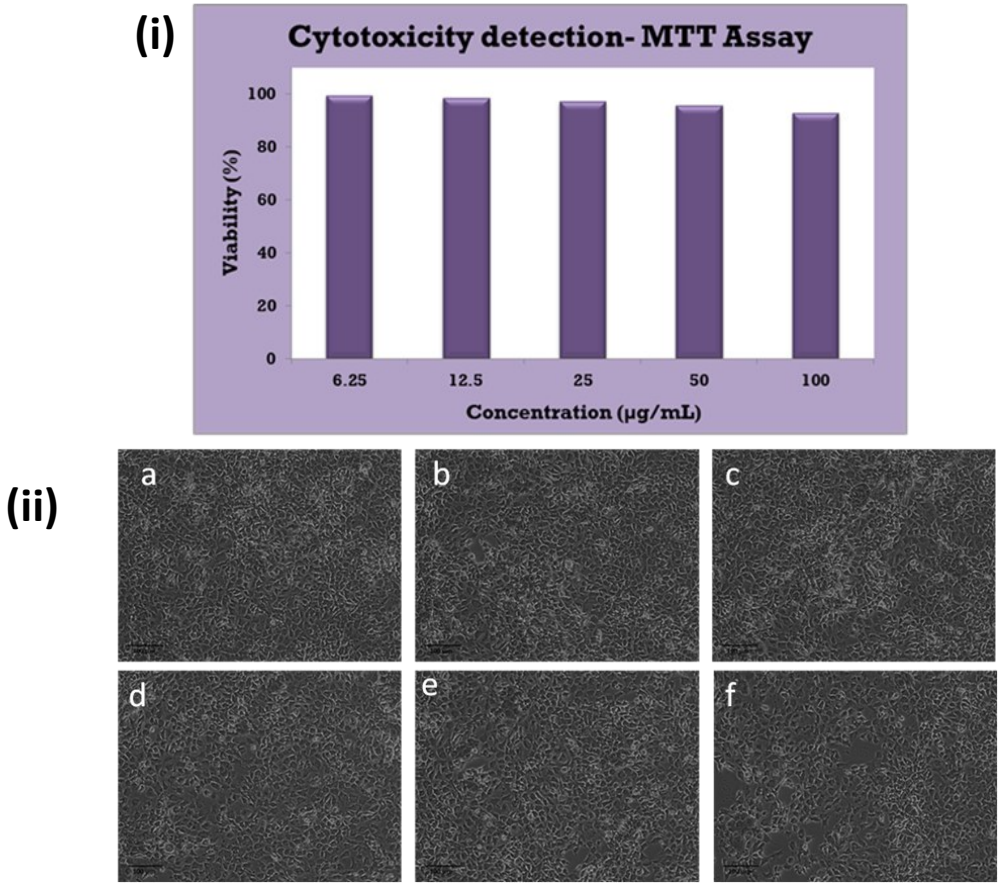

Fig. 3. (i) Graphical representation of cytotoxicity analysis of *Dactylorhiza hatagirea* root extract on TM3 cell lines for varying concentrations using MTT assay after 24 hr incubation post treatment. (ii) Microscopic images of TM3 cell lines post treatment with varying concentrations of root *Dactylorhiza hatagirea* extract; a) control, b) 6.25 µg/L, c) 12.5 µg/m, d) 25 µg/mL, e) 50 µg/ml, f) 100 µg/mL.

