ORIGINAL ARTICLE

Evaluation of Cytoprotective Effect of Silymarine, Rutin, and Catechin on Cyclophosphamide-Induced Toxicity on Brain, Heart, and Spleen in Mice

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ABSTRACT

Aim: The aim of the present study was to evaluate the cytoprotective effect of silymarine (SIL), rutin (RUT), and catechin against cyclophosphamide (CYP)-induced toxicity. **Materials and Methods:** The protocol for the study includes a total of 36 balb c mice and divided into six equal groups. These groups were: Control, CYP (25 mg/kg b.w., i.p), CYP + Amifostine (AMF) (200 mg/kg b.w., i.p), CYP + SIL (100 mg/kg b.w., oral), CYP + RUT (50 mg/kg b.w., oral), and CYP + Catechin(40 mg/kg b.w., oral). AMF, SIL, RUT, and Catechin treatments were performed for 14 days, while CYP was given till 10th day of the study. Cytotoxicity was measured followed lipid peroxidation (LPO), antioxidant markers such as superoxide dismutase, reduced glutathione, catalase, and examined the histology of brain, heart, and spleen in all groups. **Results:** During CYP treatment, all three flavonoids increased the levels of antioxidant markers and reduced the level of LPO. The CYP toxicity in brain, heart, and spleen has been reduced by flavonoids coadministration which can be clearly seen in results of LPO, antioxidant markers, and also in results of histopathology. **Conclusion:** Our study demonstrated that SIL, RUT, and catechin reduce the toxic effect of CYP and these compounds may serve as a safe medicinal supplement during CYP chemotherapy in cancer treatment.

Keywords: Catechin, Cyclophosphamide, Histopathology, Oxidative stress, Rutin, Silymarine

INTRODUCTION

Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths or one in six deaths in 2018. By 2040, those figures will nearly double, where more than two-thirds of the world's cancers will occur. Cancer is the cause of about 30% of all premature deaths from non-communicable diseases among adults aged 30–69.[1]

Cancer is an overgrowth of cells that have developed an ability to grow and divide outside the normal controls (NCs) that usually regulate the growth of cells.[2]

Cancers are caused when a series of mutations accumulate in cells during the replication of genetic material, allowing cells to transform. The mutations of three major types of genes: Oncogenes, tumor suppressor genes, and DNA repair genes, play an essential role in tumorgenesis.[3] The genetic mutations may be caused by external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism.

Most cancers can be treated and some can be cured. Treatment options include surgery,[4] radiation therapy,[5] chemotherapy,[6] hormonal therapy,[7] and other methods.[8]

The anticancer treatment depends on the character of the malignancy, for example, specific type, location, and stage of the malignant cells. Solid tumors are often treated by a combination of surgery, chemotherapy, and radiation, while

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Received: 05-04-2022
Revised: 07-06-2022
Accepted:18-06-2022

chemotherapy alone is used to treat different types of blood cancers.

Cyclophosphamide (CYP), an orally active alkylating agent which is widely used to treat various malignant and non-malignant disorders. Although it has some tumor selectivity, it also possesses a broad spectrum of toxicities.[9] The antitumor activities of alkylating agents have been attributed to their ability to cross-link the twin strands of DNA. The resulting bifunctional lesions, if not repaired, can inhibit DNA replication and transcription, eventually leading to cell cycle arrest, apoptosis, and the inhibition of tumor growth.[10]

CYP is an inactive prodrug that requires enzymatic and chemical activation to release active phosphoramide mustard.[11] Hydroxylation on the oxazaphosphorine ring by the hepatic cytochrome P450 system generates 4hydroxycyclophosphamide, which coexists with its tautomer aldophosphamide. These unstable transport precursors freely diffuse into cells, where aldophosphamide is decomposed into two compounds, phosphoramide mustard, and acrolein.[12] CYPs antineoplastic effects are associated with phosphoramide mustard, while acrolein is linked with its toxic side effects.[13] Phosphoramide mustard and acrolein are two active metabolites of CYP produced by the liver microsomal enzymes [Figure 1].[14]

Toxicity due to drugs used for neoplastic disorders is extensively documented. CYP is a widely used antineoplastic drug, which could cause toxicity to normal cells due to its toxic metabolites.[15] The wide variety of conditions in which CYP is employed puts many patients at risk, and the seriousness of CYP -induced toxicities dictates the need to limit these adverse effects whenever possible.[9] CYP chemotherapy's most common side effects include alopecia, anorexia, weight loss, nausea, vomiting, myelosuppression, and immunosuppression.

