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EXPERIMENTAL EVALUATION OF IMMUNO MODULATORY EFFECT OF *YASHADA BHASMA* (INCINERATED ZINC)

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ABSTRACT

The main aim of the study was to scientifically validate the immuno modulatory effect of *Yashada bhasma*. *Yashada bhasma*, prepared as per *Rasachandamshu*, was subjected to screening of immuno modulatory effect using three healthy adult human blood samples and with three different drug concentrations i.e. 1%, 2% and 5%. The parameters used were Nitro blue tetrazolium assay, Phagocytosis, Candidacidal assay and Chemotaxis. 5% drug suspension showed significant results in all the four parameters.

Keywords: *Yashada bhasma,* Immuno modulators, *Ayurveda*

INTRODUCTION

The present day lifestyle 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 the immune-system dysfunction due to which the mankind is prone to various major ailments and it is now proved that diseases like *Prameha* (diabetes), *Pandu* (anemia), *Vata vyadhi* (neuro muscular diseases) etc are free radical mediated ones[1]. To tackle these free radicals our body needs antioxidants and potent immuno modulators. Many herbals drugs and compound herbal preparations have been screened for their immuno modulatory properties but still there is a need for effective immuno modulators. *Yashada bhasma* prepared by following standard operative procedures laid by the classics is said to have properties like *Kapha pitta hara, Balya, Medhya, Veerya vardhaka, Parama chakshushya, Pandu, Prameha, Shwasa, Kasa, Vrana srava, Kampavatahara* etc[2]. Hence *Yashada bhasma* was prepared classically and was screened to validate its immuno modulatory effect.

AIMS AND OBJECTIVES

1. To prepare *Yashada Bhasma* according to *Rasachandamshu*
2. To screen its immuno modulatory effect

MATERIALS AND METHODS

Pharmaceutical Study:

Major Materials: *Yashada* (Zinc), *Parada* (Mercury) and *Gandhaka* (Sulphur) were the major raw materials which were collected from the department of Rasashastra and Bhaishajya kalpana according to the *Grahya Lakshnas*[3] (desired qualities) mentioned in the classics and also depending upon the % of purity.

Table 1: Analysis of Raw drugs

Drug	Test	Result (%)
Yashada[*]	% of Zn	94.42
*Parada (*Merck Co.*)*[#]	% of Hg	99.99
Gandhaka[#]	Total Sulphur	99.62

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Yashada Shodhana* and *Marana:

Yashada (Zinc metal) and the associated materials used for the preparation of *Yashada bhasma* were procured from the Dept of Rasashastra of the institution. *Samanya shodhana*[4] (general purification) was done by the *Dhalana* (liquefying and pouring) method in *Kanji* (sour gruel), *Takra* (butter milk), *Kulattha* (*Dolichus biflorus*) *kwatha* (decoction), *Gomutra* (Cow's urine) and *Tila* (Sesamum indicum) *Taila* (oil). *Dhalana* was carried out three times in each liquid media. After *samanya shodhana*, *Vishesha shodhana*[5] (specific purification) was carried out in *Churnodaka* (lime water) for seven times. After *shodhana*, the metal became more brittle and was then subjected to *Jarana*[6] (roasting) using *Apamarga panchanga churna (Achyranthes aspera).* After *Jarana*, the metal was converted into a very fine grey shining powder which was deemed fit for *Marana* (incineration). The powder was then subjected to *Marana*[7] by triturating it with *Shuddha Parada* (purified Mercury) and *Shuddha Gandhaka* (purified Sulphur) both 1/4th quantity of *Yashada*, to form a black powder, to which one *bhavana* (triturating in liquid media) each with *Kumari swarasa* (fresh juice of Aloe vera) and *Nimbu swarasa* (fresh juice of *Citrus limon*) was given and *Chakrikas* (pellets) prepared. After drying, they were kept in *sharava* (casseroles), *sandhi bandhana* (sealing) was done and subjected to *Gajaputa* (heating system). After two *Gajaputas*, *Yashada bhasma* of yellowish color was obtained.

