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Screening of Free Radical Scavenging Activity and Immunomodulatory Effect of *Haratala Bhasma*

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Abstract

Haratala Bhasma (calx of *Haratala* i.e. Arsenic Trisulphide - As_2S_3), is categorized under *Uprasa* category of the *Rasashastr*a having some peculiar attributes. *Haratala Bhasma* has its therapeutic indication in many of the diseases mainly Shwas (respiratory disease), Kasa (cough), *Apasmar* (epilepsy), *Kushta* (leprosy), *Vatarakta* (gout), *Firang* (syphilis) etc. which are now established free radical mediated diseases. It is also having properties like *Vrushya* (aphrodisiac), *Rasayana* (immunomodulator). Review of the current literature available on *Rasayanas* indicates that Anti-oxidant and Immunomodulation are the most studied activities of the *Rasayan drugs.* The effect Immunomodulation has on the human body can be compared to some extent with the effect of *Rasayan dravyas*, given in various Classical texts. Antioxidants and Immuno-modulators are talk of the hour since a decade or two. Lot of research has been carried out in this regard but the need of them is not yet fulfilled. Hence assuming *Haratala Bhasma* possesses the anti-oxidant and Immune-modulators properties the present study was carried to validate it scientifically: *Haratal* was subjected to *shodhan* (purification), *maran* (incineration) as per *Rasatarangini. Bhasma* was subjected to physico chemical analysis and advanced analytical techniques like AAS, NPST, XRD and SEM etc. Screening of free radical scavenging activity was done using albino rat liver homogenate (ex-vivo) and with standard parameters like LPO, SOD, GSH and CAT. Immuno-modulatory effect was screened using three different healthy adult human blood samples with four parameters like Nitro-blue tetrazolium (NBT) assay for respiratory burst. Phagocytosis and candidacidal assay for phagocytic function and Chemotaxis to assay motility of neutrophills. Three different concentration of the test drugs (1%, 2%and 5%) were used for both screening. The results were self conclusive and indicated that 2% and 5% *Haratala bhasma* suspensions showed better results in free radical scavenging activity in 2 days and 4 days study in all the four parameters LPO, SOD, GSH and CAT. The immuno-modulatory screening showed *Haratal bhasma* has a better effect at a concentration of 5% when compared to 1% and 2% concentration. Thus, the study revalidates the reference of *Haratala Bhasma* as a *Rasayana* and hence also establishing it as an Immunomodulator.

Keywords: *Haratala Bhasma*; Antioxidant; Immunomodulatory Effect; *Rasayana.*

Introduction

Rasashastra, one of the branches of Ayurveda, deals with the pharmacological aspects of drugs, some of which have unique attributes. These unique attributes need to be validated and explored using the scientific and technological advances of today's world, which shall open up new avenues for drug processing, development and therapeutics in Ayurveda.

The present day life style and food habits have increased the production of free radicals. These cytotoxic free radicals not only raise the oxidative stress but also play an important role in immune system dysfunction due to which mankind is prone to various major ailments and it is now proved that diseases like Shwas, Kasa, Apasmar, Kushta, Vtarakta etc. free radical mediated ones. To tackle

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these free radicals our body needs antioxidant. An antioxidant is a molecule which is capable of inhibiting the oxidation of other molecule. Oxidation reactions can produce free radicals which in turn start chain reaction that damage cells. Antioxidant terminates these chain reactions by removing free radical intermediates and inhibits other oxidation reaction.

Many herbal drugs and compound herbal preparations have been screened for their antioxidant and immuno modulatory properties but still there is a need for effective antioxidants. This dearth and also fact that *Haratala Bhasma* is being used in treating many of the free radical mediated diseases prompted us to take the present study which aims to validate the free radical scavenging activity and immune-modulatory effect of *Haratala Bhasma* scientifically and explain its probable mode of action in such diseases at the cellular level.

A number of *Rasashastra* texts including *Rasaratna samucchaya* advocate the fact that *Haratala Bhasma* acts like a *Rasayan* (Immunomodulator)[1].

Hence assuming *Haratala Bhasma* possesses anti-oxidant and Immune-modulators properties the present study was carried to understand its preclinical efficacy with modern concept of *Rasayan* (Immunomodulator).

Aims and Objectives

Screening of free radical scavenging activity and immuno-modulatory effect of *Haratal Bhasma*.