Antioxidants have been evaluated for reversal of the side effects of chemotherapy. Accordingly, combining the treatment regimen with potent and safe antioxidants could be the desirable approach to mitigate CYP-induced toxicity.[16,17] Recently, there has been an increase in interest in the search for potential drugs of plant origin capable of minimizing the toxicity induced by chemotherapy to normal cells without compromising its antineoplastic activity. The traditional system of Indian medicine extensively uses the formulations of different medicinal plants to modulate the host's immune system. The herbal formulations were found to be either less toxic or non-toxic. Nowadays, cytotoxic and other anticancerous drugs have been tried in combination with various detoxifying and protective agents to reduce or eliminate their adverse effects.[18]

This triggers interest in the present study; some flavonoids silymarine (SIL), rutin (RUT), and catechin (natural antioxidants) were evaluated and compared with amifostine (AMF) in an experimental animal mice model for cytoprotective effects against CYP -induced cytotoxicity. The experimental endpoints included survival of animals, hematological, and clinical evaluation such as blood count, oxidative stress markers (enzymatic superoxide dismutase [SOD], catalase [CAT], non-enzymatic antioxidant glutathione [GSH], lipid peroxidation [LPO], and histology of vital organs.

MATERIALS AND METHODS

Chemicals

CYP (Cyclocel) was purchased from local market. AMF was obtained as a gift sample from DRDO Gwalior. SIL, RUT, and catechin were obtained as gift samples from UIPS, PU Chandigarh. All other chemicals used in the present study were of highest purity and analytical reagent grade.

Animals

An 8-week-old adult male Balb/c mice (25 ± 05 g) were divided into groups of six each. The animals were kept at room temperature (25 ± 2°C), with 60 ± 10% humidity and an automatically controlled cycle of 12 h light and 12 h dark, standard laboratory animal feed and water were provided *ad libitum*. Animals were acclimatized to the experimental conditions for 1 week before the initiation of the experiment. All the animal experiments were carried out with the prior approval of the Institutional Animal Ethics Committee (1AEC/JU/15 Dated January 12, 2017) and were conducted strictly adhering to the guidelines of committee for the purpose of control and supervision of experiments on animals constituted by the animal welfare division of government of India.

Experimental groupings and treatments

Based on our preliminary review, the doses and duration of treatment for CYP[19] AMF, SIL, RUT, and catechin was all based on earlier reports. CYP was administered, intraperitoneally at a concentration of 25 mg/kg b.w. for 10 consecutive days 1 h after flavonoids administration. AMF, SIL, RUT, and CAT catechin treatments were performed for 14 days. AMF was administered, intraperitoneally and all the flavonoids were given through oral route.

Animals were divided as follows:

- Group-I – NC – Saline
- Group-II – CYP Control (CYP) – 25 mg/kg (i.p.)
- Group-III – Positive Control (CYP + AMF) – 200 mg/kg (i.p.)
- Group-IV – Treatment 1 (CYP + SIL) – 100 mg/kg (oral)
- Group-V – Treatment 2 (CYP + RUT) – 50 mg/kg (oral)
- Group-VI – Treatment 3 (CYP + CAT) – 40 mg/kg (oral).

Hematological analysis

Blood was collected from all the animal's retro orbital sinus on the 14th day and following hematological parameters were assessed in whole blood. (a) Total white blood cell (WBC) count, (b) Total red blood cell (RBC) count, and (c) Hemoglobin content.

Analysis of oxidative stress marker enzymes

Animals were observed for mortality, behavioral changes, and body weight changes. After the experimental period of 14 days (drug administration), animals were sacrificed by cervical dislocation. Brain, heart, and spleen tissues were isolated; the tissues were perfused with 0.9% ice-cold normal saline. Then, 10% tissue homogenates were prepared in 10 mM PBS at pH 7.4. The mixture was centrifuged at 10,000 g for 20 min at 4°C to prepare PMS. After centrifugation, the supernatant was collected and tissue protein content was quantified. Then, malondialdehyde (MDA) levels as a biomarker of LPO reduced glutathione (GSH) levels, SOD, and CAT activities as antioxidant defense system biomarkers were analyzed in the supernatant.

Tissue protein content

In an alkaline solution, a copper-protein complex is formed. This complex reduces the phosphomolybdatephosphotungstatere agent (Folin-Ciocalteus-Phenol Reagent) and forms a dark blue color. The resulting color intensity is directly proportional to the protein concentration in the medium.[20]

LPO assay

The LPO was assayed through thiobarbituric acid-reactive substances (TBARS) by Wills's method (1966).[21] Briefly, 0.5 ml of post-mitochondrial supernatant and 0.5 ml of TrisHCl were incubated at 37°C for 2 h. After incubation, 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000 g for 10 min. To 1 ml of supernatant, 1 ml of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling, 1 ml double distilled water was added and absorbance was measured at 532 nm. TBARSs were quantified using an extinction coefficient of $1.56 \times 105\ M^{1}\ cm^{1}$ and expressed as nmol of MDA per mg protein.