Experimental Study

Screening of Immuno-modulatory effect of Yashada Bhasma[8]

Materials Required: Drug: Yashada bhasma. Human blood samples – 3 (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.

Nitro blue tetrazolium test

Principle:

Neutrophils when exposed to the yellow dye of NBT, take up the dye into phagosomes and intracellular reduction of dye converts them to an insoluble, blue crystalline form (formazan crystals). Un-stimulated neutrophils do not ingest this dye. These blue crystals are visible in the light microscope and can be counted. The NBT test gives information about phagocytic function, since the dye is not taken into cells except by phagocytosis.

Procedure:

Preparation of Chemicals and Reagents:

- *E. coli* Endotoxin Standard: 20 ml of broth from each of 5 strains of *E. coli* was taken and boiled on water bath for 2 hrs. Then centrifuged at 2000 rpm for 30 min and pooled the supernatant to store it as 1 ml aliquots at –20^{0}C.
- Nitro blue tetrazolium (NBT): 30g of NBT powder added in 10 ml of sucrose solution.

- Minimum Essential Medium (MEM): 1.17g of MEM powder was dissolved in 100 ml of distilled water.

Preparation of *Yashada bhasma* suspension: Compound powder of Tragakanth was used as suspending agent. Preparation of compound powder of Tragakanth: 7.5 g of Tragakanth, 10 g each of gum acasia and soluble starch and 22.5 g of finely powdered sucrose were taken and mixed well.

1%, 2% and 5% suspensions of *Yashada bhasma* were prepared with Compound Powder of Tragacanth (CPT). 1% *Yashada bhasma* suspension was prepared by adding 1g of *Yashada bhasma* with 2g compound powder of tragacanth into 100 ml of distilled water (D.W.). 2% *Yashada bhasma* suspension was prepared by adding 2g of *Yashada bhasma* with 4g of compound powder of tragacanth into 100 ml of distilled water. 5% *Yashada bhasma* suspension was prepared by adding 5g of *Yashada bhasma* with 10g of compound powder of tragacanth 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.

Table 2: *Yashada bhasma* suspensions

Ingredients	1% YB Suspension	2% YB Suspension	5% YB Suspension
Y.B.	1 g	2 g	5 g
CPT	2 g	4 g	10 g
D.W.	100 ml	100 ml	100 ml

Estimation of NBT positive cells: Three healthy blood samples were taken viz. A, B and C. Suspension of leucocytes was made ($5x10^6$ / ml) in 0.5 ml Phosphate buffer solution (PBS). 0.1 ml endotoxin-activated plasma was added. 0.2 ml freshly made-up 0.15% NBT solution was added and incubated at 37^oC for 20 min. and then gently spun at 400 rpm for 3-4 min. The supernatant was discarded. One drop of PBS was added and gently re-suspended the cells in the small volume of fluid at the bottom of the tube. A film was prepared by allowing a drop of this fluid to dry on a microscope slide. Using an oil immersion objective, 300 neutrophils were counted and the % of NBT positive cells containing blue deposits determined.

Table 3: Filling of each test tube in NBT

Ingredients for NBT Test	1% Suspension	2% Suspension	5% Suspension	PC (Positive control)	NC (Normal control)
MEM	150µl	150µl	150µl	200µl	250µl
NBT	50µl	50µl	50µl	50µl	50µl
DRUG	100µl	100µl	100µl	-	-
BLOOD	100µl	100µl	100µl	100µl	100µl
ENDOTOXIN	-	-	-	50µl	-

All the test tubes were incubated for 20mins at 37^0C. Then kept at room temperature for 20 min. Smear was prepared. Slides were dried for 10-15 min. Methanol fixation was carried out and again slides were kept for drying. Then stained in Giemsa stain for 15 min and washed under tap water. After complete drying the slides were observed under light microscope with oil-immersion objective.

