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IMMUNOMODULATORY AND ANTI-OXIDANT ACTIVITY OF THE HERBOMINERAL FORMULATION IN ALBINO WISTER RATS

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ABSTRACT

Background: The present study was designed to evaluation of Immunomodulatory and Anti-Oxidant Activity of the Herbomineral Formulation in Albino Wister Rats. **Objective:** To evaluate the Immunomodulatory and anti-oxidant activity of the herbomineral formulation in albino wister rats. **Materials and Methods:** The herbo mineral formulations were formulated into capsule. The preliminary phytochemical investigation and thin layer chromatography were performed to identify the phytoconstituents of the formulation. Evaluation of Immunomodulatory and anti-oxidant activity from HMF in albino wister rats were evaluated by using a Neutrophil Adhesion test, Carbon clearance test, Cyclophosphamide-Induced Immunosuppressant, LPO(Lipid peroxidase estimation), SOD(Superoxide dismutase estimation), CAT (Catalase estimation), Glutathione estimation. **Results:** The aqueous extract and column fraction revealed the presence of terpenoids as phytoconstituents and The TLC analysis of Alkaloids{ n-butanol: glacial acetic acid: water (4:1:5)}, Flavonoids{ Ethyl acetate: formic acid: acetic acid: water (100:11:11:26)} , Glycosides { Ethyl acetate: Methanol: water: (100:13.5:10) } confirms that the method gave better separation and three compounds were observed with Rf value of 0.87, 0.55, 0.91. And the Immunomodulatory and anti-oxidant activity was determined by using animal model studies like Cyclophosphamide-Induced Immunosuppression. Cyclophosphamide administration caused a significant reduction blood parameters and HMF significantly prevented the myleosuppression as compared to cyclophosphamide-alone-treated rats. In the present study the test samples exhibited significant ($P<0.0001$) Immunomodulatory and anti-oxidant activity at a dose of 100, 200 mg/kg. It may due to the presence of Alkaloids, Flavonoids , Glycosides. **Conclusion:** From the present finding it has been concluded that the immunomodulatory and anti oxidant activity of HMF in animal's model of immunosuppression. Were chosen to assessed after treatment with HMF in 2 different doses (100mg/kg & 200 mg/kgp.o) and compared with toxicant agent cychophosphamide (30mg/hg I.p single dose a day for 3days) and standard during levomisole (25 mg/kg p.o. single dose a day for 28 days).In this study, showed that Herbomineral formulation is a potent immunostimulant, stimulating both the specific & non specific immune mechanisms and also possess the anti oxidant activity.

INTRODUCTION

Ayurveda, the Indian traditional system of medicine means 'science of life and longevity'. It is a time tested medical system developed over a period of time since 500 BC in Indian sub-continent with continuous use by the national and international societies. The unbeaten heritage of this system is a treasure house of knowledge for both preventive and curative health care available to humankind. It has provided treatment to many diseases using herbs, metals and minerals, formulating them into potent dosage forms. Immunomodulators are substances that have ability to influence various components af immune system. They can be used therapeutically for correcting pathological aberrations of the immune response in conditions such as immunodeficiency, chronic infections, autoimmunity, organ transplantation and neoplasia ,and soon. Such substances are often referred to as "Biological response modifiers" as well. The action of immunomodulators may

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be specific or non specific. The pharmacological modulation of the immune response is essential dependent upon the dose of the substance applied. The mode of the application and the prevailing status of the system of the host. Modified-release capsules are hard or soft capsules in which the contents or the shell or both contain excipients or are prepared by special procedures such as microencapsulation which, separately or together, are designed to modify the rate, place or time of release of the active ingredient(s) in the gastrointestinal tract. Herbo-mineral formulations of Ayurveda contain specified metals or minerals as composition, which have their beneficial effects on biological systems. These metals or minerals are transformed into non-toxic forms through meticulous procedures explained in Ayurveda. Though literature is available on quality aspects of such herbo-mineral formulations; contemporary science is raising concerns at regular intervals on such formulations. Thus, it becomes mandate to develop quality profiles of all formulations that contain metals or minerals in their composition. Alpinia galanga one of the plant in the ginger family. Phytochemical investigation carried out on *Alpina galanga* revealed the presence of constituents, such as Alkaloids, glycosides, flavonoids, saponins, tannins, Mucilage are show in the alpinia galangal extract. Many types of chemical constituents show in the aqueous extract. Some flavonoids identified as kaemperol, kaempfeide, galangin and alainia.