Materials and Methods

Haratal bhasma was subjected to the screening of free radical scavenging activity in rat's liver homogenate with four parameters like LPO, CAT, GSH and SOD. Immuno-modulatory effect was screened in human blood samples using standard parameters like Nitro-blue tetrazolium assay, Phagocytosis, Candidacidal assay and Chemotaxis. The present experimental study was carried out in the Microbiology Research Lab Belagavi.

Test Drug

Haratal Bhasma prepared by classical method following *Rasatarangini (Taranga* 11/29, Pp 248) reference.

Study Design

I. Screening of Free Radical Scavenging Activity of Haratal Bhasma

Materials Required:

Drugs: *Haratal bhasma* and Vitamin C. Rat liver homogenate.

Glassware's: Beakers, Test tubes, Pipettes (1 ml, 5 ml and 10 ml) and Stirrers.

Equipment: Micropipettes, Incubator, Centrifuge, Vortex mixture, Shimadzu UV spectrophotometer 1700 and Water bath.

Method in General: Healthy rat liver was taken. Liver homogenate was prepared. Oxidative stress was induced in the homogenate by CCl_4. Drug was added to homogenate and the parameters were assessed. One day, two days and four days study was done.

• *Lipid Peroxidation* [2]

Principle

End products of Lipid peroxidation have a property that they react with Thiobarbituric acid and produce colour. Malondialdehyde (MDA) is one of the end products of lipid peroxidation. By measuring the colour produced by photometric method we can assess the concentration of MDA that gives the extent of Lipid peroxidation. The color produced by the reaction of thiobarbituric acid with MDA was measured at 540 nm with the help of spectrophotometer. The results were expressed as nmol/ml.

Procedure

i. Preparation of 30% Rat liver homogenate in 0.15M KCl [3]: Healthy adult albino rats (170 – 200 gm) of either sex were used for the study. Rats were kept for fasting for 16 – 18 hours then sacrificed by the method of decapitation. Liver removed immediately and rinsed with cold water. Then it was perfused with ice cold normal saline to remove the excess blood. Liver were chopped into small pieces and weighed. 15 gm of liver was mixed with 50 ml of 0.15M KCl and homogenized with Remi homogeiser. This homogenate was filtered and stored in conical flask.

ii. Preparation of Haratal bhasma (H.B.) suspension: 1%, 2% and 5% suspensions of *Haratal bhasma* were prepared with Compound Powder of Tragacanth (CPT). 1% *Haratal bhasma* suspension was prepared

by adding 1 gm of *Haratal bhasma* with 2 gm compound powder of tragacanth (CPT) into 100 ml of distilled water (D.W.). 2% *Haratal bhasma* suspension was prepared by adding 2 gm of *Haratal bhasma* with 4 gm of CPT into 100 ml of distilled water. 5% *Haratal bhasma* suspension was prepared by adding 5 gm of *Haratal bhasma* with 10 gm of CPT into 100 ml of distilled water. Tablet of Vitamin C weighing 550 mg dissolved in 100 ml of distilled water and 1 ml was used. The same suspension was used in testing all the four parameters.

Estimation of Lipid Peroxidation: In 6 conical flasks of 25 ml quantity, 4 ml of liver homogenate was taken. All the test tubes were added with 6 ml of Potassium sulphate buffer (pH 7.4) and 8 ml of 0.15 M Potassium chloride solutions. Test sample was excluded in first two flasks (control groups). In test group, three different conical flasks were added with 1 ml of three different concentrations of drug like 1%, 2% and 5%. In standard group, one ml of Vitamin-C was added. Finally 60 µl of carbon tetra chloride (CCl_4) was added except second control. Totally six flasks were incubated at 37^0C in incubator.

• *Superoxide Dismutase (SOD)* [4]

Principle

This method is based on the ability of SOD to inhibit auto-oxidation of pyrogallol under specific conditions. Superoxide dismutase is an enzyme which inhibits oxidation. Pyrogallol is autooxidative substance which gets oxidized when exposed to atmosphere. When pyrogallol is kept with homogenate it starts getting oxidized. But the SOD present in homogenate inhibits oxidation of pyrogallol. So by calculating the extent of inhibition of auto-oxidation of pyrogallol we can assess the concentration of SOD. Reading was taken at 420 nm and expressed as units/ml.

