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An in-Vitro Evaluation of Immunomodulator Effect of *Shataputa Abhraka Bhasma*

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Abstract

Abhraka Bhasma (Calx of Incinerated Mica) is one among the *Maharasas* of the *Rasashastra* having some peculiar attributes. *Acharyas* have mentioned *Shataputa* and *Sahashraputa Abhraka Bhasma* which are indeed unique attributes of *Abhraka*. *Abhraka bhasma* has its therapeutic indication in many of the diseases mainly *Yakshma, Prameha, Pandu, Raktapitta, Jwara, Shwas, Kasa, Grahani etc.* which are now established free radical mediated diseases. It is also having properties like *Balya, Vrushya, Rasayan & Medhya. Shataputa Abhraka Bhasma* is regarded as a *Rasayan*, as given in *Rasa Tarangini*, which says '*Abhraka Bhasma* when given 20-100 *Putas* (incinerations) helps to eliminate ailments, when given 100-1000 *Putas* acts like a *Rasayan'*. 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 *Abhraka bhasma* possesses the anti-oxidant and Immune-modulator properties the present study was carried to validate it scientifically and statistically: Screening of immuno-modulator effect of *Shataputa Abhraka Bhasma* was carried out using three different healthy adult human blood samples and with three different drug concentrations i.e. 1%, 2% and 5%. The parameters used were Nitroblue Tetrazolium Test (NBT assay), Phagocytosis, Candidacidal assay and Chemotaxis. The results were self conclusive and indicated that *Shataputa Abhraka Bhasma* brings about stimulation of Leucocytes in concentration dependent manner. Significant results were seen with the 5% drug concentration (suspension) in all the four tests which indicate *Shataputa Abhraka Bhasma* is a potential immuno-modulator. 5% suspension of *Shataputa Abhraka Bhasma* stimulated leucocytes respectively, which is an indicator of highly significant phagocytic activity. Thus, the study revalidates the reference of *Shataputa Abhraka Bhasma* as a *Rasayan* and hence also establishing it as an Immunomodulator.

Keywords: *Shataputa Abhraka Bhasma*; *Rasayan*; Immunomodulatory Activity.

Introduction

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 *Prameha, Pandu, Kshaya, Vatavyadhi* are free radical mediated ones. To tackle 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 *Shataputa Abhraka bhasma* is being used in treating many of the free radical mediated diseases

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prompted us to take the present study which aims to validate immuno-modulator effect of *Shataputa Abhraka bhasma* scientifically and explain its probable mode of action in such diseases at the cellular level. A number of *Rasashastra* texts including *Rasa Tarangini* advocate the fact that *Abhraka* when given 20-100 *Putas*, helps in *Rognivrutti* (curing diseases) but when *Putas* are increased from 100-1000, it acts like a *Rasayan* (prevents disease) [1]. An attempt was made to understand and find a relation between the number of *Putas* and its preclinical efficacy with modern concept of *Rasayan* (Immunomodulation).

Concept of *Rasayan*

Rasayan is one of the eight major divisions (branches) of *Astanga Ayurveda*. *Rasayan* Therapy is the part of *Ayurveda* which deals with healthy life and longevity of human being [2], and fights against diseases to maintain health of a diseased person and gives strength to all *dhatu*s. *Rasayan* Therapy improves our inner immune power and keeps away the old-age decline of live. So, *Rasayana* therapy is very important to retain the youthfulness of body, mind and useful for both healthy as well as diseased people.

The word *Rasayan* is made up of two words, '*Rasa*' and '*Ayan*'. *Rasayan* means the pathway to attain best quality of *Dhatus*. It helps to maintain the health and also cures and prevents ailments [3]. In *Ayurveda* one of the major methods of presentation of positive health has been described i.e. *Rasayana.*

As we know, in this modern era, every human being is living under pressure to survive. This stressful life harms our health, happiness and inner sense of well being. Increased toxicity in society, pollution and uses of excess chemical based products are also a big degenerative factors, which leads the great health problems and they are roots of many fatal diseases. So, in this condition, *Rasayan* therapy becomes very necessary to rejuvenate the body system for a better harmony in body, mind and soul. *Ayurveda* has two main objects, first to maintain the health of healthy person and second is to cure the disease of suffering. *Rasayan* Therapy helps in maintaining health, prevention of diseases and also gives strength to the body to avoid the recurrence of diseases.