Fig 1: Various part of *Alpina galanga*

Pterocarpus marsupium family belongs to Fabaceae. The plant contain various type of pterostilbene, alkaloids, tannins, propterol-B,marsupinol, carpsuin, marsupinol and phydroxy benzaldehyde.

Fig 2: *Pterocarpus marsupium*

Dates contain nutrients as carbohydrates, dietary fibers, fats and proteins. Dates contain fatty acids as palmitolieic acid, Oleic, linoleic and linolenic acid. In dates 23 amino acids are found. Furthermore vitamin A, B1, B2 and nicotinic acid.

Fig 3: *Phoenix dactylifera L*

In this present study Herbal extract and mineral used for the immunomodulatory and anti oxidant activity. Herbal extract such as *Pterocarpus marupium* , *Alpinia galangal* and *Phoenix dactylifera* extracts and minerals such as Zinc and Selenium are conformed Immunomodulating and anti oxidant activity in previous research work study . Above known herb extract in all extracts of aqueos extracts purchased from herbal industry and mineral from local chemical agencies. In this work herbo mineral are will show good immunomadulatory and anti oxidant potential against cyclophosphamide induced immunosuppersion.

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AIM AND OBJECTIVES:

To evaluate Immunomodulatory and Anti-oxidants activity of the herbomineral formulation.

EXPERIMENTAL WORK

5.1 Collention of plant extracts: Three different types of plants extracts and two different minerals used for the immunomodulatory and anti oxidant activity such as *Alpinia galanga* , *Pterocarpus morsupium* , Dates Extract (*phoenix dactylifera*) and zinc, selenium. Above all 3 extracts are purchased from MG Naturals, Chennai and 2 minerals from online.

Preparation of Herbo-mineral formulation: The above all three plant extract and two minerals wera formulated as herbo-mineral capsule.

Procedure:

Step 1:- Mixing of three herbal extracts and two minerals

Step 2:- Above mixture heated on water bath under 600c and slowly add PEG to mixture.

Step 3:- Cooling the above mixture and fill the capsule.

Experimental work

Animals

Healthy albino Wistar rats either sex weighing between 180–200g were used for the study. The animals will be housed in sanitized polypropylene cages containing sterile paddy husk as bedding in an air conditioned room and allowed access to pellet diet and water add libitum (sainathagency, hydrabad). They will be maintained under standard conditions (temperature 22 ± 2oC, relative humidity 50±5% and 12 h light/dark cycle). All the animals are acclimatized for seven days before the actual study. The animals were randomized into experimental, normal and control groups. Animals were habituated to laboratory condition for 48hrs prior to experimental protocol to minimize if any specific stress. All the studies conducted were approved by the Institutional Animal Ethics Committee with the approval number IAEC/ANCP/2018-19/08.

EXPERIMENTAL DESIGN:

After 1 week of acclimatization, the animals will randomly divided into five groups with six animals in each group.

Group 1 (G1) will be served as normal control and will receive normal saline (10 ml/kg body weight p.o.) for 27-28 days.

Group 2 (G2) will received Cyclophosphamide (30mg per kg body weight i.p), single dose a daily for 3 days on 11, 12,13th days.

Group 3 (G3) will be treated with Levomisole (25 mg/kg body weight *p.o*). for 27-28 days .

Group 4 (G4) will be treated with low dose of Herbo-mineral formulation (100mg/kg) once in a day, for 27-28 days.

Group 4 (G5) will be treated with low dose of Herbo-mineral formulation (200mg/kg) once in a day, for 27-28 days.

The blood parameters studied on 14th day and 28th day.

