JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

IMMUNOMODULATORY ACTIVITY OF SIDDHA HERBAL FORMULATION MV KASHAYAM ON CYCLOPHOSPHAMIDE TREATED IMMUNOSUPPRESSED RATS

SP Malarkannan [1*], P Dharumarajan [2], Markandeyan Devaprasad[3] and MK Hariyohesh [4]

1. Assistant Professor, Department of Rural Development Science, Arul Anandar College, Karumathur, Madurai, Tamil Nadu. Mob: 9442355686, E-Mail: drspmalarkannan@gmail.com
2. Assistant Siddha Medical Officer, Department of Indian Medicine and Homeopathy, Government Hospital, Aruppukottai, Virudhunagar Dist, Tamil Nadu. Mob: 9442581332 E-Mail: harsanasiddhamm@gmail.com
3. Professor, Department of Neurology, Chettinad Academy of Research and Education, Rajiv Gandhi Salai, Kanchipuram, Tamil Nadu. Mob: 9884372833 E-Mail: prasad77.md@gmail.com
4. BS MD Scholar, Medical School foundation, Daevo Medical School Foundation Inc, Medical School Dr, Poblacion district, Davo City, 8000 Davo del Sur, Philippines. Mob: 9080960364 E- Mail: drhariyoheshmk@gmail.com

ABSTRACT

Aims and objectives

The important aspect of medicine to utilize as an antiviral drug is not only to cure the viral infection but also increase the immunity of the patients is the prime importance to control the infection.

Methodology

In this study, the immunomodulatory activity of purified Siddha herbal formulation MV kashayam at the concentration of 5ml and 10ml/kg b.w. was planned on cyclophosphamide (CYP) treated immuno-suppressed albino Wistar rats and compared with the standard immune-activating drug levamisole.

Results and conclusion

The result showed that the purified Siddha formulation MV kashayam treatment provides a significant increase in a protective effect on hematopoietic cells like (Haemoglobin) Hb, RBC, WBC, platelet cells as against CYP induced immune-toxicity and in case of delayed-type hypersensitivity reaction (DTH) showed 30% and 43% higher volume in the thickness of footpad. The phagocytic index level increased significantly and provides protection against oxidative stress triggered by CYP and recover the damage. The organ weight index of the spleen, liver, kidney and heart showed that they have recovered to

normal condition in a short period. CYP treatment significantly elevated the levels of macromolecule damage (MDA and PCO) and decrease the levels of enzymatic (SOD and CAT), non-enzymatic (GSH) antioxidant status in the liver. At the same time, treatment with MV kashayam reverse the effect of CYP induced damage via increasing CAT, SOD and GSH levels and thus protecting macromolecular damage and decrease MDA and PCO level. The morphological analysis also supports the above study and shows the ability of Siddha formulation MV kashayam to repair the damage caused by CYP induced oxidative stress.

Keywords: Siddha herbal formulation - MV kashayam- Immunomodulatory effect - immunosuppressed rats

1. INTRODUCTION

Herbal plants are the instrumental, incredible and traditional sources for the curability of various diseases in the form of medicines. The traditional system of medical practice in India is based on Ayurveda and the Siddha system of medicines are still extensively practised for curing a variety of infections by increasing the immunity of the patients. The beneficial power of the medicinal herbal plants' relay in their bioactive components specifically secondary metabolites viz. steroids, alkaloids, diterpenes, triterpenes, aliphatics and glycosides etc (1). The effective identification of phytochemicals and isolation of bioactive compounds from the medicinal herbal plants might help in combating such deadly infections provides a new approach to overcome viral infections and their transmission. There is an urgent need to develop vital solutions for the long sprint to overcome the global fear and to prevent further transmission of such a pandemic (2). The immune system of our body plays a major role to overcome the fear of infection to a greater extent. The dysfunction of the immune system is responsible for various diseases like cancer, allergy, arthritis, ulcerative colitis, asthma, parasitic diseases and infectious diseases (3). The first thing done in the field of immunomodulation was the search for an immunomodulatory agent for the treatment of residual cancer (4). The progression in the development of novel antiviral mediators with immunostimulant property is the foremost concern of medical research at present.

2. MATERIALS AND METHODS

The MV kashayam utilized in this study was prepared as per the procedure of Malarkannan and Dharumarajan (2020) (5). The study was carried out after obtaining proper clearance from the ethical committee of KM College of Pharmacy, Madurai.

2.1 Experimental animals

The immunomodulatory study was conducted in Wistar albino rats (180-220 g) of either sex. The experimental protocol was approved by the Institutional ethical committee. All animals were housed at 25 ± 5°C in a well-ventilated animal house under 12/12 h light/dark cycle with standard commercial diet as per the ethical guidelines. Animals were acclimatized to the experimental conditions for one week before starting the study to reduce animal stress.

Experimental design: The immunomodulatory activity of Siddha formulation MV kashayam was tested on the Cyclophosphamide-treated immune-suppressed rat model. The albino rats were divided into five groups and each group contained 5 animals.

