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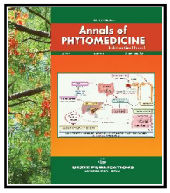

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Antioxidant and immunostimulating effects of fraction of flavonoids from *Cassia absus* L. seed in rats

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Abstract

Looking at the necessity of herbal immunostimulants along with antioxidant compounds for future use in ailments that modulate the immune system, the plant *Cassia absus* L. was explored for possessing such pharmacological properties. The study was designed to evaluate the protective effect of a flavonoid fraction isolated from *C. absus* seed (FFCA) at different doses (50, 100, and 200 mg/kg, per oral daily for 21 days) in a cyclophosphamide-induced immunosuppression model of rats. The FFCA was administered through an oral route daily to rats for 21 days, followed by the administration of cyclophosphamide (CP) on the 9th and 16th days of the study. Blood biochemical parameters, oxidative stress markers, immunity-related parameters, and histopathology of the liver and kidney were studied. The cyclophosphamide (CP) at a dose of 100 mg/kg b. wt., on the 9th and 16th days of the study, produced a negative effect on the weight of the body, feed intake, hematological profile, immune system, and oxidative stress markers in the liver and kidneys. The normal histological architecture of the liver and kidneys was also altered by CP. Pre-treatment of FFCA prevented the alterations in hematology, oxidative stress in the liver and kidneys, and immunity in rats administered with CP. Rats pre-treated with FFCA at a higher dose had fewer histological changes and less lipid peroxidation in the liver and kidneys than CP-treated rats. The chromatographic analysis revealed the presence of active phytochemicals in *C. absus* seed, including flavonoids, which may be responsible for antioxidant and immunomodulatory effects. The pharmacological potential of each of the FFCA's active ingredients has to be investigated further to find new bioactive molecules.

1. Introduction

Immunological disorders, including autoimmune diseases, allergies, rheumatic diseases, and malignancies, among others, are brought on by the immune system's interaction with bacteria and phagocytes. There are several treatment methods in which the immune system of the human or animal body has been weakened, leading to illnesses that could be fatal. Immunomodulatory medications are utilized as a therapeutic agent or a form of supportive therapy in such situations. An immune modulator alters the immunological reaction to outside substances or treatments, either directly or indirectly, and halts or delays the progression of degenerative diseases (Fagnoni *et al.,* 2000). Therapeutic drugs can occasionally lower a patient's immunity and increase their susceptibility to fatal diseases. Levamisole, L-fucose, and glucans are examples of immunomodulators that have negative side effects (Ghatak and Panchal, 2012). Significant disadvantages of synthetic immunostimulants include a larger potential for negative outcomes and long-term consequences. Patients with cancer who receive chemotherapy drugs like cyclophosphamide experience immune system impairment. The alkylating drug cyclophosphamide, which is a member of the oxazaphosphorine family, has been utilized in clinical trials for many years. In addition to its antimitotic effects, cyclophosphamide has a wealth of knowledge regarding its therapeutic usefulness in the treatment of cancer and as an immunosuppressive drug (Ahlmann and Hempel, 2016). Cyclophosphamide can weaken the immune system; however, its use in treating cancer patients cannot be disregarded. To preserve the immunological status of patients, it may be possible to combine immunostimulant medications with immunosuppressive therapeutics. Numerous factors might affect the immune system's growth, upkeep, and best performance (Sierra *et al.,* 2005). As a result, suppressing or enhancing an organism's immune response to an invasive antigen and lowering illness have long been of interest. Due to their lower risk of side effects, research on herbal immunostimulant drugs is also becoming more and more popular. The immunomodulatory properties and antioxidant activity of numerous phytochemicals found in medicinal plants (Mohideen, 2021; Bagwan *et al.,* 2022; Pawaskar and Ranade, 2022; Tamoli *et al.,* 2022; Verma *et al.,* 2022). Polysaccharides, alkaloids, and flavonoids have all demonstrated immunomodulatory properties (Kumar *et al.,* 2012) which modulate the immune system in different ways, primarily cell-mediated

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immunity, with humoral immunity being indirectly impacted (Goldsby *et al.,* 2000). Finding better medicinal plants available in nature and evaluating their immunomodulatory potential along with different types of pharmacological activities including antioxidant effect is gaining the attention of researchers (Bhatt *et al*., 2019abc; Modi *et al.,* 2019).

In Gujarati, Hindi, and Sanskrit, *Cassia absus* L. (family: Caesalpiniaceae) is also known as Chimed, Chakshu, and Chakusya. It is a lesser-known herb that is used in the winter season. It is asserted that *C. absus* seed powder enhances health and helps in the prevention of several diseases. The quaternary water-soluble alkaloids chaksine and isochaksine are abundant in the plant's seed (Ahmad *et al.,* 2018). In the seed of *C. absus*, alkaloids, flavonoids, terpenoids, glycosides, and tannins are also found (Hussain *et al.,* 2016). Upon HPTLC analysis of an alcoholic, hydroalcoholic, and n-butanol fraction of *C. absus* seed powder, several flavonoids were discovered (Pandya *et al.,* 2021). The pharmacological effects of *C. absus* seeds include antihypertensive, antifertility, antifungal, anti-inflammatory, antihyperglycemic, and antibacterial action (Ahmad *et al.,* 2018). Ethyl acetate and water extracts of *C. absus* have been shown to counteract oxidative stress-related alterations in rats with diabetes (Rashid *et al.,* 2017). Dose-dependent reduction of rats' heart rate and systemic blood pressure have been found after administration of a crude extract of *C. absus* (Aftab *et al.,* 1996). Compared to alpha-tocopherol, the *C. absus* seed extract's *in vitro* antioxidant effect was 50% higher (Jayaraman *et al.,* 2014). We have a hypothesis that the *C. absus* seed may have potential *in vivo* antioxidant and immunomodulatory effect which is still not explored. Thus, it is necessary to explore the active constituents of *C. absus* seed, particularly the flavonoids which generally may show antioxidant and immunomodulatory effects. To our knowledge, this is the first report that examines the antioxidant and immunostimulant capabilities of *C. absus* seed through an *in vivo* study. The outcome of the study would be useful for further research in the area of immunology and oxidative stress which can be ameliorated using such phytochemicals.

2. Materials and Methods

2.1 Ethical statement

The Institutional Animal Ethics Committee of the College approved the protocol of the study.