Immunomodulatory Studies:

Neutrophil Adhesion Test: The Rats will be pre treated orally with vehicle or extracts for 14 days. On 14th day of drug treatment, blood samples will be collected by puncturing retro orbital plexus into heparinised vials and analysed for total leukocyte cell (TLC) and differential leukocyte cell (DLC) counts. After initial counts, blood samples will incubated with 80 mg/ml of nylon fibres for 15 min at 37°C. The incubated blood samples will have again analyzed for TLC and DLC. The product of TLC and percent neutrophil gives neutrophil index of blood sample. The percent neutrophil adhesion was calculated as shown below:

Neutrophil adhesion (%) = [(NIu − NIt)/NIu] ×100

Where: NIu is the Neutrophil index of untreated blood Samples, **Nit** is the Neutrophil index of fibre-treated blood samples

Carbon Clearance Test: Rats will be administered HMF and vehicle orally for 10 days according to the experimental protocol. Forty-eight hours after the last dose of the drug, animals of all the groups received intravenous injection of (0.3 ml per 30 g) Indian ink (colloidal carbon) via the tail vein. Blood samples will with drawn from each animal by retro orbital plexus at an interval of 0 and 15 min after the ink injection. A 50 µl blood sample will be mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution will be determined at 660 nm using UV Visible spectrophotometer (Thermo scientific). The phagocytic index, K will be calculated using the following formula.

K = (LogeOD1- LogeOD2)/15

Where: OD1 and OD2 are the optical densities at 0 and 15 min, respectively.

Cyclophosphamide Induced Immunosuppression: Albino Wister rats will

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be treated with test drug and vehicle for 10 days as described in the experimental protocol. After the drug treatment, groups 2, 3 and 4 will be injected with Cyclophosphamide (30 mg/kg i.p.) on the 11th, 12th and 13th day. On day 14th, blood sample will be collected from the retro orbital plexus of individual animals and analyzed for haematological parameters using automated cell counter (Invitrogen).

Anti-oxidant studies:

LPO (Lipid peroxidase estimation): Two milli liters of 28% tricholroacetic acid will be add to the 2.0 ml of RBC suspention and centrifused. one ml of 1% thiobarbituric acid will be add to the supernent, heated in boiling water bath for 60 min ,cooled and absorbance will read at 532nm. Lipid peroxidation will be calculated using the molar extinction coefficient of malondialdehyde (MDA) 1.56×105 and expressed in the of nanomoles of MDA g-1 Hb.

SOD (Superoxide dismutase estimation): To 50 µl of the erythrocyte lysate prepared from the 5% RBC suspension , 75 mM of tris-HCL buffer (pH8.2) and 30 mm EDTA will be add and absorbance will be read at 429 nm ,using spectrophotometer. 2 mm of pyrogallol will be add to the reaction mixture and an increase in absorbance will be recorded at 420 nm of 3 min. one unit of enzyme activity is 50% inhibition of the rate of auto-oxidation of cyclophosphamide as determine by changing in absorbance min-1 at 420nm . The protein content of lysate using estimated by Lower's method and the activity of SOD is expressed as units' mg-1 protein.

CAT (Catalase estimation):

Catalase (CAT) activity will be determined in erythrocyte using Aeib's method with some modifitcations. The erythrocyte lysate (50 µl) will be add to a cuvette containing 2.0 ml phosphate buffer (pH7.0) and 1.0 ml of 30 mM at H2O2 and absorbance will read at 1 min 240 nm. The molar extinction coefficient of H2O2, 43.6Mcm-1 will use for determination of the catalase activity. One unit of activity is equal to one millimole of H2O2 degraded per minute and is expressed as units mg-1 of proteins.

Glutathione estimation:

Blood glutathione will measured by addition of 0.2 ml of whole blood to 1.8 ml distilled water followed by 3.0 ml of precipitating mixture (1.67 g metaphospharic acid, 2.0g EDTA and 30 g Nacl to make 100 ml of solution). It will centrifuged at 5000xg for 5 min and 1.0 ml of supernatant will be add to 1.5 ml of the phosphate solution ,followed by the addition of 0.5 ml of DTNB reageny. The optical density is measured at 412 nm using a spectrophotometer.

Statistical Analysis: All the data was expressed as mean ± SEM and analyzed using Graph Pad prism version 5.1 using ANOVA fallowed by Dennet's multiple comparison test.