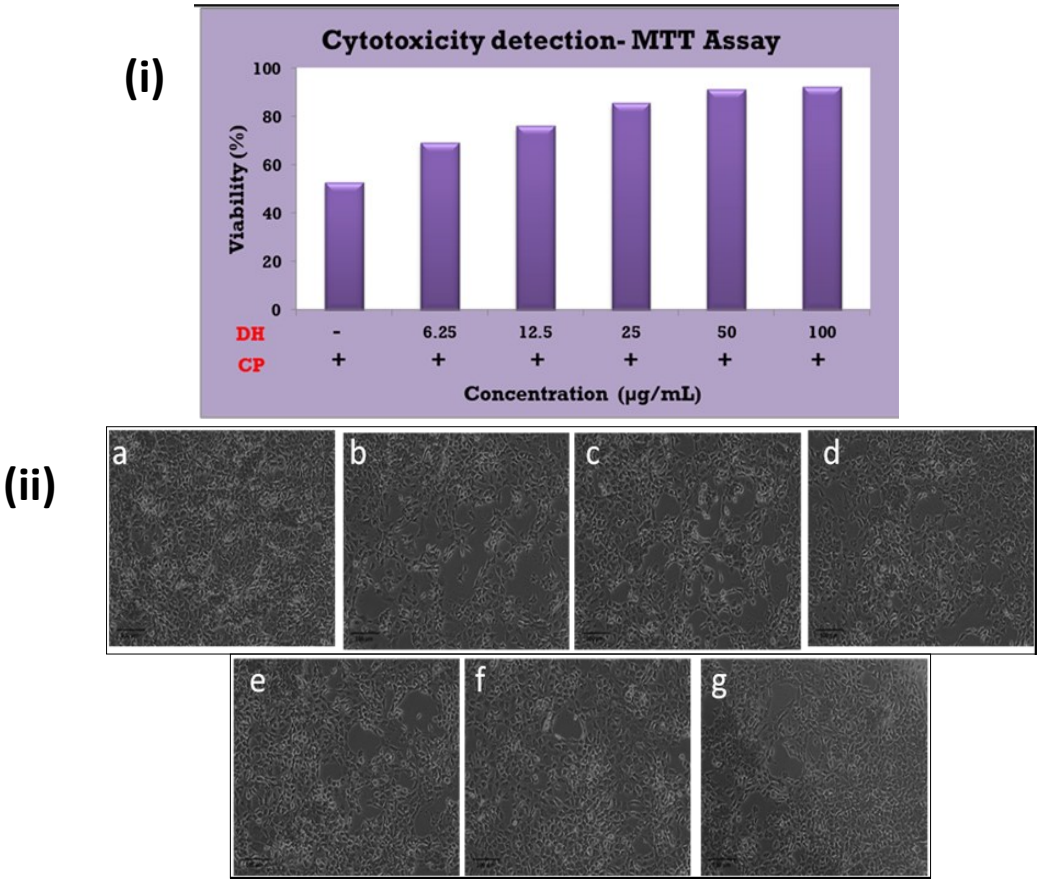

Fig. 4. (i) Graphical representation of cytotoxicity analysis of *Dactylorhiza hatagirea* root extract pre-treated on cyclophosphamide treated TM3 cell lines (ii) Microscopic images of TM3 cell lines pre-treated with root of *Dactylorhiza hatagirea* extract post treatment with CP; a) control, b) CP, c) CP + 6.25 µg/mL *D. hatagirea*., d) CP + 12.5 µg/mL *Dactylorhiza hatagirea*, e) CP + 25 µg/mL, f) CP + 50µg/mL DH, g) CP + 100 µg/mL *Dactylorhiza hatagirea.*

DCFH-DA staining

As shown in Fig. 5 CP treated cells showed higher intensity of green fluorescence indicating elevated levels of ROS upon CP treatment compared to untreated control. Pre-treatment with DH extract has reduced ROS levels in dose dependent manner where 50 µg/mL DH pre-treatment showed highest efficiency and fluorescence intensity was comparatively similar to control group.

RT-qPCR

Effect of CP treatment on steroidogenic genes such as 3βHSD, AR, Cyp11a1, Cyp17a1, Cyp19 and StAR showed a significant down-regualtion compared to control, confirming its toxicity towards reproductive system. Pre-treatment with the extract aided in up-regulation of these genes with respect to CP treatment groups suggesting its potential in mitigating damage induced due to CP in TM3 cells but less than control group. Cyp17a1 gene expression was found to be significantly upregulated in extract pre-treated group compared to both control and CP treatment showed a potential positive role of extract in steroidogenesis.