Procedure

i. Estimation of SOD: SOD was estimated on 1st day as follows: 50µl of reaction mixture from the homogenate which is kept for incubation was taken in a test tube. To it, 2.8 ml of Tris buffer solution was added. Just before checking its optical density, reaction mixture was added with freshly prepared 0.1 ml of 20mM pyrogallol. Immediately after addition of pyrogallol O.D. was seen using U.V. spectrophotometer at 420 nm at an interval of 1.5 min and 3 min. ΔA obtained for control (C) and Test (T). SOD was calculated by formula (C-T/Cx50) × 2000.

• *Reduced Glutathione (GSH)* [5]

Principle

Glutathione protects cells from the free radicals produced through oxidation. It can only do this by remaining in its naturally reduced state so that it is readily available to neutralize free radicals by bonding with them. As GSH bonds, it converts to its oxidized form, called glutathione disulfide. Then an enzyme-glutathione reductase-reverse it back to its reduced state. The ratio of reduced GSH to oxidized GSH within the cells can be used to measure cellular toxicity.

Procedure

i. Estimation of Glutathione: On 1st day 0.2ml of homogenate which was incubated was taken and mixed with 1.8ml of EDTA. To this, 3ml of precipitating reagent was added and mixed thoroughly with Vortex mixer and centrifuged for 15 min at 2800 rpm. 2ml of Supernatant was taken and added with 4ml of 0.3M disodium hydrogen phosphate solution and 1ml DTNB reagent. Then absorbance was read at 412nm inUV spectrophotometer. In 2 days and 4 days study addition of 1 ml of the different test drug suspensions and the standard drug were done to the homogenate mixture daily and the estimation of GSH was done as above at the end of 2 days and 4 days.

• *Catalase (CAT)* [6]

Principle

The enzyme catalase reacts with Hydrogen peroxide and converts it in to H_2O and O_2. In this study, liver homogenate was added with fresh solution of Hydrogen peroxide and the amount of Hydrogen peroxide utilized by the homogenate was calculated. By this we calculated the concentration of CAT present in the homogenate.

Procedure

i. Estimation of Catalase: On 1st day estimation, 10µl of reaction mixture from the homogenate which was kept for incubation was taken in a test tube and 5ml of Phosphate buffer solution was added. Just before checking its optical density, reaction mixture was added with freshly prepared Hydrogen peroxide solution. Immediately after addition of H_2O_2, O.D. was seen at 240 nm with the help of digital U.V. spectrophotometer from 0 to 60 min.

Concentration of CAT was calculated with the formula: ΔA × 4225.35. In 2 days and 4 days study addition of 1 ml of the different test drug suspensions and the standard drug were done to the homogenate mixture daily and the estimation of CAT was done as above at the end of 2 days and 4 days.

II. Screening of Immuno-modulatory effect of Haratal Bhasma [7]

• *Isolation of Neutrophils*

For any laboratory assay of neutrophils, it is preferred to isolate a relatively pure population of granulocytes or at least all white blood cells with minimum manipulation of blood unless otherwise indicated.

• *Nitro Blue Tetrazolium Test* [8]

Materials Required: Drug: Haratal Bhasma. Human blood samples – 3 samples (A, B and C). Equipments and Glassware: Micropipette, Incubator, Centrifuge, Light Microscope (having oil-immersion objective), Top pan balance, Water bath, Weighing Machine, Measuring cylinders, Test Tubes and Slides.

Procedure

- To 0.5ml of anti coagulated whole blood, add 200µl of 0.15% NBT and 0.2ml of Hanks balanced salt solution (HBSS).
- Prepare another set of tubes with above mentioned reagents and 0.05ml of endotoxin C prepared from Esch. coli.
- Incubate the tubes at 37°C for 20 mins. Mix well and prepare a thin film of smear on glass slide stain with Giemsa and study under oil immersion field of microscope.

Interpretation

- For the tube containing endotoxin, more than 80% of phagocytes cells should reduce NBT and demonstrate bluish black granules in the cytoplasm.
- For the first set of tubes without endotoxin. The percentage of NBT reducing cells should not be more than 20% in a healthy individual.

• *Phagocytosis And Candidacidal Assay* [9,10]

Principle

It is the twin method which can be performed at the same time for phagocytosis and for candidacidal assay. The cells are exposed to the candida albicans suspension. If the leucocytes are stimulated to phagocytic activity, the majority of candida cells will be engulfed by them. The leucocytes containing candida cells are clearly visible under light microscope which can be counted and MPN (Mean Particle Number) can be calculated.