IMMUNOMODULATORY STUDIES:

A. Neutrophil Adhesion test : Incubation of blood with nylon fibres produced a decrease in the neutrophil count due to adhesion of neutrophils to the fibres . Pre-treated with HMF 100 mg/kg and 200 mg/kg evoked a significant increase in the in vitro neutrophil adhesion to nylon fibres .

DISCUSSION

Immunomodulatory agents enhance the immune responsiveness of an organism against a pathogen by activating the immune system. Many plant products used in traditional medicine have been reported to have immunomodulating acitivities.While some of these stimulate both humoral and cell-mediated immunite (CMI), others activate only the cellular components of the immune system, i.e. phagocytic function without affecting the humoral immunity. In the current study, we found that HMF modulates both cellular and humoral immunity in experiment rats. Acute dose toxicity study, in which the animal treated with the HMF at a higher dose of 2000 mg/kg, did not produce any significant toxicity signs, behavioral changes, body weight changes or macroscopic findings during observational period. So the LD of HMF should be more than 2000mg/kg. In the present study three different models, each of which provides information about effect on different components of the immune system was used. Neutrophil adhesion test is widely used to check the effect of various test drugs in cell – mediated immune reactions. The adhesion of neutrophil to nylon fibres indicates the migration of cells in the blood vessels and the number of neutrophils reaching the site of HMF were found to enhance the adhesion of neutrophil in to the fibre . This might be due to the up regulation of the β2 integrins, present on the surface of the neutrophil through which they adhere firmly to the nylon fibres. The effect of HMF on the reticulo endothelial system (RES) was evaluated using the carbon clearance test. RES main consists of phagocyctic cells (macrophages),

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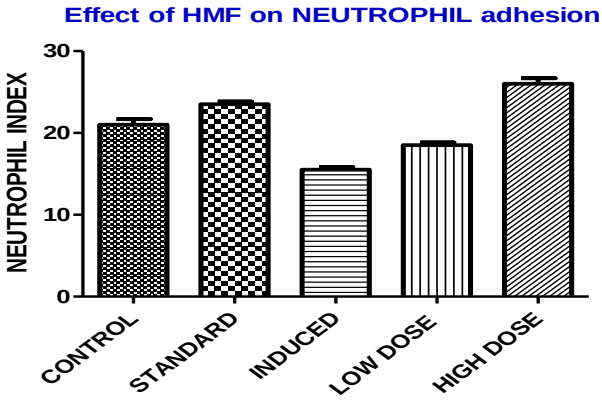
Table 1: Neutrophil Adhesion test

All values are expressed MEAN ±SEM one way ANOVA followed By Dunnets method

Groups	Treatment	Netrophil Adhesion (%)
Control	Normal Saline (Orally) 10ml/kg	21 ± 0.65
Standard	Levomisole (25 mg/kg p.o.)	23.5± 0.35***
Induced	Cyclophosphamide 30 mg/kg i.p (11,12,13th days)	15.5 ± 0.35
Low dose	HMF 100 mg/kg p.o.	18.5 ± 0.35*
High dose	HMF 200 mg/kg p.o.	26 ± 0.69***

P<0.001 and P<0.0001 Vs Control

Neutrophil Index:



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B. Carbon clearance test:

The Phagocytic activity of the reticulo-endothelium system was measured by the carbon clearance test. Both the doses HMF showed significant increase in phagocytic index as compared to the control animals.

Table 6.4.B: Effect of HMF on Carbon clearance test

Groups	Treatment	Phagocytic index
Control	Normal Saline (Orally) 10ml/kg	0.02 ± 0.0004
Standard	Levomisole (25 mg/kg p.o.)	0.04 ± 0.001***
Induced	Cyclophosphamide 30 mg/kg i.p ($11,12,13^{th}$ days)	0.0105 ± 0.0003***
Low dose	HMF 100 mg/kg p.o.	0.05 ± 0.001*** a
High dose	HMF 200 mg/kg p.o.	0.06 ±0.001*** b

All the Values are expressed as MEAN±SEM (n=6) ONE way ANOVA followed by Dunnets method
[a]*P*<0.0001, [b]*P*<0.0001 as compared to control group .