CP treatment to TM3 cell lines at 5 µg/mL for 24 hr significantly reduced expression of genes related to steroidogenesis such as 3βHSD, AR, CYP17a1, CYP19 and StAR. Expression of CYP17a1 remained unaltered with CP treatment compared to control. Pre-treatment with the root extract has elevated the levels of 3βHSD, AR, CYP17a1, CYP19 and StAR showing the positive impact of sample pre-treatment in protecting the cells against CP induced damage leading to damage in gene expression. Sample treatment has shown significant increase in expression of CYP17a1 with respect to control in both 25 and 50 µg/mL extract treated cells suggesting the independent effect of extract on leydig cells.

Quantitative real time PCR results showed that treatment with CP at 5 µg/mL for 24 hr has significantly reduced the expression of anti-oxidant marker genes such as CAT, GPx3, SOD1 and SOD2. *Dactylorhiza hatagirea* pretreated cells showed an upregulation in anti-oxidant genes such as CAT, GPx and SOD1 with respect to CP treated group but downregulated with respect to control. Expression levels of SOD2 were found to be significantly upregulated in both extract treated groups when compared to control and CP treated groups suggesting the potential anti-oxidant property of the extract via upregulation of SOD2 gene as the defence mechanism against oxidative stress induced by CP (Fig. 6).

Discussion

Cyclophosphamide is an alkylating agent and well-known for its chemotherapeutic applications. Even though it can act as both anti-tumor and immunosuppressive agent, toxicity to surrounding normal cells is considered as a severe side-effect and as a matter of concern requiring immediate remedial measure. Phenolic compounds are proven to be effective in protecting normal cells against oxidative stress induced toxicity by CP (3) or any other cellular damages caused due to toxic compounds such as zinc oxide nanoparticles, bisphenol-A (BPA), nicotine, D-galactose etc. (6,12 -15). Apart from plant-derived phenolic compounds, candidates such as Vitamin C co-treatment were also reported to have a protective effect against leydig cell damage induced via