The groups are as follows:

- Group I served as control received saline solution.
- Group II served as an immuno- suppressant group, received cyclophosphamide at the dosage of (30 mg/kg, i.p.)
- Group III served as the test groups which were immune-suppressed with cyclophosphamide (30 mg/kg, i.p) and treated with Siddha formulation MV kashayam (5ml/kg, orally).
- Groups IV served as the test groups which were immune-suppressed with cyclophosphamide (30 mg/kg, i.p) and treated with Siddha formulation MV kashayam (10ml/kg, orally).
- Group V served as the positive control which received cyclophosphamide (30 mg/kg, i.p) along with Standard drug-levamisole hydrochloride (LH) (10 mg/kg bw, i.p).

All the groups treated with Siddha formulation MV kashayam and levamisole hydrochloride were administered on daily basis for 11 days, while cyclophosphamide was given on 4, 5 and 6^{th} days of the experiment.

At the end of the experiment, the animals were sacrificed by cervical dislocation and the blood was collected using a heart puncher in 3% citrate containing tubes. The organs namely the liver, spleen, heart and kidney were immediately collected, weighed and stored at 8°C. The following tests were carried out to assess the immunomodulatory effects of the MV kashayam compared with standard drug Levamisole were as follows

2.2 Bodyweight and relative organ weight determination:

Bodyweight and relative organs (Spleen, Liver, Heart, Kidney) weight were measured for all animals and the results were expressed as mg of organ weight/g bodyweight of the animal,

$$\text{Organ weight index} = W1/W0 \times 100,$$

where
W1 is the weight of an Organ and
W0 is the weight of the body

2.3 Haematological analysis

The level of WBC, RBC, platelet and haemoglobin levels were determined using an automatic cell counter

2.4 Cyclophosphamide-Induced Myelosuppression

In cyclophosphamide-induced myelosuppression, mice were divided into five groups of five animals each. Group I (control group) and group II (cyclophosphamide group) received the vehicle for a period of 13 d.

Group III received levamisole (50 mg/kg) for 13 d. The animals of treatment group IV and V were given Siddha formulation MV kashayam at the rate of 5ml/kg and 10ml /kg respectively daily for 13 d. The animals of groups II to V were injected with cyclophosphamide (30 mg/kg, i.p.) on the 11th, 12th and 13th day, 1h after the administration of the respective treatment. Blood samples were collected on the 14th day of the experiment and the total white blood cell (WBC) count was determined by the routine haematological method using Neubauer chamber with haemocytometer [17].

2.5 Carbon Clearance Test

Mice were divided into four groups randomly with five animals in each group. The control (group I) received vehicle (normal saline), while group II received levamisole (50 mg/kg) for 14 d. The animals of treatment group III and IV were given Siddha formulation MV kashayam (5ml/kg and 10ml /kg respectively) daily for 14. On the 14th day of treatment, three hours after the last dose, mice were injected with 0.1mL of carbon suspension (Pelican ink) intravenously through the tail vein. Blood samples (25 µL) were collected from retro-orbital plexus just at 0 and 15 min after injection. Blood samples were mixed with 2 mL of 0.1% w/v Na2CO3. The carbon clearance i.e., rate of elimination of carbon from blood was determined by turbidometric spectroscopy at 650 nm using a UV spectrophotometer. The phagocytic index (K) was calculated using the formula:

$$K= (\ln OD_1 - \ln OD_2)/ t_1-t_2$$

Where

OD_1 and OD_2 are the optical densities at time t_1 and t_2 [19].

2.6. Neutrophil Adhesion Test

In the neutrophil adhesion test, the control group I received the vehicle, while group II received levamisole (50 mg/kg) for 14 d. The animals of treatment group III and IV were given with Siddha formulation MV kashayam (5ml/kg and 10ml /kg respectively) daily for 14 d. On the 14th day of the treatment, blood samples from all the groups were collected by puncturing the retro-orbital plexus under mild ether anaesthesia. Blood was collected in vials pre-treated by disodium EDTA and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with nylon fibre (80 mg/mL of blood sample) for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and per cent neutrophil gives a neutrophil index of the blood sample. The percentage of neutrophil adhesion was calculated as follows:

$$\text{Neutrophil adhesion Percentage} = \text{NIu} - \text{NIt X } 100 \div \text{NIu}$$

Where

NIu is neutrophil index before incubation with nylon fibre

NIt is neutrophil index after incubation with nylon fibre [19].

2.7. Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's test. All the values were expressed as mean ± SEM.

2.8 EFFECT OF MV KASHAYAM ON CELL-MEDIATED IMMUNE RESPONSE

2.8.1 Immunization

About 5µg of hepatitis B vaccine (Revac-B, from Bharat Biotech, India) was given as antigen on the 4th day (IM) of the experiment. The vaccine contained aluminium hydroxide as an adjuvant and was preserved with thiomersal.