Antioxidant enzymes

GSH

GSH was estimated according to the method described by Ellman (1959).[22] The supernatant (1 ml) was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for 1 h. The sample was centrifuged at 1200 g for 15 min at 4°C. To 1 ml of this supernatant, 2.7 ml of phosphate buffer (0.1 M, pH 8) and 0.2 ml of 5,5-dithiobis (2-nitrobenzoic acid) were added. The yellow color developed was read immediately at 412 nm using the Shimadzu Spectrophotometer. Results were calculated using the molar extinction coefficient of the chromophore ($1.36 \times 104M^{1}\ cm^{1}$) and expressed as a percentage of control.

SOD activity

The SOD activity was estimated by the method described by Kono (1978)[23] by observing the inhibitory rate of Nitroblue tetrazolium (NBT) reduction. One unit is referred to the quantity of enzyme which is responsible for half-maximal inhibition of NBT reduction.

Reagents

(1) Solution A consists of 50 mM Na_2CO_3 (0.52 g/100 ml) in 0.1 mM EDTA (0.003 g/100 ml) (pH = 10.0). (2) Solution B

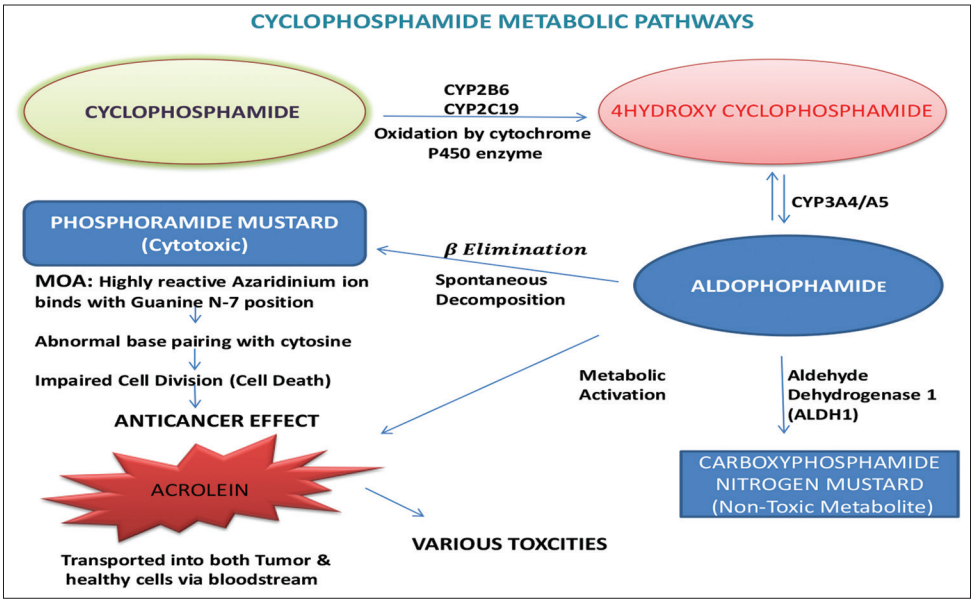

Figure 1: Cyclophosphamide metabolic pathways

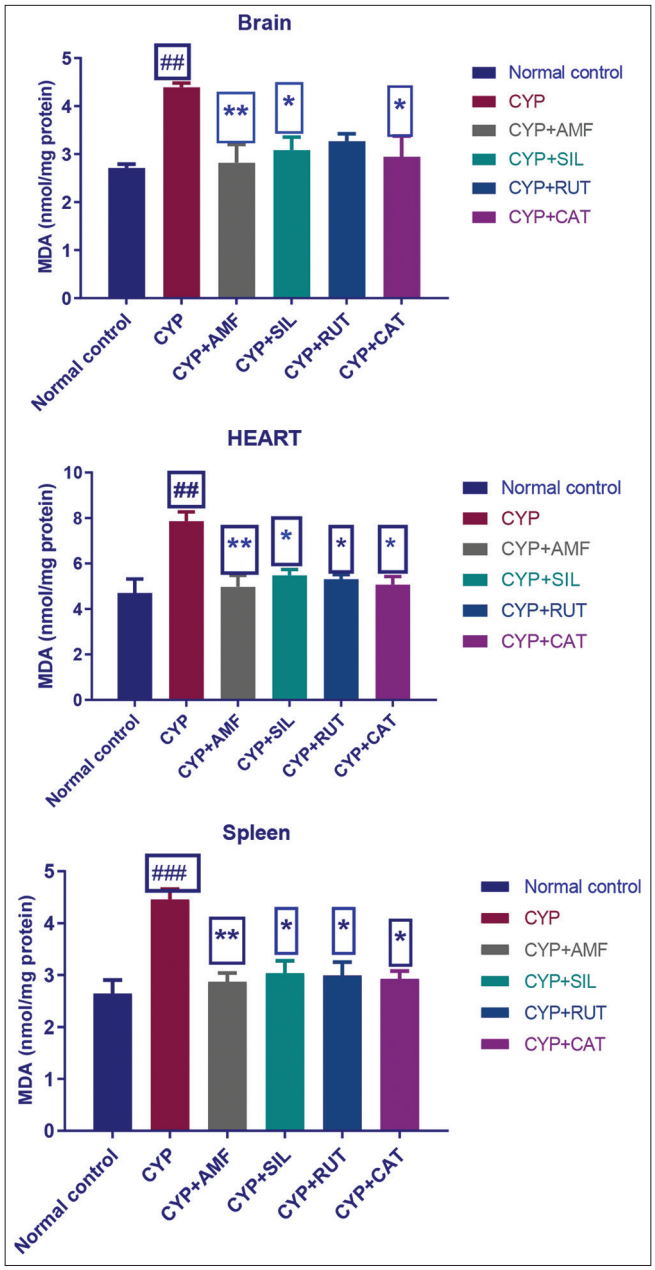

Figure 2: Effect of silymarine, rutin, and catechin on blood malondialdehyde (μmoles/mg) levels. The data are expressed as the mean ± S.E.M. (n = 6). $^{\#\#}P < 0.01$ compared with control group, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with cyclophophamide-treated animals