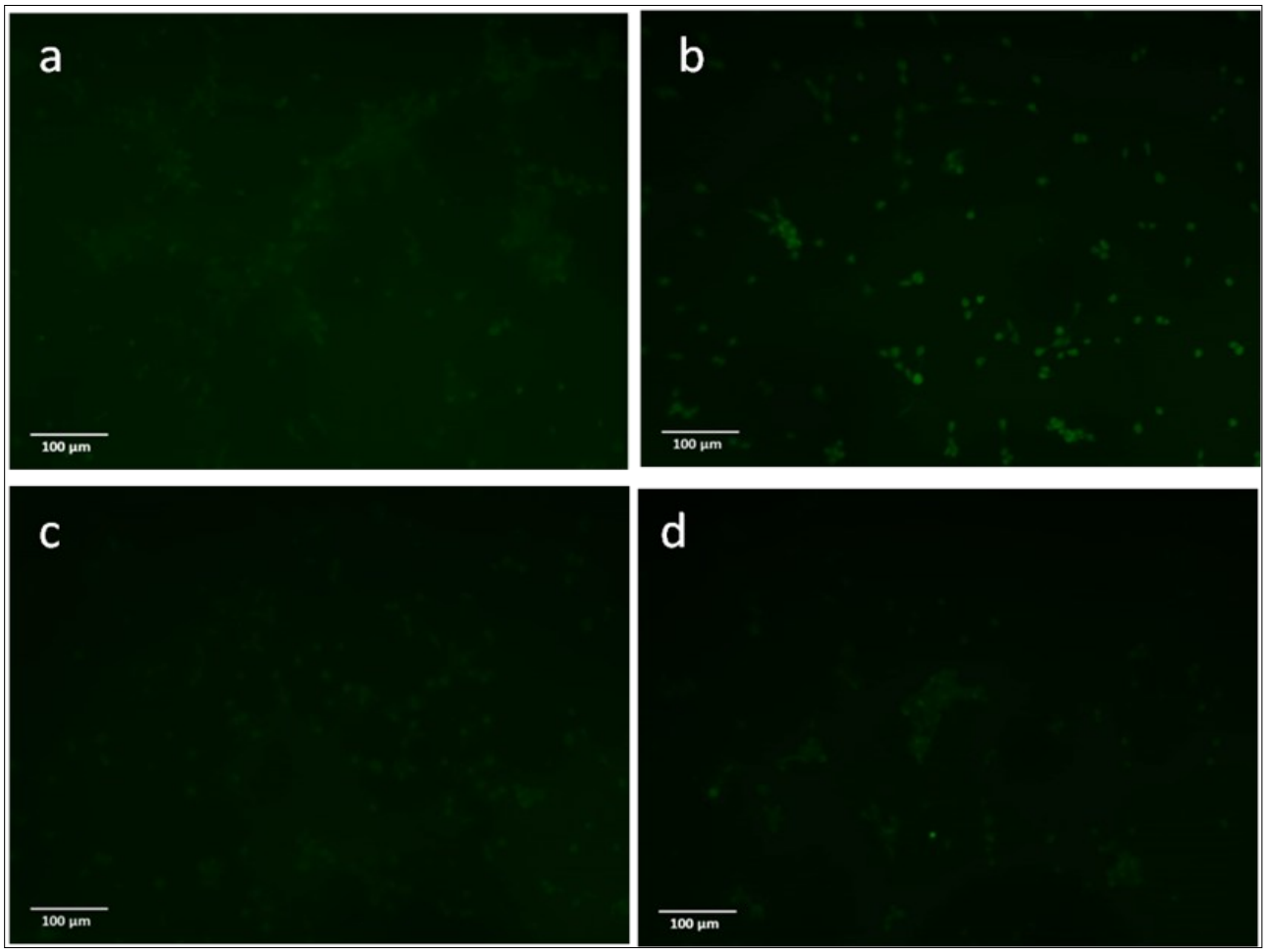

Fig. 5. ROS detection using DCFH-DA staining on cyclophosphamide induced TM3 cell line and cells pre-treated with *Dactylorhiza hatagirea* root extract prior to CP induction; a) control, b) CP, c) CP + 25 µg/mL *D. hatagirea*, d) CP + 50 µg/mL *D. hatagirea*.