2.8.1.1 Cellular immune response

2.8.1.1.1 Delayed type Hypersensitivity reaction

The cell-mediated immune response was assessed by a footpad reaction test. On the 10th day, 5µg of hepatitis B vaccine was injected in the right paw and saline was injected in the left paw. On the 11th day after 24 h, the paw volume was measured using a plethysmometer and the results were expressed as % of the increase in the paw volume.

2.8.1.1.2 Phagocytic response

The phagocytic response was determined according to the method Wang et al (2012) (11). On the 7th day of the experiment, the animals were injected with 100 µl of Indian ink via intravenous injection. 50µl of blood was collected with 5µl of 3% citrate by retro-orbital puncher at an interval of 2 and 30 minutes after the injection of ink. Then 25 µl of citrated blood was added to 3 ml of 0.1% sodium carbonate solution to lyse the RBC. The concentration of ink in the blood was read at A675nm using a spectrophotometer.

The carbon clearance rate (κ) and phagocytic index (α) were calculated by using the following formula:

Rate of carbon clearance (κ) = $(logOD1 - \log OD2)/ (T1 - T2)$

Where OD1 is the absorbance at 2 minutes; OD2 is the absorbance at 30 minutes; T1 is the time of blood collection at 2 minutes; T2 is the time of blood collection at 30 minutes

Phagocytic index α = $(\sqrt[3]{k} \times A)/ (B + C)$

Where A is the body weight, B is the liver weight, and C is the spleen weight.

2.8.2Total antioxidant status of organs

2.8.2.1 Tissue Homogenate preparation

The liver was homogenized in 50 mM phosphate-buffered saline (pH 7.4) by using chilled mortar and pestle at 4°C. The homogenate was centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was used for the determination of the antioxidant status of the organs.

2.8.2.2 Total glutathione level

Estimation of the reduced glutathione (GSH) level was done according to the method of Ellman (1959) (3). Briefly, 400µl of the tissue homogenate was treated with 400µl of 5% sulphosalicylic acid and mixed well with vortex. Then the mixture was centrifuged at 1000 x g for 10 minutes at 4°C. 100 µl of the supernatant was mixed with 400 µl of 0.3 M phosphate buffer (pH 8.4) and 400 µl of distilled water. Then 100 µl of 0.001 M freshly prepared DTNB (5,5-dithiobis (2-nitrobenzoic acid)) was added and kept at room temperature for 10 minutes. The formation of the yellow coloured product was measured at 412nm. The amount of glutathione present in the tissue homogenate was calculated by constructing a standard graph with glutathione and the results were expressed as µM/mg of protein.

2.8.2.3 Lipid peroxidation

The amount of lipid peroxide present in the tissue was estimated according to the method of Stocks and Dormandy (1971) (6). Briefly, 400 µl of the tissue homogenate was mixed with an equal volume of 10 % Trichloroacetic acid and kept at 4°C for 30 minutes. The proteins were removed by centrifugation at 2000 × g for 10 minutes at 4°C. 500 µl of 1% thiobarbituric acid was added to 500 µl of the supernatant and the mixture was kept in a boiling water bath for 30 minutes. The reaction mixture was cooled and centrifuged at 2000 × g for 10 minutes at 4°C. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The concentration of lipid peroxide was calculated using the molar extinction coefficient of MDA-thiobarbituric chromophore (1.56 x 10-5/M/cm) and the results were expressed in terms of nmol MDA/mg of protein.

2.8.2.4 Carbonyl protein

The level of protein damage was determined by carbonyl protein estimation according to the method of Reznick and Packer (1994) (4). Briefly, 200µl of the tissue homogenate was treated with 200µl of 1% trichloroacetic acid and was kept at 4°C for 30 minutes. The mixture was centrifuged at 2000 x g for 15 minutes and the pellet was re-suspended in 10 mM 2,4-dinitrophenylhydrazine in 2N HCl or with 2N HCl as a control blank. This mixture was kept at room temperature for 1 hour and then centrifuged at 2000 x g for 10 minutes. The pellet was washed three times with 1:1 ethanol/ethyl acetate solution. Finally, the carbonyl protein containing the pellet was dissolved in 6 M Guanidine. The protein hydrazones were measured at A370 nm using a spectrophotometer. The amount of carbonyl protein was calculated from the molar extinction coefficient of 22,000 M−1 cm−1 and the results were expressed as µg/mg of protein.