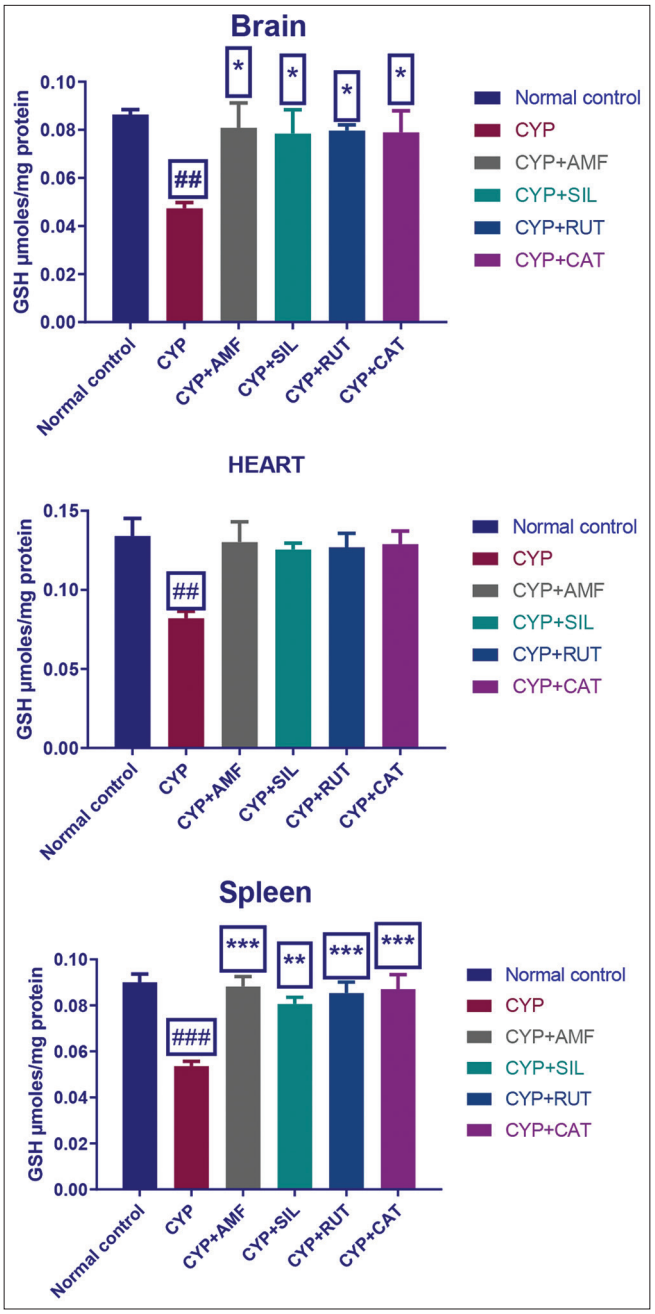

Figure 3: Effect of silymarine, rutin, and catechin on blood glutathione (μmoles/mg) levels. The data are expressed as the mean ± S.E.M. (n = 6). $^{\#\#}P < 0.01$ compared with control group, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with cyclophophamide-treated animals

consists of 96 μM NBT in solution A (0.008 g/100 ml). (3) Solution C consists of 0.6% Triton X-100 (v/v) in solution A. (4) Solution D consists of 20 mM Hydroxylamine HCl, adjust pH to 6 with 2 M NaOH.

Test method

Procedure of SOD estimation: Test solution was prepared by adding 1.3 ml Solution A to 1.0 ml double distilled water, 0.5 ml Solution B and 0.1 ml Solution C, 0.1 ml Solution D, and 0.1 ml Sample. The standard solution was prepared by adding 1.3 ml Solution A to 1.1 ml double-distilled water, 0.5 ml Solution B and 0.1 ml Solution C, and 0.1 ml Solution D. Reference (Blank) solution was prepared by adding 1.3 ml Solution A to 1.1 ml double distilled water, 0.5 ml Solution B and 0.1 ml Solution C, and 0.1 ml Sample. The change in absorbance was estimated at 560 nm at 30-s intervals for 2 min using a spectrophotometer. The reaction was started by adding hydroxylamine to the reaction mixture. The reduction of NBT occurred, which caused an increase in absorbance at 560 nm in aerobic conditions.