2.2 Collection and authentication of the plant material

C. absus seeds were obtained from the Ayurvedic shop in Junagadh, Gujarat, India (Figure 1). Dr. M. M. Jani (Botanist, Bahauddin Science College, Junagadh) validated the plant material. A specimen of the plant material was deposited in the Herbarium of the Department (JVC/VPT/SP/PS/01/18).

Figure 1: Seeds of *Cassia absus* L. used in the study.

2.3 Preparation of the extract

A 25 g of *C. absus* powder was extracted with 250 ml of 70% methanol for three days at room temperature before being filtered and dried. To produce an adequate amount of extract, this process was carried out in various batches. The extract obtained above underwent fractionation by being dissolved in distilled water at a 1:15 (extract to solvent, w/v) ratio. The solution was sonicated and stirred ferociously to create a homogenous mixture. This liquid mass was segregated in a reparatory glass funnel by making the solvents more polar. The extract was shaken with n-hexane, chloroform, ethyl acetate, and n-butanol at least three times (30-50 ml of each solvent). The upper layer of the n-butanol layer was collected, dried in a vacuum, and then used to create the flavonoid-rich fraction of *C. absus* (FFCA) (Pandya *et al.,* 2021).

2.4 HPTLC analysis of flavonoid fraction of *C. absus* seed

Qualitative evaluation of the presence of various flavonoids in an isolated fraction of *C. absus* was done using Linomat 5 HPTLC applicator. The solvent systems of n-butanol: glacial acetic acid: water (4:1:5) were used to elute the sample on a thin layer chromatography plate (Ali *et al.,* 2011). Derivatization was done using 1% 2-aminoethyl diphenylborinate in methanol followed by 5% polyethylene glycol-4000 in methanol. The plates were then visualized at 366 nm.

2.5 LC-QToF-MS/MS analysis of a hydroalcoholic extract of *C. absus* seed

Hydroalcoholic extract of *C. absus* seed was used to explore the presence of various phytochemicals. MS/MS analysis of a hydroalcoholic extract of *C. absus* seed was carried out using Ultra-High-Definition Accurate-Mass QTOF-MS coupled to the LC (Agilent Technologies, USA, Model 6540). A C18 column (4.6 × 100 mm, 3.5 ìm) column was used for separation. The mobile phase was pumped at the rate of 0.6 ml/min [Solvent A (0.1% formic acid/ water) and solvent B (acetonitrile), programmed as follows: 0 min, linear change from A–B (95:5 v/v) to A–B (5:95 v/v); 12 min, isocratic A–B (5:95 v/v); 20 min, linear change to A–B (95:5 v/v) 22 min and 25 min, linear change to A–B (95:5 v/v)].

2.6 Animals and experimental design

Amongst, various drugs or chemical-induced immunosuppression in rat models is used to explore the immunostimulant effects of drugs or herbs. The cyclophosphamide is a commonly used anticancer drug that produces immunosuppression and its use may serve an additional advantage that the studied compounds may be useful against side effects produced by it. At the age of 8 to 9 weeks, 36 male Wistar rats were procured from a registered breeder. The animals were kept in a room that was 23.0 ± 3.0ºC at 50-60% relative humidity. Throughout the trial, the 12 h light-dark cycle was maintained. Rats were divided into six distinct groups at random, each with six rats after a week of acclimation. Animals in the control group (C) received standard saline treatment. On days 9 and 16, cyclophosphamide (CP) was given intraperitoneally (i.p.) to animals in group 2 (CP) at a dose of 100 mg/kg body weight. The dose of cyclophosphamide was selected based on a previous report (Zanchi *et al.,* 2014). Levamisole (2.5 mg/kg) was given orally to animals in group 3 (LM + CP) every two days for 21 days, plus CP on the ninth and sixteenth

days. Animals in group 4 (FFCA50 + CP) received a flavonoid-rich fraction of *C. absus* (FFCA) orally twice a day for 21 days (50 mg/kg b. wt.) plus CP on the ninth and sixteenth days. Animals in group 5 (FFCA100 + CP) received FFCA 100 mg/kg b. wt. orally twice a day for 21 days in addition to CP on the 9th and 16th day. The last group's animals (FFCA200 + CP) received oral FFCA at 200 mg/kg b. wt. for 21 days, plus CP on the 9th and 16th day. The body weights of each rat were noted separately at the end of each week. The food consumed by the rats was also recorded. A serum was taken to determine the HA titer, and blood was drawn at the end of the study to evaluate hematological parameters. To assess T and B lymphocytes, spleen tissue was used in the MTT assay. After injecting sheep red blood cells (SRBCs), footpad thickness was measured to look into the delayed-type hypersensitivity reaction.

2.7 Hematological parameters

Using an automatic hematology analyser, hematological parameters were examined (Abacus Junior Vet 7, Diatron, Hungary).

2.8 Oxidative stress markers in the liver and kidney

Samples of both organs (liver and kidney) have been used to assess the oxidative stress parameters. Superoxide dismutase (SOD) activity, catalase activity (CAT), reduced glutathione (GSH) and MDA concentration was determined using a published methodology (Marklund and Marklund, 1974; Aebi *et al.*, 1984; Ellman, 1959; Lykkesfeldt, 2001).

2.9 Humoral immune response (Haemagglutination assay)

Haemagglutination assay was carried out according to the protocol described by Puri *et al.* (1994). An aliquot (25 µl) of 2-fold diluted sera in phosphate buffer saline (0.15 M, pH 7.4) was challenged with 25 µl of 1% v/v SRBC'S suspension in V-bottom microtiter plates. After 1 h of incubation, the plates were visually examined for haemagglutination. The antibody titer was calculated using the greatest dilution of the test serum that caused haemagglutination.

2.10 Cell-mediated immune response

Delayed-type hypersensitivity assay was carried out to explore the effect of different treatments on cell-mediated immunity (Doherty, 1981).

2.11 MTT assay

The removed spleens were handled in an aseptic manner. To create a homogenous splenocyte suspension by mincing, the rat spleens were removed, washed in phosphate-buffered saline (PBS, pH 7.4), and then processed as per published methods (Zheng *et al.*, 2015).