2.8.2.5 Superoxide Dismutase activity

The level of superoxide dismutase in the tissue was estimated according to the method of McCord and Fridovich (1969) (7). This method is based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol. Briefly, 100µl of the tissue homogenate was added to 700 µl of 100 mM of Tris-HCl buffer (pH 8.2) containing 30 mM EDTA. Then, 200µl of 2 mM of pyrogallol was added to the solution and measured at 420nm for 60 sec using a spectrophotometer. A blank was run without the addition of homogenate. One

unit of SOD activity is the amount of enzyme capable of inhibiting 50% of the rate of autoxidation of pyrogallol compared with the blank and are expressed as units'/mg protein/min

2.8.2.6 Catalase activity

The catalase level in the tissue homogenate was estimated according to the method of Sinha (1972) (8) with minor modification. Briefly, add 100 µl of tissue homogenate to 300 µl of 50 mM phosphate buffer pH-7. Then, 100 µl of 200mM H_2O_2 was added to the mixture, mix well and placed at room temperature for 30 sec. immediately, after 30 sec, add 500 µl of 1.5% potassium dichromate / acetic acid (weight/volume). The mixture was kept in a boiling water bath for 10 minutes and cooled. The absorbance was read at 590 nm against blank using a spectrophotometer. The different concentration of H_2O_2 (1-50 µM) was used to construct the standard graph and The catalase activity was calculated from the standard graph of H_2O_2 (1-50 µM) and results were expressed as µmole of H_2O_2 consumed/ mg of protein/ minute.

2.9 RESULTS AND DISCUSSION

The immune system plays an important role in defence mechanism and protects the body against various antigens and infectious diseases. The homeostatic balance is maintained by the stimulation or suppression of immune cells and it keeps the body in normal healthy condition. Thus the immune modulator plays a vital role in maintaining the immune system.

In this study, the immunomodulatory activity of purified Siddha formulation MV kashayam was studied on cyclophosphamide (CYP) treated immuno-suppressed albino Wistar rats. The immunomodulatory activity of Siddha formulation MV kashayam was compared with the standard immune-activating drug levamisole.

The CYP is an alkylating drug that belongs to the subclass of nitrogen mustard. It is commonly given as a chemotherapeutic drug for cancer treatment and as an immunosuppressant for organ transplantation and autoimmune disorder(9). CYP also causes some side effects such as myelosuppression, immune suppression and oxidative stress which may be life-threatening(10). The inactive form of CYP is activated by the liver enzyme cytochrome P450 to 4-hydroxycyclophosphamide which transferred to other organs as well. Then, 4-hydroxycyclophosphamide is further converted to phosphoramide mustard and acrolein. The phosphoramide mustard causes cytotoxic damage to cells and acrolein causes some side effects (12).

In this study, CYP is given to the Wistar albino rats to suppress the immune system and induce oxidative stress. The cyclophosphamide effect is expected to reduce the activity of haematological parameters, cell-mediated immune responses and macrophage production. Moreover, CYP impaired the organs through its toxic metabolites and caused oxidative stress. The effect of Siddha formulation MV kashayam on CYP induced immune-toxicity was examined through myelosuppression, immune suppression and oxidative damage.

Table no.1 Effect on Siddha formulation MV kashayam on Haemoglobin RBC level, WBC level and Platelets level

Groups	Haemoglobin (g/dl)	RBC (106/µL)	WBC (10^6/mL)	Platelets count (103/µL)
G1	13.40 ± 1.40	7.85± 0.62	7.47 ± 0.38	848.35 ± 10.50
G2	10.75 ± 0.75	6.73 ± 0.44	3.42 ± 0.15	550.90 ± 6.25
G3	12.70 ± 0.90	7.22 ± 0.50	6.56 ± 0.24	770.10 ± 8.38
G4	13.05 ± 1.10	7.52 ± 0.54	6.88 ± 0.27	789.38 ± 8.55
G5	13.30 ± 1.15	7.72 ± 0.58	7.08 ± 0.30	807.40 ± 8.78

Table no:2 Effect of Siddha formulation MV kashayam on enzymatic and non-enzymatic antioxidant status of liver tissue

GROUPS	Catalase U/mg	SOD U/mg	Glutathione U/mg	MDA ηM/mg of protein	PCO ηM/mg of protein
G1	4.93±0.39	64.05±3.78	38.60±1.75	7.38±0.39	3.32±0.23
G2	1.28±0.22	41.10±2.64	20.40±1.12	14.80±0.88	6.40±0.37
G3	3.63±0.30	52.45±2.80	29.26±1.28	12.65±0.66	5.64±0.24
G4	3.87±0.34	56.15±3.11	32.57±1.36	13.28±0.76	5.86±0.31
G5	4.26±0.37	61.10±3.27	34.70±1.45	13.87±0.88	6.17±0.33

2.9.1 Effect of Siddha formulation MV kashayam on hematopoietic function against cyclophosphamide-induced immune-toxicity

The protective effect of Siddha formulation MV kashayam on hematopoietic function against CYP induced immune-toxicity was evaluated by counting the level of haematological parameters like (Haemoglobin) Hb, RBC, WBC and platelet cells. The results of the haematological analysis showed that the level of Hb, RBC, WBC and platelet cells were significantly reduced in the negative control group of animals (G2- CYP alone treated) when compared to a normal control group of animals (G1-Saline alone) ($P<0.01$) However, these levels were raised significantly in Siddha formulation MV kashayam treated group of animals (G3-5ml/kg b.w.), and G4 = 10ml/kg b.w.) in a dose-dependent manner. The increase in the levels of haematological parameters in the group of animals of G3 and G4 showed a significant difference with the negative control group of animals (G2) ($P<0.01$). The haematological parameters in positive control groups of animals (G5) which are treated with standard drug levamisole (10mg/kg) showed significant recovery when compared to the negative control group of animals (G2) ($P<0.05$).