Table 1: Hematological profile in different groups of animals

Parameter	Units	NC	CYP	CYP+A	CYP+S	CYP+R	CYP+C
Hemoglobin	gm/dL	11.2	10.8	11.8	11.6	11.2	10.9
Packed Cell Volume (PCV)	%	37.4	33.1	35.3	34.6	36.3	33.5
WBC Count	Cells/mm^3	9700	3100	7200	6200	6400	5900
RBC Count	Millions/mm^3	7.57	6.95	7.34	7.29	7.25	7.14
Platelet Count	K/μL	1030	932	1145	1020	1050	1120

NC: Normal control, CYP: Cyclophosphamide, WBC: White blood cell, RBC: Red blood cell

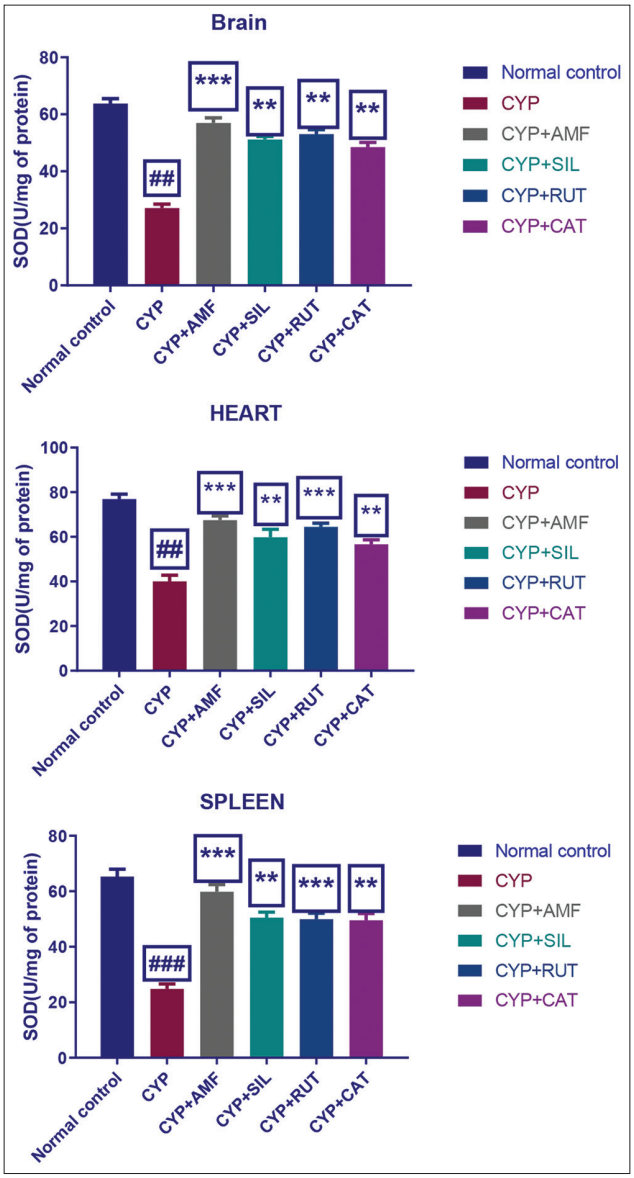

Figure 4: Effect of silymarine, rutin, and catechin on blood superoxide dismutase (μmoles/mg) levels. The data are expressed as the mean ± S.E.M. ($n = 6$). $^{\#\#}P < 0.01$ compared with control group, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with cyclophophamide-treated animals