Sushruta has Defined a Healthy Man as One who has

Equilibrium of the *Doshas (Sama dosha),* Normal functioning of *Agni (Sama Agni),* Normal condition of seven *Dhatus (Sama Dhatu),* Normal excretion of waste products *(Sama Malkriya).* Beside this *Atma* (Soul), the *Indriyas* or sense organs and *Mana* or mind should be happy and cheerful *Prasanatamendriya Mana* (Su.Su.15/15). The current modern definition of health is also same "Health is a state of complete physical, mental and social well being and not merely absence of disease (W.H.O).

Aims and Objectives

Immunomodulatory effect of *Shataputa Abhraka Bhasma* by In-vitro method was carried out by means of Nitroblue Tetrazolium Test (NBT assay), Phagocytosis, Candidacidal assay and Chemotaxis.

Materials and Methods

The present In-vitro Immunomodulatory study was carried out in the Microbiology Research Lab of Belgaum.

Test Drug

Shataputa Abhraka Bhasma prepared by classical method following *Rasa Tarangini* reference.

Study Design

In-vitro screening for Immunomodulatory activity using Nitroblue Tetrazolium Test (NBT assay), Phagocytosis, Candidacidal assay and Chemotaxis [4].

Screening of immuno-modulatory effect of *Shataputa Abhraka Bhasma* was carried out using three different healthy adult human blood samples (A, B and C) and with three different drug concentrations i.e. 1%, 2% and 5%. The parameters used were NBT (Nitroblue Tetrazolium Test) assay, Phagocytosis, Candidacidal assay and Chemotaxis.

A. NBT Test (Nitroblue Tetrazolium Test):[5, 6, 7, 8]

The NBT dye reduction test gives information about the phagocytic and intracellular killing functions of leucocytes which are necessary for normal microbiocidal activity. The dye is taken into leucocytes by phagocytosis and then stimulation of the hexose monophosphate-shunt pathway (HMP) of glucose oxidation and concomitant changes in oxidative metabolism lead to the reduction of the dye to an insoluble blue crystalline form (formazan crystals). 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.

- 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.
- *Shataputa Abhraka Bhasma* suspension of three concentrations 1%, 2% and 5% were used.

Procedure

Materials Required

- Human blood samples- 03(A,B and C), Test drug - *Shataputa Abhraka Bhasma*, 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.

Preparation of Chemicals and Reagents

- E.Coli Endotoxin Standard: 20 ml of broth from each of S strains of E. Coli was boiled on water-bath for 2 hours, centrifuged at 2000 rpm for 30 minutes and the supernatant was pooled and stored -20°C.
- 0.15% Nitroblue tetrazolium dye: One part of 0.3% Nitroblue Tetrazolium (NBT) solution, prepared in 0.34% sucrose solution, was added to one part of phosphate buffer solution (PBS) and was used fresh.
- Leucocyte suspension: A suspension of leucocytes (5×10^6/ml) was prepared in 0.5 ml PBS.
- Preparation of Test samples: *Shataputa Abhraka Bhasma* solutions were prepared in concentrations of 1 %, 2% and 5%. The solutions were centrifuged and the supernatants were used in the assay.

Nitroblue Tetrazolium (NBT) Dye Test

- Three healthy human blood samples were taken viz. A, B and C.
- The assay mixture consisted of 0.2 ml of 5×10^6/ ml of leucocytes suspension and 0.2 ml freshly prepared 0.15% NBT solution.
- 0.1 ml of test substance at different concentrations was added to the reaction mixture.
- 0.1 ml of endotoxin-activated plasma was added to the 0.15% NBT solution and leucocytes which served as a positive control (standard).
- A normal control was maintained in another test tube with leucocytes suspension, distilled water and NBT solution.
- All the test tubes were incubated separately at 37°C for 20 min and centrifuged gently at 400 rpm for 3-4 min. The supernatant was discarded.
- A drop of PBS was added and the cells were gently re-suspended at the bottom of the test tube.
- A film was prepared by allowing a drop of this fluid to dry on a microscope slide.
- Slides were dried for 10-15 minutes.
- Methanol fixation was carried out and again slides were kept for drying purpose.
- Slides were further stained in Giemsa stain for 15 minutes and washed under tap water.
- After complete drying, the slides were observed under light microscope with oil immersion objective.
- 300 neutrophils were counted and the % of NBT positive cells containing the blue spots (stimulated) were determined.

Observation and results were given in Table 1 and statistical analysis in Table 2.