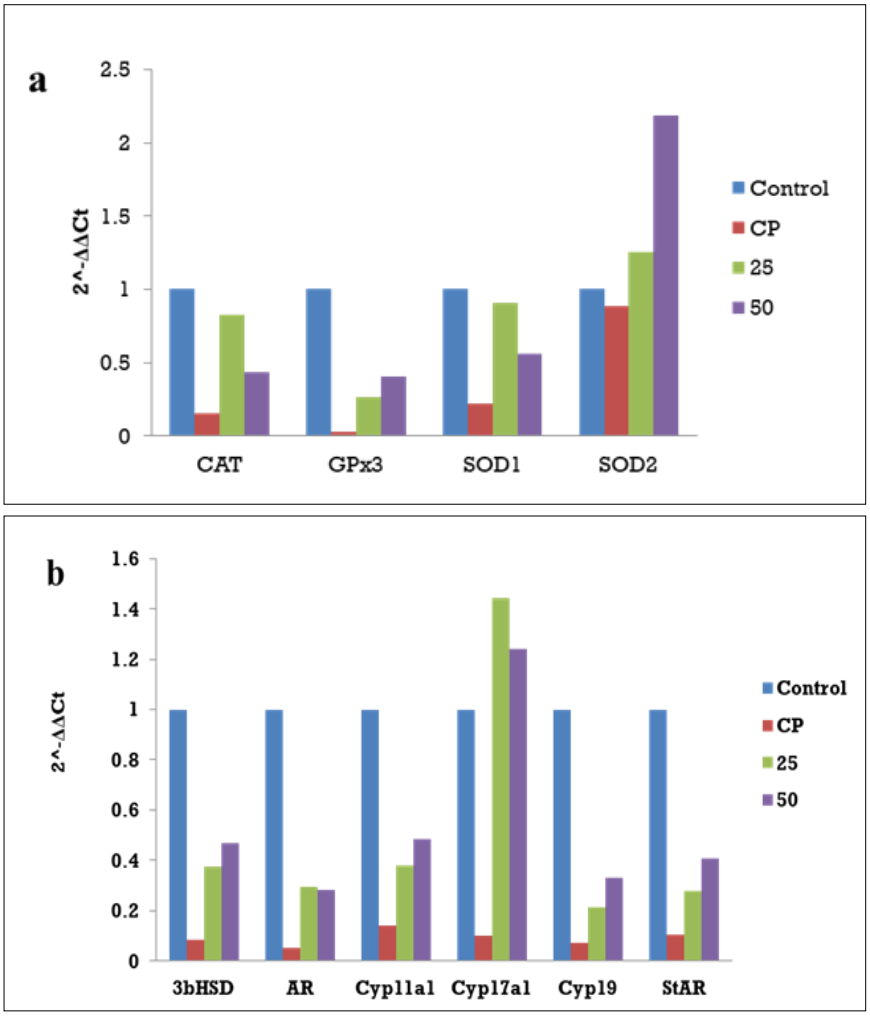

Fig. 6. Effect of CP and DH pre-treatment on a) antioxidant markers (CAT, GPx3, SOD1, SOD2) and b) steroidogenesis markers (3bHSD, AR, Cyp11a1, Cyp17a1, Cyp19, StAR) at gene level.

glycidamide or lead nitrate treatment (16, 17). Traditional ayurvedic knowledge suggests its potential properties to stimulate male reproductive system with its underlying mechanism remains unknown (9).

Previous reports suggest that CP treatment can damage leydig cells via SMAD pathway and sertoli cells responsible for male secondary sex characteristics (18, 5). An *in vivo* study proved that CP treatment can significantly reduce the density of Leydig cells in the interstitial space which are responsible for production of testosterone (1, 3). It plays a vital role in spermatogenesis progression and production of sperms in testis (3). Alterations in levels of testosterone can also reduce sensitivity of leydig cells towards leutinizing hormone (LH) (3). In the current study, we aimed to elucidate the protective effect of the extract in CP induced reproductive toxicity and its underlying mechanism using TM3 leydig cells as *in- vitro* cell line model.

GC-MS analysis revealed presence of two major compounds and among the two compounds 2-Mercaptophenol, which is a major constituent in the crude extract is known to possess several biological activities including antioxidant potential (19). GC-MS analysis of the raw extract indicated 3-fluorobenzyl alcohol (20.75 %) and 2-mercaptophenol (79.25 %) to be present in considerable amounts. To our knowledge, this is the first publication describing these 2 compounds to be principal constituents in this specific plant extract. While both molecules have established bioactivity elsewhere, their synergistic coexistence in this extract and presumed function in modulating antioxidant activity is a new finding. This adds to the chemical characterization of the extract and provides a platform for further research into their pharmacological relevance. Nonetheless, it is interesting to note that the radical scavenging activity observed cannot be explained solely by the presence of these two compounds. Though they occupy the cumulative area of peaks identified by means of GC-MS, the method is more suited for analysis of volatile and semi-volatile compounds. Non-volatile phenolics or flavonoids such as dactylorhins, hydroquinone and resveratrol, already known to reverse oxidative stress may also be present in the extract and be responsible for the overall antioxidant action (11). The isolated compounds therefore most likely are responsible, but the contribution of other bioactive constituents cannot be ruled out.

CP treatment generates ROS and thereby damaging the tumour cells. It has several shortcomings such as neurotoxicity and reproductive toxicity. ROS generation in the surrounding normal cells are the major reason for normal cellular damage. Compounds with antioxidant potential may be used to provide a protective effect to the normal cells. Since DH root extract showed presence of a compound with antioxidant potential further evaluation for its protective nature against reproductive toxicity induced due to CP treatment was carried out.