Observations and Results

Table 4: Results of NBT (Stimulated cells in %)

Blood sample	Blood sample - A			Blood sample - B			Blood sample – C		
Concentration of drug	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.
Stimulated cells	65%	73%	82%	68%	70%	85%	66%	69%	80%
NC	19%			20%			23%		
PC	78%			79%			77%		

Statististical Analysis

Table 5: Kruskal Walli's test for NBT results

Mean of 1 %	66.3 ± 1.52
Mean of 2 %	70.6 ± 2.08
Mean of 5%	82.3 ± 2.57
Chi square	7.2
DF	2
P	0.027
Result	Significant

Maximum stimulation was seen with 5% Yashada bhasma suspension and we can infer that stimulation increased with the increase in concentration of the drug in our study.

Phagocytosis and Candidacidal Assay

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.

In candidacidal assay, sodium deoxicholate and Methylene Blue are added to the same cells containing candida albicans. Sodium deoxycholate lyses the leucocytes but do not damage candida cells. Methylene blue is used for staining of dead candida cells. Using an improved Neabauer counting chamber the proportion of dead cells i.e. those which have taken up the methylene blue can be determined.

Procedure:

Preparation of chemicals and reagents: Minimum Essential Medium (MEM): was prepared same as in the NBT test. Sodium deoxicholate 2.5% in distilled water, pH = 8.7. Methylene Blue solution: 0.01% in 0.15 M NaCl.

Isolating Neutrophils by Dextran sedimentation method:

Materials:

i) Dextran solution – 6% in 0.15 M NaCl
ii) MEM – As tissue culture medium
iii) Preservative free heparin – 15U/ml blood

Heparinized blood sample, 3 ml, was diluted with 3 ml of MEM. Diluted blood was then mixed with 1.5 ml of dextrone carefully by rotation to avoid air bubbles in which red blood cells could be trapped. Incubation was carried out at 37^0C for 25 min without disturbing. The supernatant was

removed into 3 to 4 centrifuge tubes and an equal volume of MEM was added.

The tubes were then centrifuged at 500 rpm for 10 min. The supernatant was discarded and the cells were carefully flicked up. A small volume of MEM was used to wash the contents of 2 tubes into one and then was filled to 10µl with MEM. The liquid was centrifuged at 500 rpm for 10 min. Above step i.e. pooling the neutrophils into one tube was repeated, centrifuged further at 500 rpm for 10 min, supernatant liquid was then discarded after which cells were flicked up and finally 850µl of MEM was added to make the volume up to 1 ml.

Candida albicans suspension: Candida albicans were grown on glucose peptone agar. A culture suspension of 24 hour old was prepared and used for test.

Table 6: Filling of test tubes for Phagocytosis and Candidacidal Assay

Ingredients	1% of Y.B.	2% of Y.B.	5% of Y.B.	PC	NC
MEM	100µl	100µl	100µl	100µl	200µl
Neutrophil Suspension	100µl	100µl	100µl	100µl	100µl
Candida suspension	100µl	100µl	100µl	100µl	100µl
Test drug	100µl	100µl	100µl	-	-
Serum	-	-	-	100µl	-

All the test tubes were kept in an incubator for 30 min at 37^0C. Then 0.1 ml from each test tube was taken and smear prepared on glass slides for phagocytosis assay. Further all test tubes were incubated in an incubator for 30 minutes at 37^0C for candidacidal assay. Meanwhile, for phagocytosis assay, methanol fixation and Giemsa staining were carried out and slides were kept for drying. After 30 min, 250 µl of 2.5% sodium deoxycholate and 1 ml of methylene blue indicator were added to each tube. Centrifugation was done for 10 min at 1000 rpm. Supernatant liquid was discarded and only the sediment part was collected. One drop from each test tube was taken on separate slides. Cover slips were placed over that drops and each slide was then observed under light microscope for dead candida cells and counted at least 300 cells per slide using Neubauer's chamber.