2.12 Histopathological evaluation of organs

Both organs (liver and kidney) were placed into 10% buffered formalin after being isolated from four animals of each group. Standard procedure was followed for processing tissue and making blocks as well as section cutting. Hematoxylin and eosin (H & E) stains were applied to sections of each tissue that were cut at a thickness of 5 microns. Pathological lesions were noted after microscopically examining the stained slides.

2.13 Statistical analysis

GraphPad Prism 9.0 was used for statistical analysis. Data were screened for normal distribution or equal variances, and parametric (Tukey's HSD test) or non-parametric test (Dunn's test) were applied. The data of change in paw thickness were compared with control using the Mann-Whitney test.

3. Results

3.1 HPTLC and LC-QTOF-MS analysis

A flavonoid-rich fraction of the *C. absus* seed utilized in the experiment, which was analyzed using HPTLC, exhibited the presence of six distinct flavonoids (Figure 2). A hydroalcoholic extract of *C. absus*, however, revealed 58 components, including flavone-type flavonoids, after being subjected to LC-QTOF-MS analysis. Twelve substances have been found among the fifty-eight, and they may be the reason for their potent antioxidant activity and other pharmacological qualities (Table 1, Figure 3).

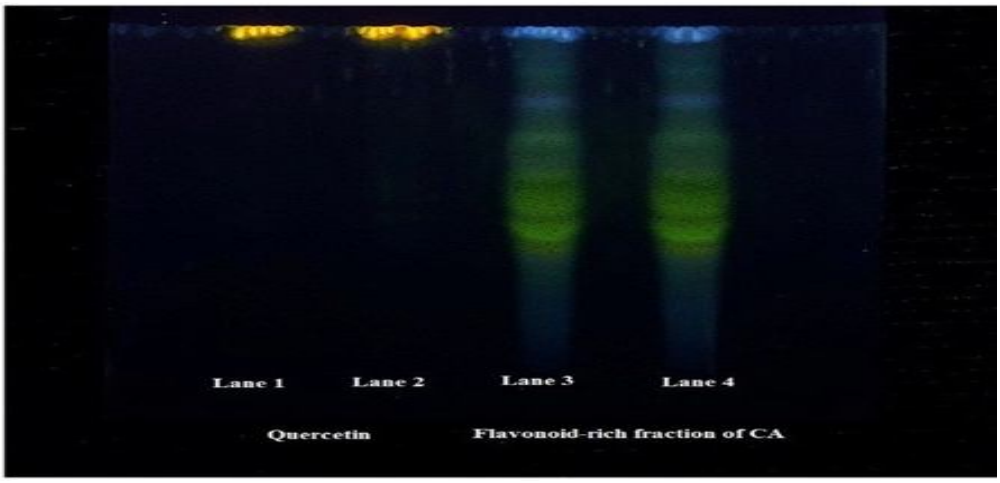

Figure 2: Detection of flavonoids in a flavonoid-rich fraction of *C. absus* seed (FFCA). Lanes 1 and 2 show standard flavonoid (quercetin) and lanes 3 and 4 show different flavonoids in the fraction of *C. absus* seed used in the experiment.

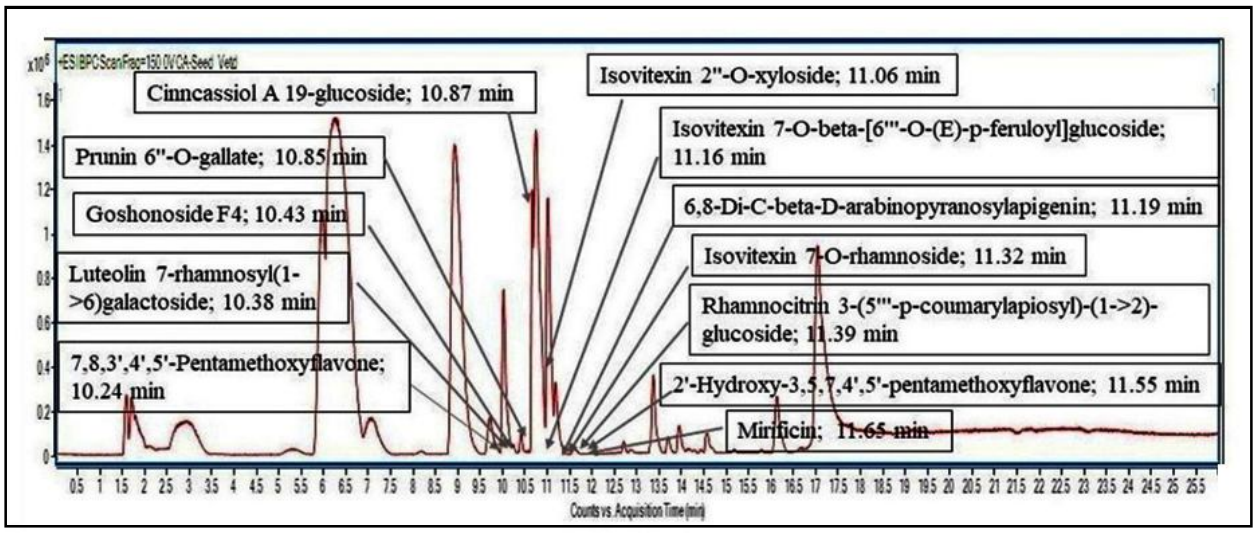

Figure 3: Chromatogram (LC-QToF-MS) of hydroalcoholic extract of *C. absus*.

Table 1: Major antioxidant compounds identified in the extract of *C. absus* seed