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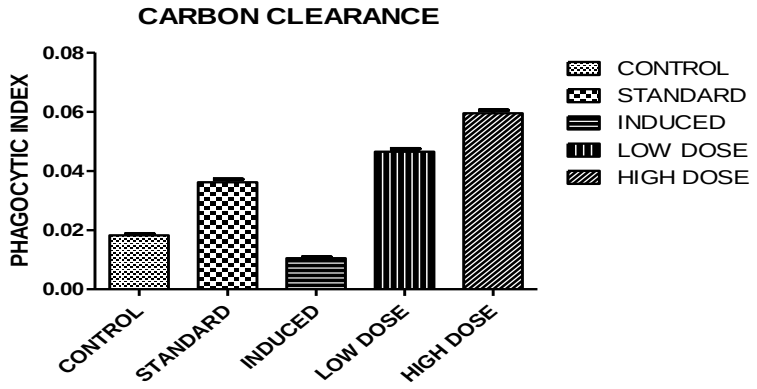


C. Cyclophosphamide-Induced Immunosuppression:

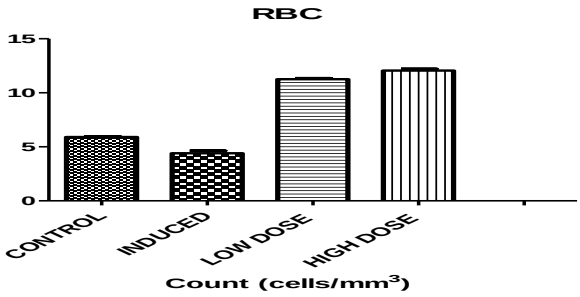
Cyclophosphamide administration caused a significant reduction in RBCs, WBCs and platelets count. Pre-treatment with HMF (both doses) significantly prevented the myleosuppression as compared to cyclophosphamide-alone-treated rats.

Table6.2.3 : Cyclophosphamide-Induced Immunosuppression :

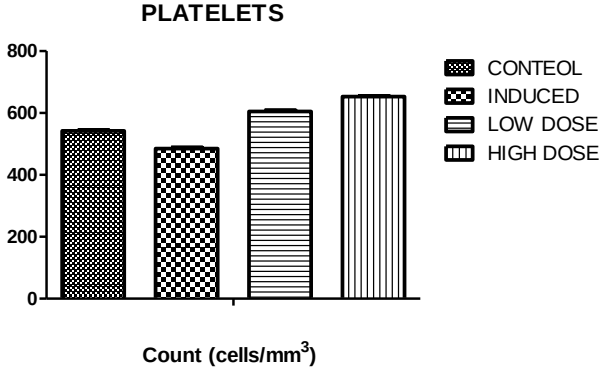
	RBC ($\times10^6$)	**Platelets ($\times10^3$)**	**WBC ($\times10^3$)**
Control (saline 10ml/kg)	6 ± 0.07	542.5 ± 1.707822	10.2 ± 0.18
Induced Cyclophosphamide (30 mg/kg)	4.400 ± 0.2386 ***	485 ± 3.41 ***	4.5 ± 0.35 ***
Low dose (30 mg/kg + HMF 100 mg/kg)	11.25 ± 0.103 ***b	605 ± 3.41 ***b	10.6 ± 0.069 ***b
High dose (30 mg/kg + HMF 200 mg/kg)	12.05 ± 0.17078 ***a	653 ± 2.05 ***a	12.15 ± 0.102 ***a

All the Values are expressed as MEAN±SEM (n=6) ONE way ANOVA followed by Dunnets method [a]*P*<0.0001, [b]*P*<0.0001 as compared to control group .

a.RBC:



b.Platelets :



c.WBC:

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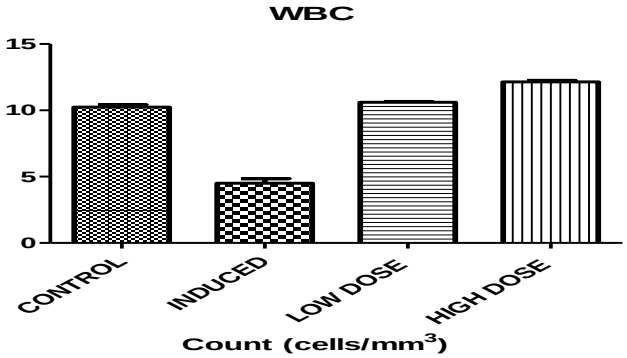