The alkylating nature of the CYP alkylates the DNA and interferes in the synthesis and proliferation of hematopoietic cells leading to myelo suppression. Myelo suppression is the process of decreasing the

production of immune cells (leukocytes), oxygen-carrying cells (erythrocytes) and the cells responsible for the blood clot (thrombocytes) (13). The results of this study showed that the groups treated with Siddha formulation MV kashayam have improved the production of hematopoietic cells like RBC, WBC, platelets and haemoglobin. The hematopoietic stem cells possess multipotentiality and have the capacity to renew the haematological parameters such as RBC, WBC and platelets. The increase in levels of Hb, RBC, WBC and platelets might have taken place due to the induction of hematopoietic stem cells by Siddha formulation MV kashayam (14) have reported induction of mobilization of hematopoietic progenit1or cells and boost the immunity by fucoidan from brown algae. The haematological results reveal that the Siddha formulation MV kashayam has a protective effect against CYP induced myelosuppression.

2.9.2 Effect of Siddha formulation MV kashayam on cell-mediated immunity against cyclophosphamide -treated immunosuppressed rats

The cell-mediated immune response is induced by T lymphocytes and their products (lymphokines). These cells are involved in the effector mechanism which provides defence against infectious organisms, foreign grafts, cancer cells and are also involved in delayed-type hypersensitivity reaction (15). The cell-mediated immune response was determined by delayed-type hypersensitivity reaction (DTH) by measuring their footpad thickness. A 36% decrease in the paw volume of the negative control group of animals (G2) was observed when compared to the normal control group of animals (G1) ($P< 0.05$). The thickness of the footpad increased significantly in the groups (G3 and G4) treated with Siddha formulation MV kashayam in a dose-dependent manner in comparison with the negative group of animals (G2). The footpad volume of (5ml/kg b.w.) (G3) and (10ml/kg b.w.) (G4) treated group of animals were 30% and 43% higher, respectively ($P < 0.01$ in every group), when compared to the negative control group of animals (G2). The levamisole treated group also showed similar results (i.e) 36% higher footpad volume than the control group ($P<0.01$). The group of animals treated with Siddha formulation MV kashayam alone (G3) showed 33% higher footpad volume than the normal control group of animals. The above results also supported by (16) who demonstrated cytotoxic T cells enhancing the property of fucoidan from *C.okamuranus*. The immune-boosting property of fucoidan from *L. japonica* on immune-suppressed mice by activating the macrophage and T- lymphocyte (17). In this study, the Siddha formulation MV kashayam has enhanced the cell-mediated immune response through the activation of T-cell.

2.9.3 Effect of Siddha formulation MV kashayam on phagocytic response against cyclophosphamide -treated immunosuppressed rats

Phagocytosis is a process by which the immune system effectively removes or engulf microorganisms, cancer cells, inorganic particles and tissue debris. The phagocytic test is used to evaluate the non-specific immunity of the system. The immune cells involved in phagocytosis are called phagocytes (15). The macrophages are the major phagocytic cells (neutrophils, monocytes and macrophages). The phagocytic index is calculated from the rate of clearance of colloidal carbon particles from the circulatory system.

The phagocytic index (α) of the CYP treated group of animals showed a significant decrease (39%) when compared to the normal control group of animals (P< 0.05). It indicates that the effect of CYP impaired the immune system and suppressed the levels of phagocytes. The Siddha formulation MV kashayam significantly restored the level of the phagocytic index when compared with the negative control group of animals (G2) (P< 0.05 with each group). The Siddha formulation MV kashayam at the concentration of (5ml/kg b.w), (10ml/kg b.w) increased the phagocytic index up to 11% and 29% when compared to the negative control group of animals (G2).

The standard drug levamisole showed 11.5% increased phagocytic activity than the normal control group. Siddha formulation MV kashayam was 4 times higher than that of the normal control group at a dose of 5ml and 10ml/kg (18). Several authors reported that fucoidan from brown algae has the potency to activate and proliferate the phagocytic system (17,19,20). The results carbon clearance test indicates that Siddha formulation MV kashayam can enhance the non-specific immune response against CYP induced immunosuppression.

2.9.4 Effect of Siddha formulation MV kashayam on organ weight index against cyclophosphamide - treated immunosuppressed rats:

The weight index of organs like the spleen, liver, kidney and heart reflects the health of the organism. The toxic metabolite produced from CYP is initially metabolized in liver and produces toxic metabolite which is further transferred to other organs. The toxic metabolites primarily impair the immune organs such as the liver, spleen and merely affect the kidney and heart (12). In the present study, it was shown that the weight index of the organs like the spleen and liver involved in the immune system was significantly reduced in CYP alone treated group of animals (G2) compared to the normal control group of animals (G1) (P<0.05). The weight index reduction of organs like the kidney and heart is less when compared to the weight index reduction of the liver and spleen. The CYP imposes a higher impact on the reduction of weight index of liver than on other organs like kidney, heart and lungs (21). These findings integrate with the results of the present study.