S.No.	Name	Formula	M. Wt	t_R (Min.)	Height	Peak area	CAS ID	Metlin ID
1	7,8,3',4',5'-pentamethoxyflavone	$C_{20}H_{20}O_7$	372.12	10.24	13459	65354	133342-97-9	48514
2	Luteolin 7-rhamnosyl(1->6)galactoside	$C_{27}H_{30}O_{15}$	594.16	10.38	46931	256078	160698-21-5	49141
3	Goshonoside F4	$C_{32}H_{54}O_{12}$	630.36	10.43	48005	520378	90851-27-7	93085
4	Prunin 6''-O-gallate	$C_{28}H_{26}O_{14}$	586.13	10.85	20321	154728	54835-98-2	52732
5	Cinncassiol A 19-glucoside	$C_{26}H_{40}O_{12}$	544.25	10.87	5481	24081	73599-12-9	90493
6	Isovitexin 2''-O-xyloside	$C_{26}H_{28}O_{14}$	564.15	11.06	13306	49866	53609-37-3	48672
7	Isovitexin 7-O-beta-[6'''-O-(E)-p-feruloyl]glucoside	$C_{37}H_{38}O_{18}$	770.21	11.16	17738	111423	212271-12-0	48759
8	6,8-Di-C-beta-D-arabinopyranosylapigenin	$C_{25}H_{26}O_{13}$	534.14	11.19	59175	416257	107911-03-5	48670
9	Isovitexin 7-O-rhamnoside	$C_{27}H_{30}O_{14}$	578.16	11.32	52211	296039	NA	48722
10	Rhamnocitrin 3-(5'''-p-coumarylapiosyl)-(1->2)-glucoside	$C_{36}H_{36}O_{17}$	740.19	11.39	11597	54379	147899-34-1	50984
11	2'-Hydroxy-3,5,7,4',5'-pentamethoxyflavone	$C_{20}H_{20}O_8$	388.12	11.55	30130	138717	NA	50945
12	Mirificin	$C_{26}H_{28}O_{13}$	548.15	11.65	59659	367363	103654-50-8	47531

3.2 Symptoms during the treatment period

All of the animals in the various treatment groups underwent daily assessments for physical and behavioral changes throughout the experiment. On the ninth and sixteenth days after receiving 100 mg/kg b.w., i.p. of cyclophosphamide (CP), the rats in the toxicity group had symptoms of weakness, decreased mobility, and hair loss. Rats administered a flavonoid-rich fraction of *C. absus* at doses of 50, 100, and 200 mg/kg body weight displayed significantly less hair loss. It was reported to be more active than the toxicology group.

3.3 Effects of FFCA on body weight gain and feed consumption

Table 2 displays the bodyweight growth in rats across several treatment groups. Body weight growth in the CP- and LM-treated rats was negative (substantially reduced) during the second week compared to the control group. However, all three FFCA treatments considerably increased the rats' body weight gain. The second dose of CP caused a significant decrease in body weight in all groups as compared to the control group. Rats given FFCA at a dose rate of 100 mg/kg demonstrated a modest improvement over the toxicity group. Additionally, throughout the second and third weeks of the trial, feed consumption in the rats treated with CP and LM was significantly lower than in the control rats (Table 2). Rats given FFCA at any dose used in the study showed an increase in feed consumption, while rats given FFCA at a dose rate of 100 mg/kg showed a considerable improvement.

Table 2: Effect of different treatments on body weight gain and feed consumption

Period	Treatment groups					
	C	CP	LM + CP	FFCA50 + CP	FFCA100 + CP	FFCA200 + CP
	Body weight gain (g)					
Week 1	33.25 ± 5.97	21.83 ± 1.50	29.93 ± 3.48	36.63 ± 6.41	34.97 ± 22.26	37.90 ± 6.14
Week 2	22.70 ± 3.12	-7.92 ± 5.85*	-9.40 ± 5.97*	13.07 ± 5.19#	22.80 ± 17.32#	13.27 ± 5.89#
Week 3	4.12 ± 3.09	-11.65 ± 4.42*	-5.22 ± 6.45	-10.33 ± 4.89*	-2.77 ± 6.95#	-8.97 ± 5.48*
	Feed consumption (g/day/rat)					
Week 1	23.58 ± 0.50	21.70 ± 0.66	22.27 ± 0.58	20.38 ± 1.37	25.67 ± 1.65	24.82 ± 1.29
Week 2	20.44 ± 0.73	17.31 ± 1.53*	17.68 ± 2.16*	21.17 ± 0.53	21.45 ± 0.60	18.21 ± 1.12*
Week 3	19.20 ± 0.92	12.10 ± 1.64*	10.82 ± 1.63*	16.25 ± 1.23	18.15 ± 1.09#	16.29 ± 1.30

Indicates a significant difference from the normal control group, while # indicates a significant difference from the toxicity group (#: $p< 0.05$).

3.4 Effects of FFCA on hematology

In CP-treated rats, there was a non-significant decrease in lymphocytes (%) but a significant decrease in Hb, PCV, RBC count, WBC, and platelets (Table 3). The hematological profile was improved after levamisole treatment, indicating the drug's preventive effect against CP. WBC and lymphocyte count both significantly improved after treatment with FFCA. When compared to rats treated with CP, the FFCA therapy did not affect the levels of Hb, PCV, or RBC. The platelet count was significantly higher in the rats treated with CP and FFCA at doses of 50 and 100 mg. The treatment with FFCA improved WBC, lymphocyte (%), and platelet counts.

Table 3: Effect of different treatments on hematological parameters in rats

Parameters	Treatment groups					
	C	C P	LM + CP	FFCA50 + CP	FFCA100 + CP	FFCA200 + CP
Hb (g/dl)	14.70 ± 0.09	11.40 ± 0.14*	12.97 ± 0.47	11.67 ± 1.12	12.50 ± 0.30	11.00 ± 1.16*
PCV (%)	48.05 ± 0.58	36.65 ± 0.46*	40.17 ± 1.59	37.25 ± 3.77	41.10 ± 0.89	35.73 ± 4.11*
RBCs (10^6/cmm)	8.27 ± 0.11	6.83 ± 0.06	7.53 ± 0.44	6.39 ± 0.59	6.76 ± 0.10**	6.00 ± 0.65*
WBCs (10^3/cmm)	11.78 ± 0.78	1.21 ± 0.15*	4.93 ± 1.09*#	8.47 ± 1.79*#	7.65 ± 0.93#	7.87 ± 1.42*#
Lymphocytes (%)	75.83 ± 1.03	33.67 ± 3.87*	47.90 ± 5.81*	53.35 ± 5.46*#	49.08 ± 4.03*	44.32 ± 5.28*
Platelets (x10^9/l)	688.67 ± 33.15	131.50 ± 7.83*	330.17 ± 12.39*#	643.50 ± 73.95#	603.33 ± 72.03#	557.50 ± 43.29

Indicates a significant difference from the normal control group, while # indicates a significant difference from the toxicity group (#: $p< 0.05$).