• *Phagocytosis* [9]

Procedure

- Add 0.25ml of HBSS, 0.25ml of leukocyte suspension and 0.25ml of heat killed C. albicans to three sets of tubes.
- To one set of tubes, add 0.25ml of patient's serum. To second set, add pooled human serum from healthy individuals. To a 3rd set, do not add serum. Mix and incubate at 37°C for 30 minutes.
- Centrifuge, and prepare smears from the deposit count at least 100 phagocytes and count the number of Candida ingested per cell and express as mean particle number (MPN).

• *Candidacidal Assay* [10]

- Essentially, the basic procedure is same as for the phagocytosis. Only the dead Candidas are replaced with a suspension of live Candida cells.
- Mix and incubate for a period of 1hr. at the end of incubation, add 2.5% sodium deoxycholate to each tube and mix. This lyses leukocytes without damaging Candida cells. Later, add 4ml of 0.01% methylene blue carefully to each tube, mix and centrifuge for 10 minutes. Resuspend the deposit in 0.5ml of the material, prepare a film and count the % of dead cells i.e those which have taken up methylene blue and appear blue in colour.
- Maximum activity was seen with 5% *Haratal bhasma* suspension and we can infer that activity increased with the increase in concentration of the drug in our study. The candidacidal activity was seen to be better in all the concentrations when compared to the positive control used.

• *Neutrophil Locomotion And Chemotaxis Test*

Principle

When the cells are placed in a gradient of chemo attractant, the cells change their shape as they orient and migrate in unison towards the source of stimulus, a process called as "chemotaxis". Most of neutrophil locomotion assesses the behaviour of a population of cells moving through cellulose nitrate filters or under agarose. The cells are allowed to move a set time period then fixed, stained and assessed.

Procedure

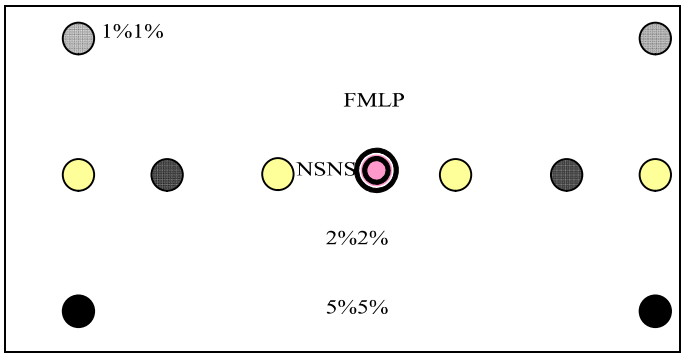
Three samples of neutrophil suspensions from blood were taken viz A, B and C. The method for neutrophil isolation was carried out according to previous method i.e. phagocytosis and candidacidal assay. Later the wells in agarose were filled.

- *Chemotaxes* [10]

Procedure

Prepare 1.2% agarose gels containing Minimum Eagles medium, pooled human serum and sodium bicarbonate, after the gels are set, cut series of 3 wells, 3mm in diameter and 3mm apart. Add FMLP a known chemo attractant to the central well and the test samples to the peripheral wells. Incubate for 2hrs. Fix the plates in methanol followed by formalin. Remove agarose layer and stain cells with Giemsa stain. Calculate the distance the cells have traveled from the edge of the peripheral wells towards wells containing FMLP.

Observations and Results



Same design was fallowed for all the samples A,B and C

- Fmlp (known chemo attractant) - Neutrophil suspension (NS)

- 1%, H.B. -2% H.B -5% H.B

Fig. 1: Filling of wells for Chemotaxis Test

Screening of Free Radical Scavenging Activity of Haratal Bhasma

In lipid peroxidation, the concentration of MDA was found to be increased in homogenate treated only with CCl_4 i.e 38.24, 40.15and 39.56 nmol/ ml in 1 day, 2 days and 4 days study respectively. This is because of oxidative stress induced by CCl_4 and lack of protective agents against oxidation. Maximum decrease in concentration of MDA was seen with 2% and 5% drug suspension in 1 day, 2 days and 4 days study. 1% suspension showed no decrease in concentration of MDA as compared to other two concentrations. 5% suspension was effective preventing lipid peroxidation (Table 1).