Observations and Results

Table 7: Results of Phagocytosis (mean particle number)

Neutrophil Suspension	Suspension A			Suspension - B			Suspension - C		
Conc. of drug	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.
Candida engulfed	4	5	5+	5	5+	5+	4	5	5+
NC	3			4			3		
PC	6			5			6		

Table 8: Results of Candidacidal assay (Dead candida cells in %)

Neutrophil Suspension	Neutrophil Suspension A			Neutrophil Suspension - B			Neutrophil Suspension - C		
Concentration of drug	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.
Dead candida cells	38%	42%	48%	36%	40%	44%	40%	45%	46%
NC	19%			17%			18%		
PC	33%			34%			32%		

Statistical Analysis

Table 9: Kruskal Walli's test for phagocytosis and candidacidal assay

Test	Phagocytosis	Candidacidal assay
Mean of 1 %	4.3 ± 0.28	38 ± 2
Mean of 2 %	5 ± 0	42.3 ± 2.57
Mean of 5%	5 ± 0	46 ± 2
Chi square	7.714	6.118
DF	2	2
P	0.021	0.047
Result	Significant	Significant

Maximum activity was seen with 5% Yashada 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:

Preparation of chemicals and reagents:

a) 0.024 gm/ml agarose: 0.24 gm of agarose was dissolved in 10 ml of distilled water by heating on water bath for 10-15 min and then cooled.

b) Supplemented MEM:

- 2 ml MEM
- 2 ml heat inactivated pooled human serum
- 0.2 ml of 7.5% sodium bicarbonate
- 5.8 ml of sterile distilled water

c) 10^{-8} M. Fm-leu-phe (As known chemo attractant)

d) Staining reagents per plate

- 3-5 ml of methanol
- 3-5 ml of formaline
- Giemsa stain

Preparation of agarose culture plates: 10 ml of pre-warmed supplemented MEM was added to 10 ml of 0.024 gm/ml of agarose and around 5 ml of this mixture was added to each culture plate. The medium was then allowed to solidify. After solidifying, appropriate wells in agarose were made.

Arrangement of wells in agarose plates: Using Pasteur pipette, wells were prepared carefully measuring 3 mm in the diameter and spaced apart.

Neutrophil locomotion and chemotaxis test: 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. 0.01 ml of Fm-leu-phe was added to the most centrally located well of the slides. 0.01 ml of neutrophil suspension was added to each of the blank well as shown in Fig 3. Lastly, 0.01 ml of 1%, 2% and 5% suspension of Yashada bhasma were added to the shaded well in three direction of one side of slide. Same was repeated on the

other end of the slides. After putting all the suspension, slides were kept for charging i.e. kept in an incubator at 37^0C for 2 hrs. Then the slides were flooded in methanol for 30 min. The slides were kept flooding in formaline for 30 min. Agarose gel was removed carefully from slides. Giemsa staining was carried out and slides were kept for drying purpose. After complete drying, slides were observed under light microscope. A distance traveled by the cells (distance of cell's migration) was noted.

Observations and Results

Table 10: Results of Neutrophil Locomotion and Chemotaxis test (In mm)

Neutrophil Suspension	Neutrophil Suspension - A			Neutrophil Suspension - B			Neutrophil Suspension - C		
Concentration of drug	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.	1% Y.B.	2% Y.B.	5% Y.B.
Movement	1.2	2.0	2.3	1.4	2.2	2.2	1.1	2.2	2.0
PC	3.0			3.2			3.0		

Statistical Analysis

Table 11: Kruskal Walli's test for chemotaxis

Mean of 1 %	1.23 ± 0.15
Mean of 2 %	2.13 ± 0.11
Mean of 5%	2.16 ± 0.15
Chi square	5.728
DF	2
P	0.057
Result	Non significant

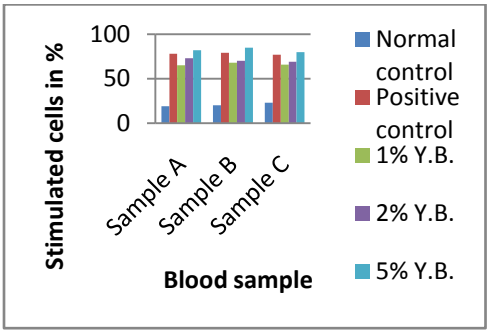
No much difference was seen in the results of 2% and 5% drug suspension. But mean value indicates 5% suspension has better result comparatively.