3.5 Effects of FFCA on oxidative stress parameters

Oxidative stress markers in the liver and kidney of rats of various groups are depicted in Table 4. When compared to control rats, the SOD activity in the liver of CP-treated rats was significantly reduced. Other treatment groups have slightly increased SOD activity levels (Non-significantly). When compared to normal control rats, the catalase activity in the liver of rats in the toxicity group and other treatment groups was reduced non-significantly. The amount of GSH in the liver of CP-treated rats did not differ significantly from that of normal control rats. GSH levels in the liver of rats treated with levamisole were significantly greater. When compared to the control and toxicity groups, the levels of GSH in the liver of rats treated with FFCA at all dosages were significantly higher. In rats of all treatment groups, there was no significant difference in MDA levels in the liver. In the kidneys of rats treated with CP, SOD activity was considerably lower than in normal control rats. SOD activity in the kidney of rats given FFCA at all doses, on the other hand, was considerably higher than in the toxicity group. The activity of catalase in the liver of rats from all toxicity groups was not significantly different. When compared to normal control rats, the amount of GSH in CP-treated rats was insignificantly lower. When compared to the toxicity and LM-treated groups, the level of GSH in the kidneys of rats treated with FFCA at a 200 mg/kg dosage was considerably greater. On the other hand, the MDA level in the kidneys of CP-treated rats did not differ significantly from that of control rats. Surprisingly, animals given FFCA at a dose of 200 mg/kg exhibited much lower levels of MDA than those in the toxicity group.

3.6 Effects of FFCA on immune parameters

Figure 4 depicts the effects of therapy on key immunity-related parameters in rats. By detecting antibody titer against SRBCs using a haemagglutination assay, the humoral immune response was seen. The antibody titer against SRBCs in rats treated with CP was significantly lower as compared to the healthy control group. In comparison to the toxicity group, rats receiving FFCA therapy at 200 mg/kg had a significantly higher antibody titer against SRBCs. In comparison to the untreated control rats, the T and B lymphocytes' capacity to multiply was somewhat diminished in the CP-treated rats. In all treatment groups, there was no noticeable difference in

the rate of B cell proliferation. However, T cell proliferation was significantly affected by the highest dose of FFCA. The paw thickness of rats given CP decreased non-significantly at 24 and 48 h after being challenged with SRBCs. On the other hand, the paw thickness of the rats given levamisole plus FFCA at 100 mg/kg was greater than that of the toxicity group.

Table 4: Oxidative stress markers in the liver and kidneys of rats of different groups

Parameters	Treatment groups					
	C	CP	LM + CP	FFCA50 + CP	FFCA100 + CP	FFCA200 + CP
	Liver					
SOD activity (U/mg tissue)	65.97 ± 2.26	34.03 ± 3.64*	41.67 ± 1.08	48.61± 4.39	53.47 ± 6.84	49.31 ± 4.62
CAT activity (U/mg protein)	57.42 ± 5.99	49.08 ± 4.26	30.65 ± 8.04	24.89 ± 6.90	24.31 ± 7.24	27.47 ± 5.19
GSH level (µg/mg tissue)	13.01 ± 1.07	8.96 ± 0.40	68.96 ± 2.12*#	22.24 ± 5.32	18.13 ± 3.88	20.57 ± 5.26
MDA level (µM/mg tissue)	5.01 ± 0.34	6.14 ± 0.81	13.53 ± 5.78	5.98 ± 1.60	6.55 ± 1.08	9.90 ± 0.99
	Kidney					
SOD activity (U/mg tissue)	65.97 ± 3.94	41.67 ± 3.57*	45.83 ± 1.52*	80.56 ± 1.76#	79.58 ± 3.84#	83.33 ± 4.44#
CAT activity (U/mg protein)	16.71 ± 9.48	7.33 ± 0.88	13.25 ± 2.57	14.36 ± 2.47	14.64 ± 1.46	14.22 ± 3.14
GSH level (µg/mg tissue)	13.31 ± 0.89	9.02 ± 0.67	8.19 ± 1.11	16.40 ± 2.91#	12.71 ± 0.49	17.89 ± 1.45#
MDA level (µM/mg tissue)	12.28 ± 0.71	15.02 ± 1.00	14.66 ± 1.78	14.34 ± 1.32	14.70 ± 0.90	7.43 ± 1.50#

Indicates a significant difference from the control group, while # indicates a significant difference from the toxicity group (#: $p < 0.05$).