6.5 Anti-oxidant studies:

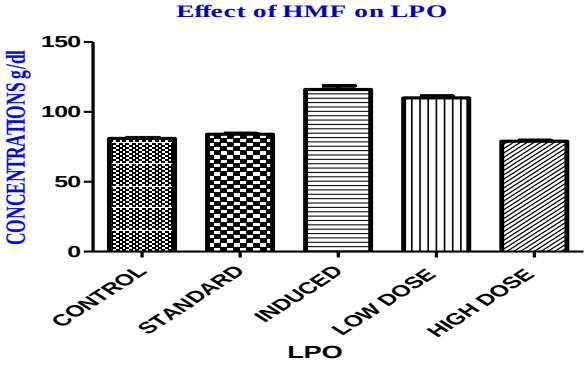
1. LPO (Lipid peroxidase estimation)
2. SOD (Superoxide dismutase estimation)
3. CAT (Catalase estimation)
4. Glutathione estimation

Effect of Herbo-mineral formulation on LPO, GSH, SOD, and CATALASE in Blood: In this study ,in the cyclophosphamide control animals , there was increase in LPO and decrease in SOD , CAT, GSH levels .In animals pre treated HMF the endogenous antioxidants such as SOD.CAT,GSH levels were increased and these increased endogenous antioxidants scavenge activity the reactive oxygen species so that the LPO levels were decreased in dose dependent manner . These results are comparable with that of Levomisole.

Effect of Herbo-mineral formulation on LPO, GSH, SOD, and CATALASE in Blood

S.no	Treatment	LPO1 (MMDA g-1Hb)	SOD (U mg-1 protien)	CAT (U mg-1 protien)	GSH (µmol g-1 Hb)
1	Saline 10ml/kg p.o	81 ± 0.683	23.500±0.341	222.5±1.707	3.4±0.0683
2	Levamisole (25 mg/kg) p.o.	84 ± 0.683 ***	21 ± 0.68312 ***	249 ± 0.683***	4.25±0.03415 ***
3	Cyclophosphamide 30 mg/kg i.p (11,12,13th days)	116 ± 2.732 **	15.5 ±0.3415 **	173 ± 0.683 ***	2.9±0.0683** *
4	Formulation 100 Mg HMF /day p.o	110 ± 1.366 ***	18.5 ±0.3415 ***	256.66 ± 1.264 ***	4.85 ± 0.0341 ***
5	Formulation 200 Mg HMF/day p.o	79 ± 0.683 ***	26 ± 0.6831 ***	285 ± 3.415 ***	5 ± 0.1366 ***

All the Values are expressed as MEAN±SEM (n=6) ONE way ANOVA followed by Dunnets method $^{***}P<0.0001$ vs Cyclophosphamide, $^{**}P<0.001$ vs Cyclophosphamide as compared to control group .