Cytotoxicity evaluation of the extract towards TM3 cell lines proved the non-toxic nature of extract towards the normal

leydig cells. No alteration in cell morphology was observed in extract treated cells when compared with untreated cells. CP treatment to TM3 cell lines showed a significant decrease in viability confirming its toxicity towards the cells. Pre-treatment with different concentrations of the facilitated enhanced cell viability than CP alone treated cells showcasing its protective nature towards decreasing the effect of CP toxicity. Viability of cells treated with 50 and 100 µg/mL extract pre-treated cells showed similar percentage viability. This might be due to the slight toxicity at higher concentrations as evidence from previous cytotoxic studies. Thus, 25 and 50 µg/mL extract pre-treatments were further analysed for the underlying mechanism behind the improvement in cell viability in extract pre-treated cells.

Formation of ROS is considered to be the major causative for cellular damage in normal cells thus the potential of the extract in scavenging ROS was analysed by using DCFH-DA staining. As evidenced from the fluorescence intensity, it is clear that CP treatment has enhanced ROS levels and extract pre-treatment has helped in reducing the ROS levels in dose-dependent manner. 50 µg/mL extract pre-treated cells showed highest similarity of ROS intensity with untreated control group.

GC-MS analysis showed presence of phenolic compounds in the crude extract and it has mediated in the radical scavenging activity. Earlier reports suggest that flavones and other phenolic compounds such as chrysin, resveratrol, rutin etc., has the potential to ameliorative the damage induced due to oxidative stress (20).

CP treatment tends to sensitize spermatogonic cells to oxidative stress via down-regulation of antioxidant genes such as SOD, GPx, CAT and GSH (3). Thus, we analysed the effect of CP treatment and extract pre-treatment on antioxidant marker genes such as SOD1, SOD2, CAT and GPx. Our results were in concordance with previous reports where CP treatment significantly down-regulates all antioxidant genes and thus making the cells devoid of any antioxidant enzymes to counter-act the ROS produced due to CP treatment and eventually leading to cell death (3). Our findings also suggest that maximum effect of extract pre-treatment was observed in expression of SOD2 genes where the gene expression was significantly higher compared to control. Thus, we postulate that the effect of *D. hatagirea* on SOD2 might be the possible reason for protection of TM3 cells against oxidative stress induced damage in cells. SOD2 is a mitochondrial matrix enzyme that scavenges oxygen radicals produced by the extensive oxidation-reduction and electron transport reactions that occur in mitochondria (21). SOD mediates in conversion of oxygen radicals to hydrogen peroxide which is also an unstable form. It is then converted by using CAT enzyme to non-toxic products. Our results suggest an elevation in SOD2 and CAT gene expression in extract pre-treated cells suggesting its possible role in mitigating oxidative stress induced by CP treatment. Leydig cells are more prone towards oxidative stress mediated cell damage due to relatively higher content of polyunsaturated fatty acids in its cell membrane (22). Compounds such as lycopene, kefir, capric acid and melatonin with strong antioxidant potential has shown similar findings where ROS induced damages to reproductive cells were mitigated via inhibition of ROS pathway (23-26). Whereas another research finding suggested that treatment with Açai berry has the potential to mitigate reproductive damage induced via CP through Nrf-2/HO-1 pathway (27). Omega 3 fatty acids have alleviated CP induced damages via immunomodulation and reduction of oxidative stress in pigs (26). Other compounds such as hesperidin were also shown to possess a testicular damage protection property via immunomodulation and reduction of oxidative stress (28). Oxidative stress induced damage by hydrogen peroxide to TM3 cells was found to be reduced in *Moringa officinalis* extract treated groups (29).

Experimental data suggests that pre-treatment of extract prior to CP treatment enhance expression of steroidogenic markers compared to CP treated groups. AR is required for development of adult leydig cells which is absent in foetal cells. AR directly influences expression of Cyp17a1 which is essential for conversion of progesterone to dehydroepiandrosterone and also precursor for testosterone synthesis. Expression of steroidogenic genes follows a sequential order where StAR expression initiate transport of cholesterol from outer to inner mitochondrial membrane where further it is cleaved by pregnenolone by Cyp11a1 followed by conversion to progesterone through activity of 3βHSD. Cyp17a1 and 17β-HSD is finally activated and mediated testosterone synthesis. Our results show an increase in expression of the aforementioned genes in the same sequential order suggesting that the cells were in the last stages of production of testosterone. A detailed time dependent effect on these genes might provide a better insight on molecular level effect of extract pre-treatment on steroidogenesis (1). *Huangqi-Guizhi-Wuwutang* and *Achilleamille folium* extract has also shown a similar cytoprotective effect on CP treated mice against reproductive damage (30, 31).