Table 1: Estimation of lipid peroxidation

Sl. No	Groups	Concentration of MDA in nmol/ml		
		1 day	2 days	4 days
1	Control	25.36	30.24	24.15
2	Homogenate with CCl_4	38.24	40.15	39.56
3	Standard with CCl_4	21.03	22.32	20.36
4	1% *Haratal Bhasma with* CCl_4	27.30	25.23	24.58
5	2% *Haratal Bhasma* with CCl_4	21.03	20.35	23.54
6	5% *Haratal Bhasma* with CCl_4	20.15	19.23	18.23

The concentration of SOD (units/ml) was found to be less i.e. 20.21, 21.02 and 19.23 in homogenate treated only with CCl_4 in 1 day, 2 days and 4 days study respectively. This is due to oxidative damage caused by CCl_4. Test drug with concentration of 1%, 2% and 5% significantly increased the concentration of SOD in 4 days study. Comparatively 2% and 5% suspensions were found significant in increasing concentration of Superoxide dismutase in 2 days and 4 days study. No activity was observed in 1 day study at the concentration studied (Table 2).

Maximum concentration of GSH (units/mg protein) with 2% drug suspension was 13.56,12.85 and 11.22 in 1 day, 2 days and 4 days study respectively and with 5% suspension it was 14.23, 13.25 and 12.77 respectively. The effect on 1st day was not considerable in any of the concentrations. Though the activity was not as much as the standard drug, it can be seen that the action of test drug is dose and time dependent (Table 3).

Maximum decomposition of H_2O_2 i.e activity of Catalase enzyme was observed with 2% and 5% drug suspension in 2 days and 4 days study. The results also showed better results for even 1% concentration. With 1% drug suspension the readings (units/mg protein) were 124.58 and 119.65 on 2 days and 4 days study respectively (Table 4).

Table 2: Estimation of superoxide dismutase (SOD)

Sl. No	Groups	Concentration of SOD (units/ml)		
		1 day	2 days	4 days
1	Control	31.25	29.36	30.21
2	Homogenate with CCl_4	20.21	21.02	19.23
3	Standard with CCl_4	25.03	24.16	26.38
4	1% Test drug with CCl_4	18.23	23.25	26.35
5	2% Test drug with CCl_4	23.87	23.56	27.23
6	5% Test drug with CCl_4	21.03	25.35	24.56

Table 3: Estimation of reduced glutathione (GSH)

Sl. No.	Groups	Concentration of GSH (Units/mg protein)		
		1 day	2 days	4 days
1	Control	12.03	13.25	13.86
2	Homogenate with CCl_4	15.26	18.59	19.35
3	Standard with CCl_4	10.23	9.23	10.74
4	1% *Haratal Bhasma* with CCl_4	11.23	12.32	11.85
5	2% *Haratal Bhasma* with CCl_4	13.56	12.85	11.22
6	5% *Haratal Bhasma* with CCl_4	14.23	13.25	12.77

Table 4: Estimation of catalase

Sl. No	Groups	Catalase (Units/mg protein)		
		1 day	2 days	4 days
1	Control	135.26	140.23	138.59
2	Homogenate with CCl_4	96.35	89.56	92.12
3	Standard with CCl_4	155.28	159.36	157.45
4	1% *Haratal Bhasma* with CCl_4	125.23	124.58	119.65
5	2% *Haratal Bhasma* with CCl_4	134.26	132.02	137.25
6	5% *Haratal Bhasma* with CCl_4	142.03	146.35	144.96

III. Screening of Immuno-modulatory effect of *Haratal Bhasma*

Table 5: Results of Immuno-modulatory Study

	NBT (%)	Phagocytosis (MPN)	Candidacidal Assay (%)	Chemotaxis (mm)
100	58%	3MPN	37%	2.0mm
50	58%	3MPN	36%	1.6mm
25	54%	2MPN	20%	1.2mm
12.5	37%	2MPN	18%	1.0mm
5	21%	2MPN	16%	0.6mm
				FMLP-2.2mm
				Media 0.6mm

Discussions

• *Free Radical Scavenging Activity*

Free radical scavenging activity of *Haratal bhasma* was assessed with 1 day; 2 days and 4 days study in rat liver homogenate in which oxidative stress was induced with Carbon tetra chloride ($CCl_{4)}$. CCl_4 is hepatotoxic due to its ability to destruct liver cells, possibly by a free radical mechanism. Lipid per oxidation reaction, a type of oxidative degeneration of polyunsaturated fatty acids, has been linked with altered membrane structure and enzyme inactivation. Exact mechanism may be by inhibiting mitochondrial respiration i.e. inhibition of consumption of O_2 by mitochondria. Initially 40 µl of CCl_4 was tried to induce oxidative stress but the results were not satisfactory. So 60 µl was used and it was sufficient to induce the oxidative stress.