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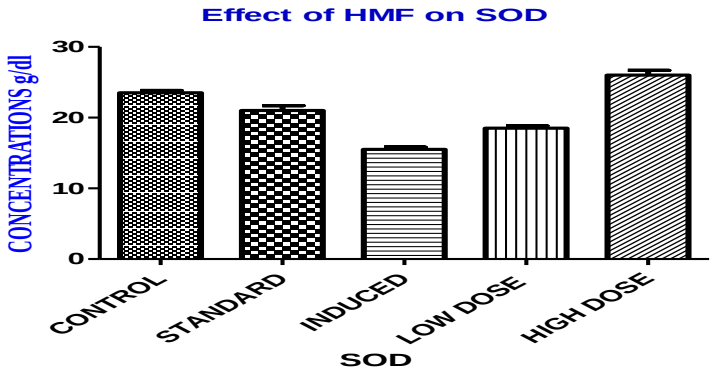
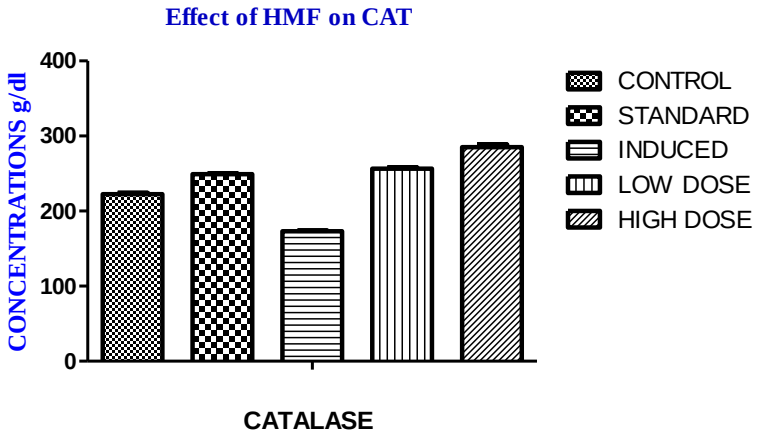
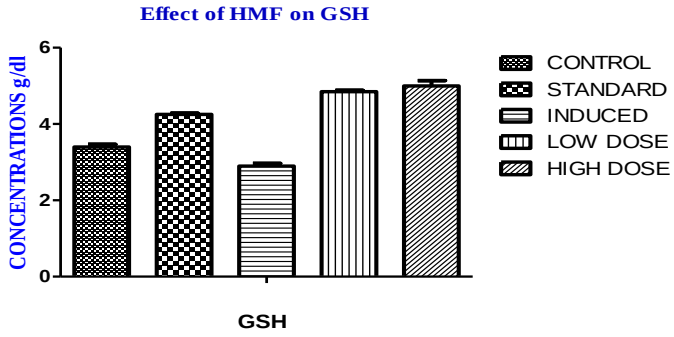
SOD:



CATALASE:



GSH:



Which specialized in the removed of forigen substances from the blood stream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon the blood by macrophage is governed by an exponential equation. Since both doses of HMF augmented the phagocytic index, it can conclude that RES was activated with 200 mg/kg then 100 mg/kg i.e . The immunomodulatory effect of HMF was also checked in cyclophosphamide include myeslosuppression animals modal. Cyclophsphamide is a nitron mustard subclass alkylating agent and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. The results showed that cyclophospamide 30mg/kg lowered the RBCs, platelets and total WBCs counts in the cyclophosphamide alone treated group.

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Interestingly, pre-treatment with HMF prevented the changes in haematoiogical parameters. The prevention of myelosuppression induced by cyclophosphamide may be through activation of marcrophages, which secrete a large number of substances including colony stimulating factor and interleukin-1. From the phytochemical analysis suggest that HMF exerts immunolatory activity, through the combined action of alkaloids, glycosides,carbohydrats, Flavonoids and tannis are known to possess immunostimulating activities. The well-known ayurvedic formulation, Herbo-mineral formulation consists of *Pterocarpus marsupium, Alpinia galangal, Pheonix dectylifera*, Zinc and selenium. The LPO, SOD, CAT, GSH levels were decreased in cyclophosphamide control rats as compared tocontrol. In HMF treated animals the LPO level was decreased and SOD, CAT, GSHlevels were increased in dose dependent manner as compared to cyclophosphamide control. These results are comparable with that due to standared, Levomisole. In the study, in the Cyclophophamide control animals, there was increase in LPO and decrease in SOD, CAT, GSH levels. In animals pre-treated with HMF the endogenous antioxidant such as SOD, CAT, GSH levels were increased and these increased endogenous antioxidants scavenge the reactive oxygen species so that LPO levels were decreased in dose dependent manner. These results are comparable with that of Levamisole.

CONCLUSION

In conclusion the present research works investigating the immunomodulatory and anti oxidant activity of HMF in animal's model of immunosuppressant. Blood parameters such as RBC, WBC & Platelets and antioxidant parameters in blood LPO, SOD, CAT & Glutathione. Were chosen to assess after treatment with HMF in 2 different doses (100mg/kg & 200 mg/kg p.o) and compared with toxicant agent cychophosphamide (30mg/hg I.p single dose a day for 3 days) and standard during levomisole (25 mg/kg p.o. single dose a day for 28 days). In this study, showed that Herbomineral formulation is a potent immunostimulant, stimulating both the specific & non specific immune mechanisms and also possess the anti oxidant activity.

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