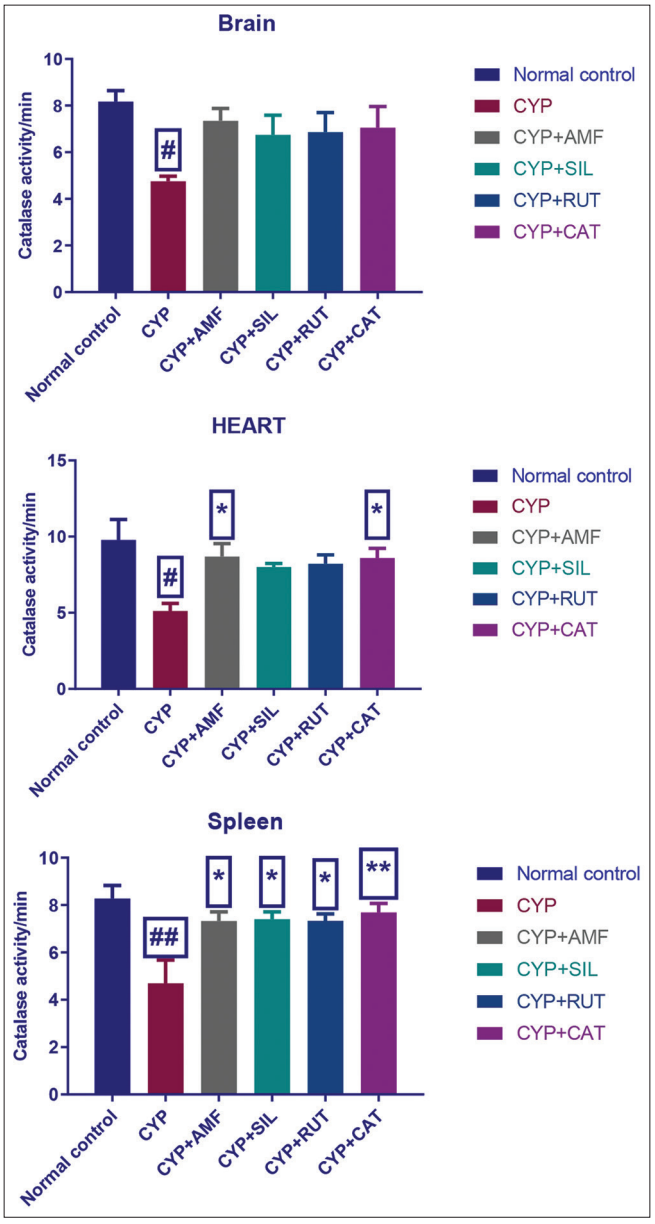

Figure 5: Effect of silymarine, rutin, and catechin on blood catalase (μmoles/mg) levels.The data are expressed as the mean ± S.E.M. ($n = 6$). $^{\#\#}P < 0.01$ compared with control group, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with cyclophophamide-treated animals

Estimation of CAT

CAT activity was assayed by Luck's method (1971),[24] wherein the breakdown of H_2O_2 was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer (1.25 102M H_2O_2) and 0.05 ml of supernatant of the

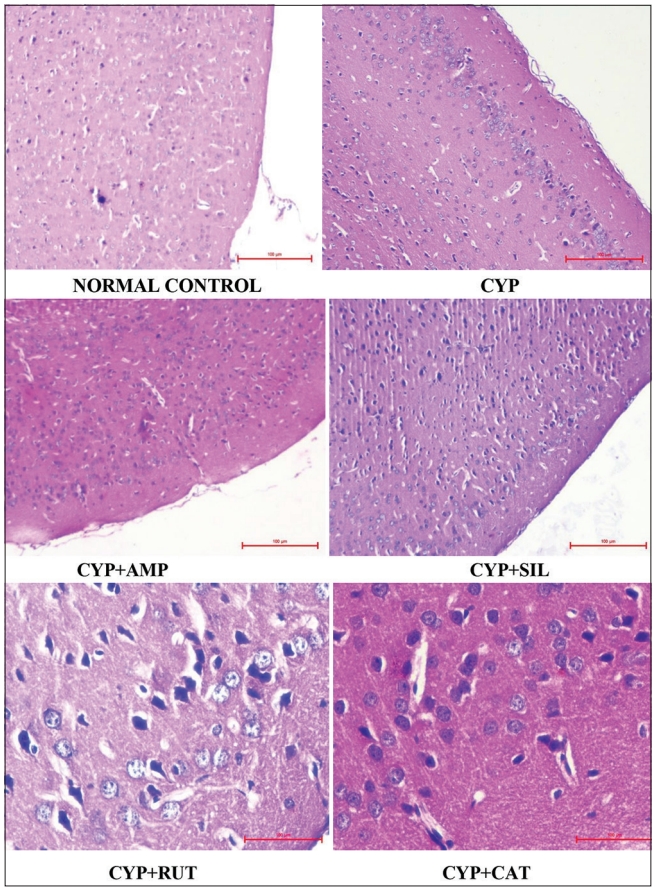

Figure 6: Photomicrographs of effect of silymarine, rutin, and catechin treatments on cyclophosphamide-induced histological alterations on mice brain tissues

brain homogenate (10%), and the change in absorbance was recorded at 240 nm using the Shimadzu Spectrophotometer. Enzyme activity was calculated using the millimolar extinction coefficient of H_2O_2 (0.07). The result was expressed as micromoles of H_2O_2 decomposed/min/mg protein.

Histopathological analysis

Parts of the vital tissue of each animal were fixed in 10% formalin solution, dehydrated in ascending grades of ethanol, and embedded in paraffin. Sections at 4–6 µm were stained with hematoxylin and eosin and examined under a light microscope.

RESULTS

Hematological parameters

CYP administration reduced the hemoglobin, WBC count, RBC count, and platelet count in mice. The animals in all treatment groups showed an increment in the hemoglobin, WBC count, RBC count, and platelet count [Table 1].