Lipid Per Oxidation

In lipid per oxidation, the concentration of MDA was found to be increased in homogenate treated only with CCl_4 i.e. 38.14, 40.15 and 39.56 nmol/ ml in 1 day, 2 days and 4 days study respectively. This is because of oxidative stress induced by CCl_4 and

lack of protective agents against oxidation. Maximum decrease in concentration of MDA was seen with 2% and 5% drug suspension in 1 day, 2 days and 4 days study. 1% suspension showed no decrease in concentration of MDA as compared to other two concentrations. 5% suspension was effective preventing lipid per oxidation.

Superoxide Dismutase (SOD)

The concentration of SOD (units/ml) was found to be less i.e. 10.66, 9.45 and 8.66 in homogenate treated only with CCl_4 in 1 day, 2 days & 4 days study respectively. This is due to oxidative damage by CCl_4. It should be remembered that CCl_4 was added only on 1st day.

The concentration of SOD (units/ml) was found to be less i.e. 20.21, 21.02 and 19.23 in homogenate treated only with CCl_4 in 1 day, 2 days and 4 days study respectively. This is due to oxidative damage caused by CCl_4. Test drug with concentration of 1%, 2% and 5% significantly increased the concentration of SOD in 4 days study. Comparatively 2% and 5% suspensions were found significant in increasing conc of SOD in 2 days and 4 days study.

Reduced Glutathione

Maximum concentration of GSH (units/mg protein) with 2% drug suspension was 13.56, 12.85 and 11.22 in 1 day, 2 days and 4 days study respectively and with 5% suspension it was 14.23, 13.25 and 12.77 respectively. The effect on 1st day was not considerable in any of the concentrations. Though the activity was not as much as the standard drug, it can be seen that the action of test drug is dose and time dependent.

Catalase

Maximum decomposition of H_2O_2 i.e. activity of Catalase enzyme was observed with 2% and 5% drug suspension in 2 days and 4 days study. The results also showed better results for even 1% concentration. With 1% drug suspension the readings (units/mg protein) were 124.58 and 119.65 on 2 days and 4 days study respectively.

- *Immuno Modulatory Effect of Haratal Bhasma*

NBT Test: Stimulated neutrophils for phagocytosis were counted under light microscope using NBT dye in three blood samples named A, B and C. E-coli endotoxin was used as standard or positive control drug. *Haratal bhasma* suspension of three concentrations 1%, 2% and 5% were used

Phagocytosis

Here neutrophils suspension from three different blood samples A, B and C were exposed to candida albican cells and the mean particle number (MPN) of candida engulfed by the neutrophils was calculated. Pooled human blood serum was used as positive control. *Haratal bhasma* suspensions of three different concentrations 1%, 2% and 5% were used as test drug.

Candidacidal Assay

Dead candida cells (in %) were calculated in this assay. Pooled human blood serum was used as positive control. *Haratal bhasma* suspensions of three different concentrations 1%, 2% and 5% were used as test drug.

Chemotaxis

The distance traveled (in mm) by the neutrophils under the influence of a known chemo attractant and the three concentrations 1%, 2% and 5% of *Haratal bhasma* was noted. Fm-leu-phe was used as chemo attractant which acted as positive control.

Conclusion

For preventing lipid peroxidation (LPO) concentration of 2% *Haratal bhasma* was sufficient and in 4 days study the results were slightly better than standard drug which indicates that the action of drug increased as the duration of the drug administration increased.

In SOD, GSH and CAT study 5% *Haratal bhasma* suspension showed better results which indicate that as the dose increases the action also increases.

Immuno-modulatory study also revealed similar results in which 5% drug suspension showed significant results in all the four parameters i.e. NBT, Phagocytosis, Candidacidal assay and Chemotaxis.

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