The treatment with Siddha formulation MV kashayam (G3 and G4) improved the spleen and liver weight significantly compared to a negative control group of animals (G2) (P<0.05). The rate of the index of organ weight recovery was based on their concentration. The results of the organ weight index showed that the suppressed health due to CYP induced oxidative stress have recovered to normal after Siddha formulation MV kashayam treatment.

2.9.5 Effect of Siddha formulation MV kashayam on antioxidant status of organs against cyclophosphamide-induced oxidative stress -:

The antioxidant status acts in connection with many diseases results in immune dysfunction. The drugs like cisplatin, cyclophosphamide or corticosteroids used for immunosuppression generates free radicles (Reactive Oxygen Species) and toxic metabolites [22]. The imbalance between the reactive oxygen

and antioxidant defence mechanism causes oxidative stress exhibiting inflammatory response and resulting in tissue and cell injury (23). The increased generation of ROS will damage macromolecules like lipid, DNA and protein in the tissue. (24). The immunomodulator from plant origin can reduce oxidative stress through antioxidant mechanism (25). Moreover, Siddha formulation MV kashayam has shown comparably good superoxide radical scavenging capacity and other antioxidant activities.

The effect of Siddha formulation MV kashayam against oxidative stress caused by CYP treatment was assessed by the determination of enzymatic and non-enzymatic antioxidant status and levels of macromolecular damage.

2.9.6 Effect of Siddha formulation MV kashayam on non-enzymatic antioxidant status of liver organ

The non-enzymatic antioxidant status plays a major role in maintaining the innate antioxidant status (26). Glutathione belongs to non-enzymatic antioxidant and also repairs immunological and neurodegenerative disorders (27). Reduced glutathione level is a suitable indicator for overall antioxidant defence which maintains alpha-tocopherol and ascorbic acid and is also a coenzyme for glutathione S-transferases and glutathione peroxidases (28).

The level of glutathione was significantly reduced in the liver of the negative control group of animals (G2) (liver = 20.50±1.05) compared to the normal control group of animals ($P<0.05$). The Siddha formulation MV kashayam treatment on CYP intoxicated group of animals (G3 and G4) directly increased the glutathione levels significantly based on the dose concentration in liver organs.

2.9.7 Effect of Siddha formulation MV kashayam on enzymatic antioxidant status of organ

The groups of enzymes which are involved in the conversion of active oxygen molecule into non-toxic molecules are superoxide dismutase (SOD) and catalase (CAT). These enzymes maintain the enzymatic antioxidant status of the tissue. The antioxidant enzyme superoxide dismutase converts the superoxide to water peroxide whereas catalase converts hydrogen peroxide to water and oxygen. They are mainly located in peroxisomes, cytoplasm and mitochondria (29). In this study, the levels of CAT significantly reduced in the liver of the negative control group of animals (G2) compared to the normal control group (G1) ($P< 0.05$).

The Siddha formulation MV kashayam treatment to the animal (G3 and G4) significantly restored the decreased CAT level to an equal or above the CAT levels of the normal control group of animals in a dose-dependent manner ($P < 0.05$). (G5) of levamisole treated group of animals that are intoxicated with CYP showed 7.2% higher CAT levels than the normal control group of animals (G1).

Similarly, were significantly reduced in the liver negative control group of animals (G2) when compared to the normal control group of animal (G1) (P<0.05). Like that of CAT, the level of SOD significantly increased upon the dose-dependent manner treatment with Siddha formulation MV kashayam and attained the level of the normal control group of animals at the concentration of 5ml and 10ml/kg (P< 0.05).

2.9.8 Effect of Siddha formulation MV kashayam on macromolecular damage of organ

The important markers of oxidative stress are macromolecular like lipid and protein damage which produce lipid peroxide (MDA) and carbonyl protein (PCO) (30,31). The MDA and PCO levels were found to be significantly increased in all the organs of the negative control group of animals (G2) compared to the normal control group of animals (G1). The increased levels of MDA and PCO indicate that toxic metabolite produced from CYP induced oxidative stress damage the lipids and proteins present in the organ tissue. The liver of the negative control group of animals (G2) showed almost 1.5- 2.2 times higher MDA level than that of the normal control group (G1) (P <0.05).