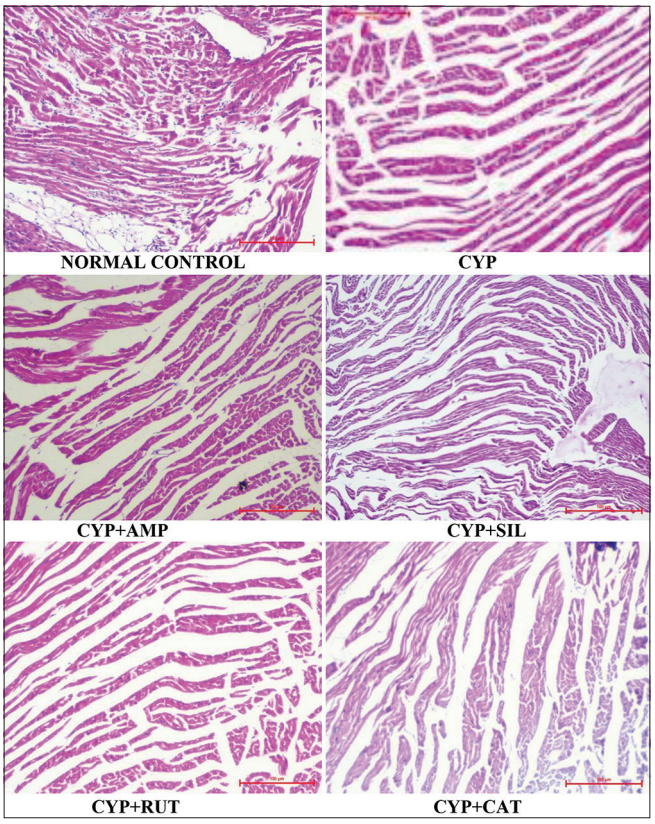

Figure 7: Photomicrographs of effect of silymarine, rutin, and catechin treatments on cyclophosphamide-induced histological alterations on mice heart tissues

Analysis of oxidative stress marker enzymes

The levels of oxidative stress marker enzymes such as GSH, MDA, SOD, and CAT were analyzed in the brain, heart, and spleen tissue of all experimental group animals. The CYP administered group of animals also showed a significant reduction in the antioxidant parameters such as SOD, CAT, and GSH levels in the brain, heart, and spleen tissues and an increase in MDA levels.

Tissue protein content

CYP administration decreased the protein content in most vital organ tissues compared to NC animals. Coadministration of interventional drug molecules has improved the protein content in the brain, heart, and spleen tissues.

LPO assay

CYP increased the hepatic TBARS concentration. LPO level of tissue homogenates of all vital organs, namely, brain, heart, and spleen was significantly reduced by flavonoids such as SIL, RUT, and catechin treatment when compared with control [Figure 2].

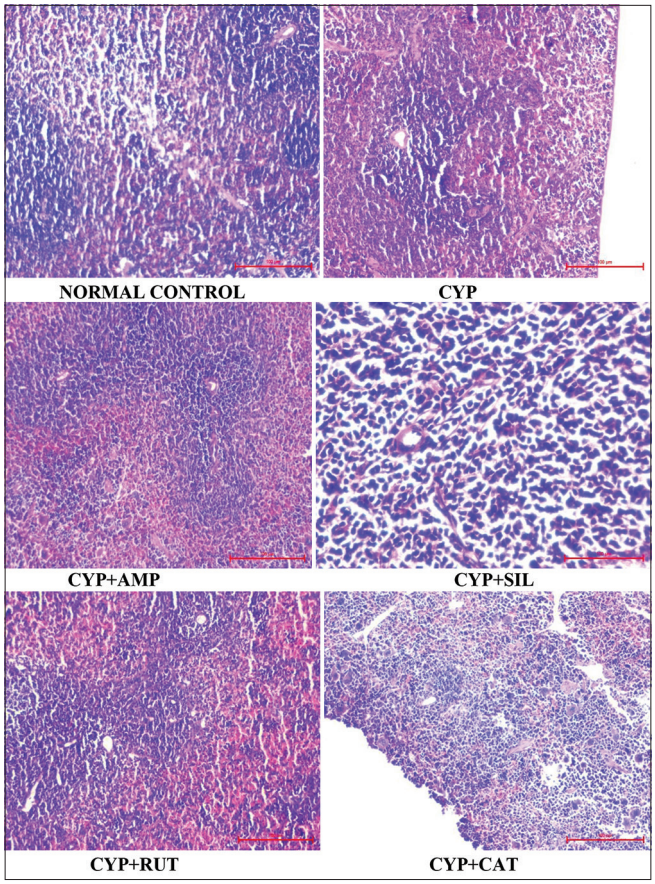

Figure 8: Photomicrographs of effect of silymarine, rutin, and catechin treatments on cyclophosphamide-induced histological alterations on mice spleen tissues

Antioxidant enzymes

Estimation of GSH

The results revealed that administering SIL, RUT, and catechin in treatment groups increased the GSH levels in the brain, heart, and spleen tissues [Figure 3].