AR negative cells usually undergo apoptosis (32) as evidenced from the viability results of CP treated TM3 cells. Reduction in expression of AR negatively regulates expression of genes such as Cyp17a1, HSD17β3, HSD3β6 (32). This evidence further supports our findings where CP treated cells showed very minimal levels of Cyp17a1 gene expression compared to control whereas extract pre-treatment aided in upregulating Cyp17a1 in both groups. Flavones such as apigenin were reported to positively regulate androgen production via upregulation of StAR gene expression (33). It is also reported that COX2 enzyme inhibition can also facilitate in upregulation of StAR gene expression (33). ROS generation due to chemical inducers such as Benzo(a)Pyrene also showed a similar toxicity pattern to steroidogenic gene expression of Cyp11a1, 3βHSD, 17βHSD and StAR (34, 35). Apart from cytoprotective effect against CP induced reproductive toxicity, plants such as *Sarsa borealis* and *Taraxacum officinale* has been reported to upregulate genes involved in steroidogenesis whereas effect of *D. hatagirea* on sterodogenic genes is not yet reported (36, 37).

Furthermore, *D. hatagirea* as a potential protective agent in CP-induced reproductive toxicity has also been supported directly and indirectly by certain studies. It has been predominant in traditional ayurvedic medicinal systems with respect to its professed aphrodisiac and restorative properties (38). Contemporary scientific studies have backed up this professed characteristic by validation through laboratory studies. The therapeutic potential has largely been due to its rich content of phytochemicals. A number of bioactive molecules like flavonoids, polyphenols and alkaloids have been found in DH that actively possess antioxidant, anti-inflammatory and immunomodulatory activity (39). Moreover, a number of other phytochemicals like coumarins, tannins and glycosides have also been extracted that confer medicinal potential to *D. hatagirea* (40). These are compounds that have been reported

to fight oxidative stress, a major mechanism of cell damage induced by chemotherapy drugs like CP (41). Beside from its effects on reproductive health, is also reported to have noteworthy neuroprotective and cardioprotective effects (42). The neuroprotective activity of has been attributed to its antioxidant property which reduce the neuronal cell damage and protect against neurotoxocity induced by various stressors (43). It acts in a similar way to reduce damage in cardiac muscle tissues, indicating towards a broader protective role against systemic toxicity. These are closely related to its possible antioxidant and immunomodulatory activities. In another similar study, its extracts were found to be scavenging free radicals and enhancing the activity of endogenous antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) (44). This scavenging process efficiently plays a pivotal role in the neutralization of ROS produced by CP treatment, thus protecting against cellular and DNA damage (45). Overall, this binomial action of sheltering the cells from oxidative stress efficiently and modulating the immune system, makes it an ideal species for the development of supportive therapies to decrease the side effects of chemotherapy.

Conclusion

Our research finding suggests that CP induces cell damage due to enhanced ROS generation leading to reduced cell viability and down-regulation of genes responsible for steroidogenesis. The ethanolic extract of *D. hatagirea* is found to be beneficial in mitigating the adverse effect caused due to CP in leydig cells as evidenced from enhanced viability and reduced ROS generation. Presence of phenolic compounds in the extract might be the factor towards its antioxidant activity. The extract also showed effect in upregulation of steroidogenic genes suggesting its therapeutic potential in treatment of disorders related to defects in steroidogenesis and as a protective factor to mitigate toxicity imparted due to chemotherapeutic drugs. An up-regulation in steroidogenic genes with respect to control suggests its potential to be explored further in areas related to disorders due to decrease in levels of testosterone synthesis in males.

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Authors' contributions

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

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest

Ethical issues: None

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