The levels of MDA were found to decrease significantly in Siddha formulation MV kashayam treated group of animals (G3 and G4) in a dose-dependent manner compared to the negative control group, G2(P <0.05) Similarly, PCO levels significantly increased 1.5-2 times higher in the liver of negative control group of animals (G2) compared to the normal control group of animals (G1). The increased levels of PCO on CYP toxicity decreased significantly on Siddha formulation MV kashayam treatment in a dose-dependent fashion (G3 and G4) (P<0.05)

The CYP toxicity induced high MDA and PCO levels were found normal at a concentration of 5ml and 10ml/kg b.w.) and 10 (mg/kg b.w) of Siddha formulation MV kashayam and levamisole, respectively. The fucoidan significantly reduced MDA levels against 2,2-azobis dihydrochloride induced oxidative stress in the zebrafish model (32). The fucoidan from *C. okamuranus* also reduces the MDA levels in Sprague-Dowley (SD) rats (33).

The present study showed that the purified Siddha formulation MV kashayam provide protection from oxidative stress triggered by CYP and recover the damage. CYP treatment significantly elevated the levels of macromolecule damage (MDA and PCO) and decrease the levels of enzymatic (SOD and CAT), non-enzymatic (GSH) antioxidant status in the liver. At the same time, Siddha formulation MV kashayam treatment at the concentration of 5ml and 10ml/kg b.w. reverse the effect of CYP induced damage via increasing CAT, SOD and GSH levels and thus protecting macromolecular damage and decrease MDA and PCO level. The morphological analysis also supports the above study and shows the ability of Siddha formulation MV kashayam to repair the damage caused by CYP induced oxidative stress.

2.10 CONCLUSION

The present study has proved that Siddha formulation MV kashayam not only enhance the immune system but also protect the organs against oxidative stress. The results of the present study recommend that

the Siddha formulation MV kashayam could be used as a prominent natural immunomodulating molecule with therapeutic value.

2.11 ACKNOWLEDGEMENT The authors are thankful to Tamil Nadu State Council for Science and Technology, Chennai and the Principal, Arul Anandar College, Karumathur for providing necessary permission, infrastructures and equipments.

2.12 CONFLICTS OF INTEREST

No conflicts of interests.

2.13 FUNDING INFORMATION: Heartfelt gratitude is extended to Tamil Nadu State Council for Science and Technology, Chennai for providing financial help.

2.14 AUTHORS CONTRIBUTION: All authors in the present study made substantial contributions towards the collection of herbs, preparation of Kashayam, maintenance of lab animals and conduction of various tests, analysis of data and interpretation of results.

REFERENCES

1. Chikezie, P.C., Ibegbulem, C.O. and Mbagwu, F.N. 2015. Bioactive principles from medicinal plants. Res. J. Phytochem. 9 (3), 88–115.
2. Balachandar, V., Mahalaxmi, I., Kaavya, J., Vivekanandhan, G., Ajithkumar, S., Arul, N., Singaravelu, G., Kumar, N.S. and Devi, S.M. 2020. COVID-19: emerging protective measures. Eur. Rev. Med. Pharm. Sci. 24 (6), 3422–3425.
3. Ellman, GL 1959, 'Tissue sulfhydryl groups', Archives of biochemistry and biophysics, vol. 82, no. 1, pp. 70-77.
4. Reznick, AZ and Packer, L 1994, '[38] Oxidative damage to proteins: Spectrophotometric method for carbonyl assay', Methods in enzymology, vol. 233, no. pp. 357-363
5. Malarkannan, S.P. and Dharumaraj, P. 2020. Anti-Inflammatory Activity of Siddha Preparation ModiVaer Kashayam as a Proposed remedy for Management and Treatment of Covid 19.Int J Res Analytical Rev. 7 (3): 756-759.
6. Stocks, J and Dormandy, T 1971, 'The autoxidation of human red cell lipids induced by hydrogen peroxide', British journal of haematology, vol. 20, no. 1, pp. 95-111
7. McCord, JM and Fridovich, I 1969, 'Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein)', Journal of Biological Chemistry, vol. 244, no. 22, pp. 6049-6055
8. Sinha, AK 1972, 'Colorimetric assay of catalase', Analytical biochemistry, vol. 47, no. 2, pp. 389-394
9. Moore, MJ 1991, 'Clinical pharmacokinetics of cyclophosphamide', Clinical pharmacokinetics, vol. 20, no. 3, pp. 194-208
10. Wang, Q, Song, Y, He, Y, Ren, D, Kow, F, Qiao, Z, Liu, S and Yu, X 2014, 'Structural characterisation of algae Costaria costata fucoidan and its effects on CCl 4-induced liver injury', Carbohydr Polym, 2011, vol. 107, no. pp. 247-254