SOD activity

The results revealed that administering SIL, RUT, and catechin in treatment groups increased the SOD levels in the brain, heart, and spleen tissues [Figure 4].

Estimation of CAT

The results revealed that administering SIL, RUT, and catechin in treatment groups increased the SOD levels in the brain, heart, and spleen tissues [Figure 5].

Histopathological analysis

The histopathological analysis was carried out to assess the protective effect of SIL, RUT and catechin on CYP-induced toxicity in the brain, heart, and spleen. The histopathological changes in the brain, heart, and spleen tissues were evaluated as described in the materials and methods section. The histopathology results indicate that the control group animals showed normal, well-organized, and closely arranged red and white pulp structure in each organ. On the other hand, the CYP administered group animals showed irregular shape, a significant decrease in the white pulp, and there was congestion in the red pulp region compared to the control group animals in each organ. Moreover, compared with CYP administered group animals, SIL, RUT, and catechin treated group animals. The congestion in the red pulp region decreased and the architecture of the white pulp region was somehow similar to that in the untreated control group. Still, there was a prominent decrease in the frequency of pathological results [Figures 6,7 and 8].

DISCUSSION

CYP is a well-known pro-oxidant molecule that can generate ROS. The excess production of ROS leads to the depletion of cellular detoxifying thiols and antioxidantenzymes.[25] In this study, CYP -treated animals showed a significant reduction in activities of CAT and SOD as well as GSH levels, along with increased levels of MDA in the brain, heart, and spleen. In general, the SOD plays an essential role in removing O_2^- by converting it to $H_2O_2$and rapidly transforming it into water and oxygen by CAT.[26] Here, the decreased levels of SOD and CAT production were observed in CYP-treated animals due to the frequent attack on the brain, heart, and spleen by O_2^-, H_2O_2, and hydroxyl radical-induced by the CYP caused oxidative stress.[19] The GSH, an antioxidant enzyme, is the primary defense against oxidation and acts as the free radical scavenger in cells and tissues. In this study, CYP administration significantly reduced the GSH production in the brain, heart, and spleen tissue, which occurred due to the promoting effect of CYP on decreasing the activity of GSH by oxidative stress and tissue damage.[27] In addition, high levels of MDA were formed due to free radicals in the animals treated with CYP. The MDA is a consistent indicator of oxidative stress and tissue damage. The high level of MDA can alter the structure and function of tissue membrane. Thus, the results suggested that the toxicity of CYP mediated oxidative stress and tissue injury. However, the administration of SIL, RUT, and catechin attenuated the oxidative stress caused by CYP, which was reflected by the increased levels of GSH and high activities of CAT and SOD as well as decreased levels of MDA in animals administered with SIL, RUT, and catechin. A previous study reported that gallic acid (20 mg/kg) protected against the oxidative damage caused by CYP in rats by increasing the levels of GSH and activities of CAT and SOD as well as by decreasing the levels of MDA.[28]

Histological studies were carried out in the brain, heart, and spleen to analyze the impact of CYP on these tissues. The previous reports have also demonstrated that the effect of oxidative stress of CYP induces injury to brain, heart,

and spleen tissues.[29,30] In the present investigation, it was observed that CYP destroyed myocardial tissues in the heart, neurons in the brain, and splenocytes in spleen tissue due to oxidative damage. Moreover, the oxidative damage of CYP administration on the tissues is significantly reduced by the SIL, RUT, and catechin treatments.

CONCLUSION

The therapeutic effects of anticancer agents are associated with adverse side effects due to the toxicity they cause. Even though CYP is very commonly preferred as achemotherapeutic agent, its toxic side effects limit high-dose use. Thus, new effective agents are required to protect the normal tissue from chemotherapy-related toxicity without protecting the tumor and tumor growth stimulation properties. Therefore, the combination of the treatment regimens with antioxidant and cytoprotective properties can be useful in the protection of healthy cells and tissue against CYP-induced oxidative damage. Overall, our study concludes that SIL, RUT, and catechin pre-treatment attenuate CYP-induced oxidative stress in the brain, heart, and spleen and the subsequent damage in mice through scavenging of free radicals. The bioactive antioxidant principles in SIL, RUT, and catechin could be responsible for the cytoprotective effect.

ACKNOWLEDGMENTS

The authors express their gratitude to the Dean of UIPS, Punjab University, Punjab, for permitting us to utilize their laboratory facilities for some part of the present study.

AUTHOR'S CONTRIBUTIONS

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2. Second author – Dr. Manoj Sharma – Associate Professor, SOS in Pharmaceutical Sciences, Jiwaji University, Gwalior.

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Source of Support: Nil. **Conflicts of Interest:** None declared.