DISCUSSION

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.

Graph 1: Results of NBT test

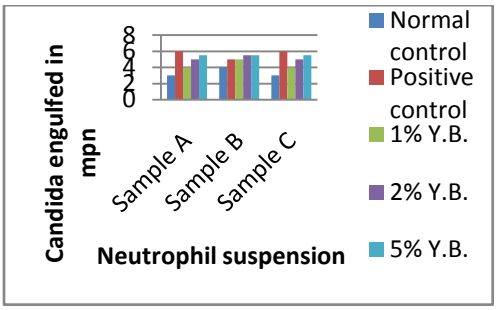


With P value 0.027, the results obtained were significant. We can infer that activity increased with the increase in the concentration of the drug. Maximum stimulation was seen with 5% *Yashada bhasma* suspension (mean 82.3 ± 2.57).

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.

Graph 2: Results of Phagocytosis (mpn)

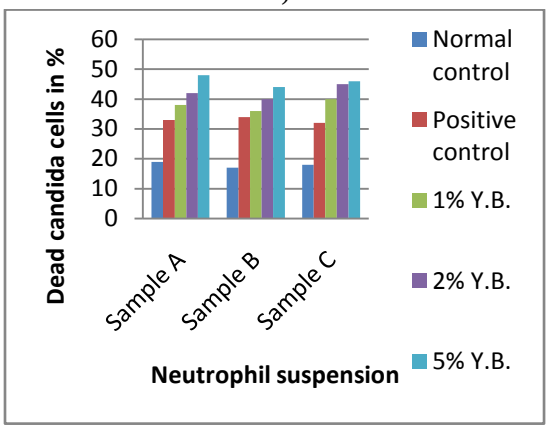


With P value 0.021 the result was significant and it was seen that the activity increased with the increase in the concentration of the drug. Maximum activity was seen with 5% *Yashada bhasma* suspension and it was almost equal to the positive control values.

Candidacidal assay

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

Graph 3: Results of Candidacidal assay (dead cells in %)

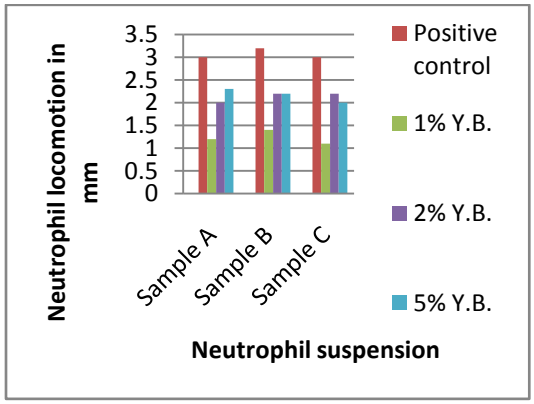


With P value 0.047 the result was significant and it was seen that the activity increased with the increase in the concentration of the drug. Maximum activity was seen with 5% *Yashada bhasma* suspension. The activity was seen better in all the three concentrations when compared with the positive control used.

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 *Yashada bhasma* was noted. Fm-leu-phe was used as chemo attractant which acted as positive control.

Graph 4: Results of Chemotaxis



With P value 0.057 the result was non – significant. There is no much difference in the result of 2% and 5% drug suspension. May be the known chemo attractant which was used was more powerful which attracted most of the cells towards it. But the results were good in 2% and 5% when compared to 1% drug suspension.

CONCLUSION

Samanya Shodhana by *Dhalana* in *Kanji*, *Takra*, *Kulattha kwatha*, *Gomutra* and *Tila taila* for 3 times in each media in the specified order, *Vishesha Shodhana* by *Dhalana* in *Churnodaka* for 7 times, *Jarana* by using *Apamarga panchanga churna* and *Marana* by two puta were sufficient to obtain yellow colored *Yashada bhasma* which passed all the classical *bhasma parikshas*. Immuno-modulatory study revealed 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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