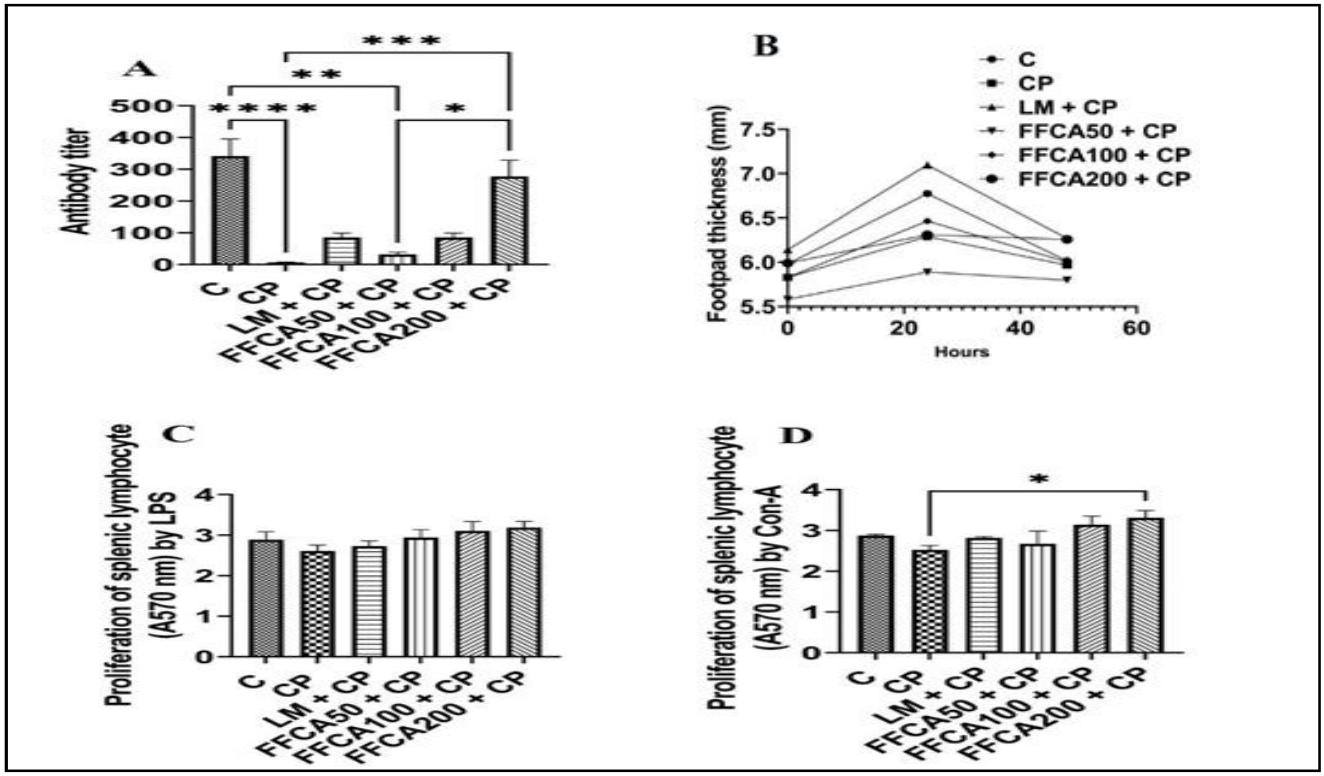

Figure 4: Effect of different treatments on immunity of rats. C: Control, CP: Cyclophosphamide, LM: Levamisole, FFCA: Flavonoid-rich fraction of *C. absus*. *Indicates significant differences between the groups (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$, ****: $p < .0.001$).

3.7 Effect of FFCA on histological changes in liver and kidney

Microscopic views of the liver of rats of different treatment groups are shown in Figure 5. Control rats showed intact architecture with normal hepatocytes but with mild fatty changes; CP-treated rats exhibited vacuolar degeneration and mild sinusoidal congestion; rats treated with levamisole + CP showed mild fatty changes with congestion; rats treated with FFCA at 50 mg/kg + CP exhibited mild to moderate degenerative changes as evident by swollen hepatocytes with cloudy cytoplasm; rats treated with FFCA at 100 mg/kg + CP showed mild degenerative changes with swollen hepatocytes; rats treated with FFCA at 200 mg/kg + CP exhibited normal architecture with radially arranged hepatic cords, but with foci of mild degenerative changes. Microscopic views of the kidneys of rats of different treatment groups are shown in Figure 6. The Control group showed the normal structure of the renal cortex; CP-treated rats exhibited moderate glomerular atrophy and the presence of proteinaceous casts in some of the tubules; levamisole + CP-treated rats showed normal architecture of glomerulus and mild congestion in tubules; rats treated with FFCA at 50 mg/kg + CP (FFCA50 + CP) exhibited mild congestion with moderate glomerular atrophy along with proteinaceous casts in few tubules; rats treated with FFCA at 100 mg/kg + CP showed mild glomerular atrophy with near to normal architecture of tubules with mild congestion; rats treated with FFCA at 200 mg/kg + CP exhibited normal architecture of glomerulus (G) but with mild congestion in few tubules.