Maximum stimulation was seen with 5% *Shataputa*

Table 1: Results of Immunomodulatory Effect (NBT test) Results of NBT (Stimulated cells in %)

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

A.B.- *Shataputa Abhraka Bhasma*

Table 2: Statistical Analysis (NBT) 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

Table 3: Results of Phagocytosis Results of Phagocytosis (mean particle number)

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

Table 4: Results of Candidacidal assay (Dead candida cells in %)

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

Table 5: Statistical Analysis (Phagocytosis and Candidacidal assay) 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

Table 6: Results of Neutrophil Locomotion and Chemotaxis test (In mm)

Neutrophil Suspension	Neutrophil Suspension - A			Neutrophil Suspension - B			Neutrophil Suspension - C		
Concentration of drug	1% A.B.	2% A.B.	5% A.B.	1% A.B.	2% A.B.	5% A.B.	1% A.B.	2% A.B.	5% A.B.
Movement of neutrophils	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	

Table 7: Statistical Analysis (Chemotaxis test) 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.

Abhrakaa Bhasma suspension and we can infer that stimulation increased with the increase in concentration of the drug in this study.

B. Phagocytosis and Candidacidal Assay

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.
- *Shataputa Abhraka 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.
- *Shataputa Abhraka Bhasma* suspensions of three different concentrations 1%, 2% and 5% were used as test drug.

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 Required

- Dextran solution – 6% in 0.15 M NaCl
- MEM – As tissue culture medium
- 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 minutes 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 minutes. 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 minutes, 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.

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

2. Phagocytosis & Candidacidal Assay:

Here the test tubes were filled with samples. 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 ml of 2.5% sodium deoxycholate and 1 ml of methylene blue indicator were added to each tube. Centrifugation was done for 10 minutes 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.

Observation and results given in Table 3 and 4, statistical analysis in Table 5.

Maximum activity was seen with 5% *Shataputa Abhraka Bhasma* suspension and we can infer that activity increased with the increase in concentration of the drug in this study. The candidacidal activity was seen to be better in all the concentrations when compared to the positive control used.

C. Neutrophil Locomotion and Chemotaxis Test:

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 *Shataputa Abhraka Bhasma* was noted.
- Fm-leu-phe was used as chemo attractant which acted as positive control.

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 formalin
- 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.

Procedure

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, Lastly, 0.01 ml of 1%, 2% and 5% suspension of *Shataputa Abhraka 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 formalin 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.

Observation and results given in Table 6, statistical analysis in Table 7.

Results and Discussion

i. 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.
- *Shataputa Abhraka bhasma* suspension of three concentrations 1%, 2% and 5% were used.
- 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% *Shataputa Abhraka bhasma* suspension (mean 82.3 ± 2.57) and it was better than the positive control (E. coli endotoxin).

ii. 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.
- *Shataputa Abhraka bhasma* suspensions of three different concentrations 1%, 2% and 5% were used as test drug.
- 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% *Shataputa Abhraka bhasma* suspension and it was almost equal to the positive control values.

iii. Candidacidal Assay

- Dead candida cells (in %) were calculated in this assay.
- Pooled human blood serum was used as positive control.
- *Shataputa Abhraka bhasma* suspensions of three different concentrations 1%, 2% and 5% were used as test drug.
- 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% *Shataputa Abhraka bhasma* suspension.
- The activity was seen better in all the three concentrations when compared with the positive control used.

IV. 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 *Shataputa Abhraka bhasma* was noted.

- Fm-leu-phe was used as chemo attractant which acted as positive control.
- 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.
- Significant results were seen with the 5% suspension in all the four tests which indicate *Shataputa Abhraka Bhasma* is a potential Immunomodulator.

Conclusion

- Thus *Shataputa Abhraka Bhasma*, exhibits a potent In-vitro Immunomodulatory (stimulant) activity in a concentration dependent manner.
- The results are self conclusive and indicate that *Shataputa Abhraka Bhasma* brings about stimulation of Leucocytes and thus in turn leads to highly significant phagocytic activity which is evident from the In-vitro NBT test.
- The 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.
- The Immunomodulatory activity of *Shataputa Abhraka Bhasma* is concentration dependent. The activity of *Shataputa Abhraka Bhasma* at 5% is even higher than the positive control group (Endotoxin Activated Plasma). Thus it can be concluded that *Shataputa Abhraka Bhasma* which is highly rated as a *Rasayan* by various *Rasa Shastra Granthakaras*, has a significant In-vitro Immunomodulatory activity in concentration dependent manner. This also validates the principle that *Shataputa Abhraka Bhasma* acts as a *Rasayan* after subjecting to 100 *Putas*. Animal and Clinical trials shall further consolidate the above results and shall help to find mechanism of action of Immunomodulation of *Shataputa Abhraka Bhasma*.

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