11. Wang, M, Meng, XY, Yang, RL, Qin, T, Wang, XY, Zhang, KY, Fei, CZ, Li, Y, Hu, Yl and Xue, FQ 2012, 'Cordyceps militaris polysaccharides can enhance the immunity and antioxidation activity in immunosuppressed mice, Carbohydr Polym, vol. 89, no. 2, pp. 461-466
12. Sun, H-X and Peng, X-Y 2008, 'Protective effect of triterpenoid fractions from the rhizomes of Astilbe Chinensis on cyclophosphamide-induced toxicity in tumour-bearing mice, Journal of Ethnopharmacology, vol. 119, no. 2, pp. 312-317
13. Urabe, A 2003, '[Bone marrow suppression]', Nihon rinsho. Japanese Journal of clinical medicine, vol. 61, no. 6, pp. 949-953
14. Byon, Y-Y, Kim, M-H, Yoo, E-S, Hwang, K-K, Jee, Y, Shin, T and Joo, H-G 2008, 'Radioprotective effects of fucoidan on bone marrow cells: improvement of the cell survival and immunoreactivity', Journal of veterinary science, vol. 9, no. 4, pp. 359-365
15. Miller, LE and Peacock, JE. 1991. Manual of laboratory immunology. Lea and Febiger.
16. Kim, M-H and Joo, H-G 2008, 'Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells, Immunology Letters, vol. 115, no. 2, pp. 138-143
17. Wang, W-T, Zhou, J-H, Xing, S-T and Guan, H-S 1994, 'Immunomodulating action of marine algae sulfated polysaccharides on normal and immunosuppressed mice', Chinese Journal of Pharmacology and Toxicology, vol. 8, no. pp. 199-199
18. Chen, X, Nie, W, Fan, S, Zhang, J, Wang, Y, Lu, J and Jin, L 2012, 'A polysaccharide from Sargassum fusiforme protects against immunosuppression in cyclophosphamide-treated mice', Carbohydr Polym, vol. 90, no. 2, pp. 1114-1119
19. Teruya, T, Takemoto, H, Konishi, T and Tako, M 2009, 'Structural characteristics and in vitro macrophage activation of acetyl fucoidan from Cladosiphon okamuranus', Glycoconjugate Journal, vol. 26, no. 8, pp. 1019-1028
20. Yang, X, Sun, J and Xu, H 1995, 'An experimental study on immunoregulatory effect of fucoidan', Chin. J. Marine Drugs, vol. no. pp. 9-13
21. Kanno, TYN, Sensiate, LA, Paula, NAd and Salles, MJS 2009, 'Toxic effects of different doses of cyclophosphamide on the reproductive parameters of male mice', Brazilian Journal of Pharmaceutical Sciences, vol. 45, no. 2, pp. 313-319
22. Gate, L, Paul, J, Ba, GN, Tew, K & Tapiero, H 1999, 'Oxidative stress-induced in pathologies: the role of antioxidants', Biomedicine and Pharmacotherapy, vol. 53, no. 4, pp. 169-180
23. Cotran, S, Kumar, C, Collins, T and Robbins, W 1999, 'Pathologic Basis of Disease. ed', Philadelphia: Saunders Co, vol. no. pp.
24. Colvin, O 1999, 'An overview of cyclophosphamide development and clinical applications, Current pharmaceutical design, vol. 5, no. pp. 555-560
25. Joharapurkar, A, Wanjari, M, Dixit, P, Zambad, S and Umathe, S 2004, 'Pyrogallol: A novel tool for screening immunomodulators', Indian journal of pharmacology, vol. 36, no. 6, pp. 355
26. Williams, AT and Burk, RF 1990, Carbon tetrachloride hepatotoxicity: an example of free radical-mediated injury. Seminars in liver disease. © 1990 by Thieme Medical Publishers, Inc. pp. 279-284.

27. Raghavendra, V and Kulkarni, SK 2001, 'Possible antioxidant mechanism in melatonin reversal of ageing and chronic ethanol-induced amnesia in plus-maze and passive avoidance memory tasks', Free radical biology and medicine, vol. 30, no. 6, pp. 595-602
28. Jones, DP 2002, '[11] Redox potential of GSH/GSSG couple: assay and biological significance, Methods in enzymology, vol. 348, no. pp. 93-112
29. Halliwell, B, Halliwell, B and Gutteridge, JM 1999, Free Radicals in Biology and Medicine. 0198500459.
30. Levine, RL, Garland, D, Oliver, CN, Amici, A, Clement, I, Lenz, A-G, Ahn, B-W, Shaltiel, S and Stadtman, ER 1990, '[49] Determination of carbonyl content in oxidatively modified proteins. Methods in enzymology, vol. 186, no. pp. 464-478
31. Reddy, ACP and Lokesh, B 1992, 'Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes', Molecular and cellular biochemistry, vol. 111, no. 1-2, pp. 117-124
32. Kim, E-A, Lee, S-H, Ko, C-i, Cha, S-H, Kang, M-C, Kang, S-M, Ko, S-C, Lee, W-W, Ko, J-Y and Lee, J-H 2014, 'Protective effect of fucoidan against AAPH-induced oxidative stress in zebrafish model', Carbohydr Polym, vol. 102, no. pp. 185-191
33. Thomes, P, Rajendran, M, Pasanban, B and Rengasamy, R 2010, 'Cardioprotective activity of Cladosiphon okamuranus fucoidan against isoproterenol-induced myocardial infarction in rats, Phytomedicine, vol. 18, no. 1, pp. 52-57