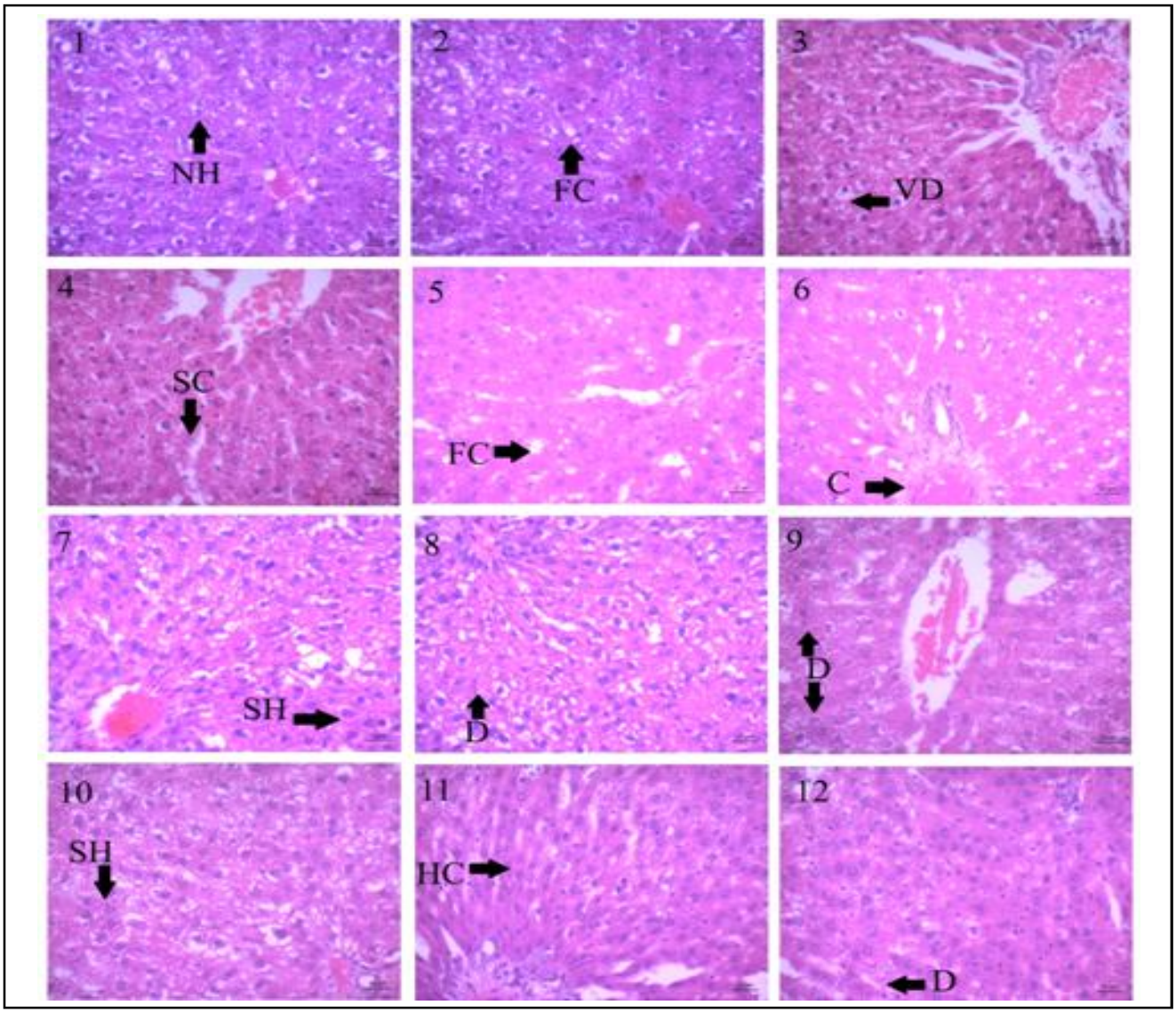

Figure 5: Microscopic view of liver of rat of different treatment groups (400X, H & E). 1 & 2): Control group showed normal hepatocytes (NH) with mild fatty changes (FC); 3 & 4): cyclophosphamide-treated group exhibited vacuolar degeneration (VD) and mild sinusoidal congestion (SC); 5 & 6): levamisole + cyclophosphamide-treated group showed mild fatty changes (FC) with congestion (C); 7 & 8): FFCA at 50 mg/kg + cyclophosphamide-treated group exhibited swollen hepatocytes (SH) with cloudy cytoplasm and mild to moderate degenerative changes (D); 9 & 10): FFCA at 100 mg/kg + cyclophosphamide-treated group showed mild degenerative changes (D) with swollen hepatocytes (SH); 11 & 12): FFCA at 200 mg/kg + cyclophosphamide-treated group exhibited normal architecture with radially arranged hepatic cords (HC) and foci of mild degenerative changes (D).

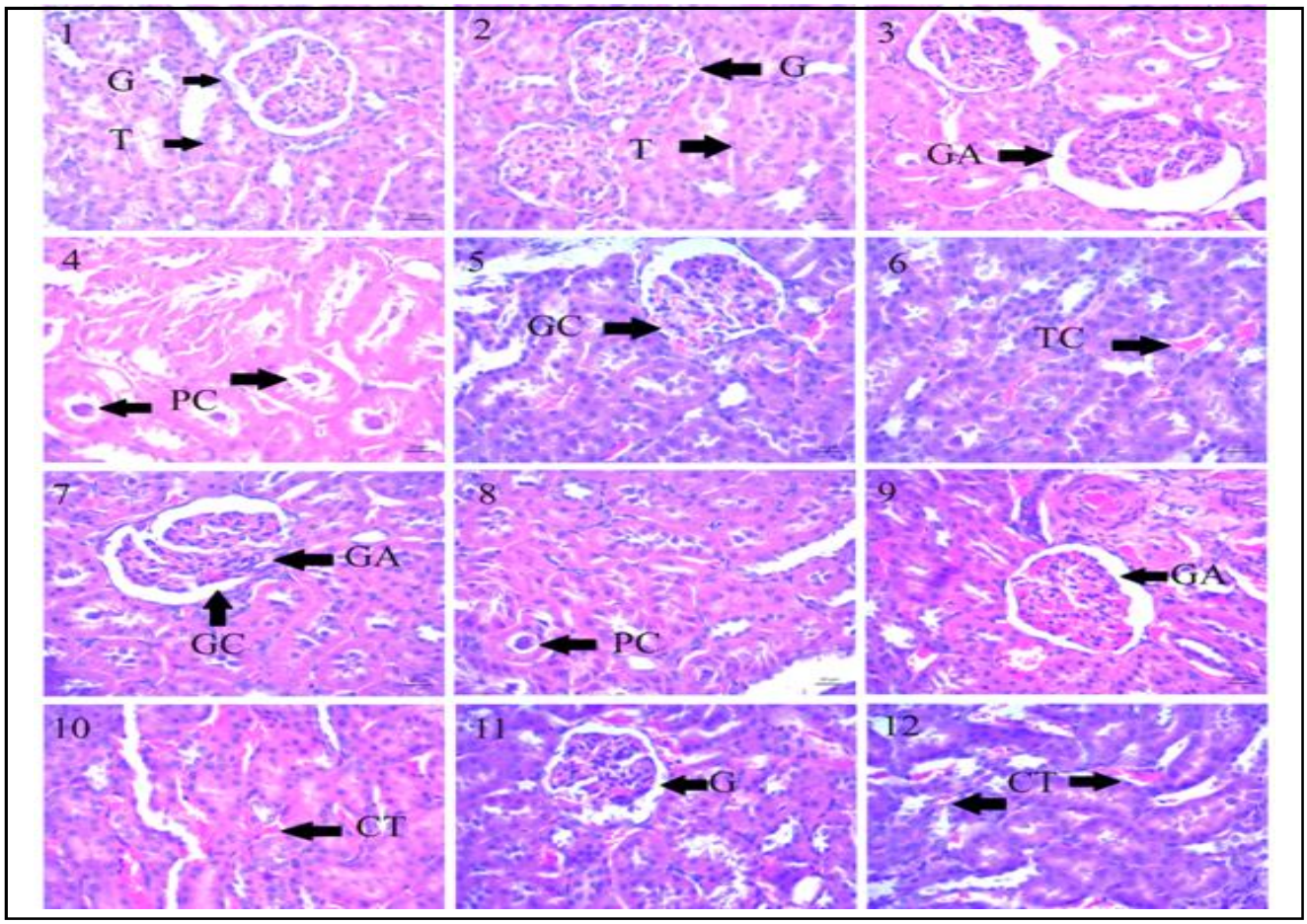

Figure 6: Microscopic view of kidney of rat of different treatment groups (400X, H & E). 1 & 2): Control group showedthe normal structure of renal cortex comprised of glomeruli (G), proximal and distal convoluted tubules (T); 3 & 4): cyclophosphamide-treated group exhibited moderate glomerular atrophy of (GA) and the presence of proteinaceous casts (PC) in some of the tubules; 5 & 6): levamisole + cyclophosphamide-treatedgroup showed normal architecture of glomerulus (G) and mild congestion in tubules (CT); 7 & 8); FFCA at 50 mg/kg + cyclophosphamide-treated group exhibited mild glomerular congestion (GC) with moderate glomerular atrophy (GA) and proteinaceous casts (PC) in few tubules; 9 & 10): FFCA at 100 mg/kg + cyclophosphamide-treated group showed mild glomerular atrophy (GA) and with near to normal architecture of tubules with congestion (CT); 11 & 12) FFCA at 200 mg/kg + cyclophosphamide-treated group showed apparently normal architecture of glomerulus (G) and mild congestion in tubules (CT).

4. Discussion

This is the first study that, as far as we are aware, demonstrates the ability of *C. absus* seed to counteract oxidative stress and improve immune status in immunocompromised rats. Many active phytochemicals including flavonoids have been identified in *C. absus* mainly 7, 8, 3', 4', and 5'-pentamethoxy flavone, luteolin, prunin, 32'-hydroxy-3,5,7,4',5'-pentamethoxyflavone, goshonoside F4 and isovitexin derivatives which might be responsible for the antioxidant and immunomodulatory effects of *C. absus*. Previous reports demonstrated pharmacological effects of a few phytochemicals identified in *C. absus* such as the antioxidant and anticancer properties of methoxy flavones by modulating apoptosis (Wudtiwai *et al.,* 2011), antioxidant effect of luteolin (Madhesh *et al.,* 2012), oxidative damage protection and anti-inflammatory activity of goshonoside F4 (Yu *et al.,* 2019), potent anti-inflammatory and antioxidant activity of derivatives of isovitexin (Lv *et al.,* 2016) and antidiabetic and antioxidant effect of prunin (Jung *et al.,* 2017). The seed of *C. absus* can't be ignored as it contains such active phytochemicals having a variety of pharmacological effects.

In several disease conditions, weakened immunity requires therapy with immunostimulant drugs. Immune suppression in cancer patients under therapy of CP requires immunostimulant drugs sometimes. Herbal-based therapeutic approach to promote immune-mediated responses may help manage the condition with fewer or minimum side effects. The fact that *C. absus* seeds have been used for many years shows how important it is for maintaining people's overall health without causing any obvious adverse effects. The isolated flavonoid fraction of *C. absus* (FFCA) had a protective effect against decreased body weight growth and feed consumption in CP-treated rats, suggesting that FFCA may have an impact on appetite and digestion. The liver as a metabolising organ produces bile and digestive enzymes which might be damaged by CP and FFCA treatment prevented such effects of CP which ultimately contributed to body weight gain in FFCA-pretreated rats when administered with CP.

Numerous hematological variables, including WBC, lymphocytes, and particularly platelets, were affected by cyclophosphamide. Similar to what we observed, pre-treatment with CP in mice has been reported to reduce white blood cells and platelets as well as alter the histomorphology of the liver and kidney (Sabry *et al.,* 2015). CP administration in mice has also been shown to affect leukocyte counts, body weight, and the spleen coefficient along with increased oxidative stress in the liver and kidney (Zhang *et al.,* 2021). According to previous reports and observations of the current study, *C. absus* seed extract possesses antioxidant, hepatoprotective, renal-protective, and lipid-lowering properties in addition to an antidiabetic potential (Rashid *et al.,* 2017). To investigate this effect of FFCA in various models for typical situations, a thorough investigation is needed. Additionally, the findings concerning the hematological measures show how safe the flavonoid-rich fraction of *C. absus* is. The impact of this proportion on biochemical markers, however, is yet unknown and may provide a clear indication of the safety of *C. absus* when used regularly.

According to Zhang *et al.* (2021), CP causes oxidative stress-mediated alterations in organs like the liver and kidney, which are also found in the current investigation. CP administration in rats resulted in significant and insignificant effects on oxidative stress-related markers in the liver and kidney, respectively. Except for the MDA level, the FFCA therapy had a favorable effect on such altered parameters. Similarly, the greater dose of FFCA, to some extent, reverses the effects of the CP-induced decrease in SOD, CAT, GSH, and elevated MDA levels. These results unambiguously show that the flavonoid-rich fraction of *C. absus* seed has the antioxidant activity that is often associated with flavonoids.

In the current investigation, the proliferation of B and T lymphocytes was reduced, which had a significant impact on antibody titer in CP-treated rats. The decreased DTH reactivity in CP-treated rats indicated the altered cell-mediated immunity. The considerable effect of CP on the spleen may be the cause of such findings. In mice exposed to CP, significantly decreased leukocyte count along with atrophic spleen has been reported (Zhang *et al.,* 2021). The increased antibody titer in the FFCA-treated rats suggested that FFCA can prevent the effect of CP on humoral immunity. Even a significantly increased delayed-type hypersensitivity reaction in rats treated with FFCA at 100 mg/kg/daily as compared to that of the toxicity group suggests that FFCA has a positive effect on cell-mediated immunity.

The histopathology of the liver and kidneys in the CP-treated mice revealed significant structural changes. However, treatment with levamisole and CA, particularly at higher doses, led to mild lesions, demonstrating the preventive effects of the investigated herbal fraction of CA. According to previous research (Bhat *et al.,* 2018), CP produces dose-dependent toxic effects The potential of CP (200 mg/kg, single dosage) to induce free radical production and deplete the antioxidant defense system in rats has been associated with periportal inflammation, hemorrhage, and congestion in hepatic tissue (Oyagbemi *et al.,* 2016), which is consistent with our findings. Histological changes in kidneys like glomerular inflammation, edema, congestion, tubular degeneration, reduced Bowman's gap, and brush edges of the proximal tubular epithelium collapsing into the tubule lumen have been reported in rats treated with CP (Cuce *et al.,* 2016). While vitamin E supplementation significantly reduced such alterations. A recent study demonstrated that melatonin; an antioxidant compound ameliorated the CP-induced histological alterations in the liver and kidneys of rats (Olukole *et al.,* 2020). Similarly, FFCA showed an ameliorating effect against CP-induced alterations in the current study which might be due to the antioxidant properties of phytochemicals present in CA. The overall findings of this study are therefore encouraging and helpful in understanding the pharmacological potential of the flavonoid fraction from *C. absus* seed.

Further study with a focus on the in-depth evaluation of the pharmacological activity of individually identified compounds in the FFCA in various other models along with the involvement of molecular mechanisms may be helpful for confirmation of the outcome of this study. Using a single model in the *in vivo* study may have limitations as compared to knowledge and data generated using various kind of animal models.

5. Conclusion

The findings demonstrate that daily oral administration of a flavonoid fraction of *Cassia absus* seed (FFCA) can reduce the functional and structural changes in the liver and kidney caused by cyclophosphamide-induced oxidative stress. In immunocompromised rats, the pre-treatment of FFCA demonstrated an immunostimulant effect. It may be useful to do an additional study that focuses on the mechanisms of action of the active components found in *C. absus* seed to find novel herbal antioxidants with immunostimulating properties.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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