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Research Article

IMMUNOMODULATORY EFFECT OF *BHRINGRAJ SWARAS SHODHIT GANDHAK* AND *GODUGDHA SHODHIT GANDHAK*: A COMPARATIVE INVITRO STUDY

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KEYWORDS: *Gandhak, Shodhan, Bhringraj Swaras, Godugdha, Rasayana,* Immunomodulatory effect.

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ABSTRACT

Introduction: *Gandhak* is one among the mineral drug explained in *Uparasa Varga*. It possesses *Rasayana* property. It attains therapeutic properties with proper *Shodhana* processes by *Godugdha* and by *Bhringraj Swaras*. Assessment of immunomodulatory effect of *Bhringraj Swaras Shodhit Gandhak* and *Godugdha Shodhit Gandhak* may provide evidence base for its textual reference and analysis of these *Shodhit Gandhak* contributes to establish standards for quality control.

Method: *Shuddha Gandhak* by *Godugdha* and by *Bhringraj Swaras* prepared as per *Rasaratnasamuchchaya* and subjected to Immunomodulatory activity by Neutrophil Function Assay test with four parameters NBT test, phagocytosis, candidacidal assay and Neutrophils locomotion (chemotaxis) test.

Results: In Immuno-modulatory assay different concentrations of *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhaka* shown significant increase in phagocytic activity, candidacidal capacity, locomotion and activation of Neutrophils for phagocytosis.

Conclusion: *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhaka* shown significant immunomodulatory effect. Statistically there is no significant difference between *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhaka*.

INTRODUCTION

The medicinal system of India is of great antiquity. Ayurveda originating from the *Vedas* documented around 5000 years ago. This is a classical system of healthcare and currently recognized and practiced all over India.

In the modern Ayurvedic practice the *Rasasastra* has been considered more useful and effective when compared to the herbal preparations. It is said that the *Rasashastra* has immense therapeutic applications some of which are prevention of ageing and reduction in age-related disorders. The *Rasausadhis* are known for smaller dosage. They do not cause any nauseating sensation during consumption. These medicines provide quick results and they are useful in majority of difficult to cure disease conditions.[1]

Rasasastra or the Ayurvedic alchemy is an important branch of Ayurvedic pharmacology. This branch deals with the use of metals, minerals, gemstones and their processing. In the ancient Ayurveda the emphasis has been over the herbs and their therapeutic usages. Later on, the animal products, metals and minerals started to find favour of the Ayurvedic practitioners. The minerals and metals are very effective and potent for immunization, rejuvenation and elimination of the diseases. The modern Indian Ayurveda makes an extensive use of *Rasashastra* so much so that it has become the vital or inseparable component of the therapeutic process.[2]

Amongst *Uparasa-Dravyas*, *Gandhak* is first explained.[3] Mineral origin and mythologically considered as '*Raja* of goddess *Parvati*'. It possesses *Rasayana* property and in text, it is explained as *Gandhashmo-Atirasayan.* Apart from this, it is also

indicated in various diseases like *Kandu*, *Kushta* etc.[4]

Health is "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.[5] For being healthy, a strong immune system is needed, so that body can resist infections from bacteria and viruses easily. If immune system of body is disturbed or do not function properly, a body catches infections and can cause severe illness. The purpose of the immune system is to keep infectious microorganisms, such as certain bacteria, viruses, and fungi, out of the body, and to destroy any infectious microorganisms that do invade the body.[6]

Immunomodulator are drugs that either suppress or stimulate the immune system. Immunostimulants stimulate the immune system. Immunosuppressants suppress the immune system.[7] Immunomodulators modulate immunity, means *Rasayanas* acts as immunomodulators. The Immunomodulatory effect means (a chemical agent as methotrexate or azathioprine) that modifies the immune response or the functioning of the immune system by the stimulation of antibody formation or the inhibition of white blood cell activity.[8]

As per *Prayojan* of *Ayurveda* i.e. *Swasthasya Swasthya Rakshanam Aturasya Vyadhi Parimokshaha*, and *Jara–vyadhividhwansa* i.e., prolonged life and cure disease, if we want to achieve this, once we should use drugs having *Rasayana* property.[9] *Gandhak* possess *Rasayana* property.

Gandhak Shodhan can be done by different media e.g. by *Bhringraj Swaras* and by *Godugdha* etc. To assure best *Rasayana* property, we have access the significant effect of *Shodhit Gandhak* in particular media.

Hence to provide a scientific data and statistical validation a study on comparative Immunomodulatory effect of *Bhringraj Swaras Shodhit Gandhak* and *Godugdha Shodhit Gandhaka* has been undertaken.

Objectives of the Study

To evaluate comparative immunomodulatory effect of *Bhringaraj Swaras Shodhit Gandhaka* and *Godugdha Shodhit Gandhaka.*

Materials and Methods

Gandhaka Shodhana by *Godugdha*[10]

Raw *Gandhaka* taken in *Khalva Yantra* and was powdered. Cow milk taken in another container and it is covered neatly by cotton cloth. *Goghrita* taken in Iron pan container and is melted by heat. After melting of *Goghrita*, powdered *Gandhaka* was added in it and stirred well until *Gandhaka* completely melts in it**.** Then melted *Gandhaka* poured into the container which contains milk through cloth. Remove the cloth after pouring of *Gandhaka*. Solid slab form was obtained. The slab is again converted into multiple small pieces and subjected to dry in open air for overnight. This procedure is repeated for three times.

Gandhaka Shodhana By *Bhringraj Swaras*[11]

Raw *Gandhaka* taken in *Khalva Yantra* and made powder. *Bhringraj Swarasa* taken in another container and it is covered neatly by cotton cloth. Powdered *Gandhaka* taken in Iron pan container and is kept for melting by slow heat. After melting of *Gandhaka*, is poured into the another container which contains *Bhringraj Swarasa* through cotton cloth. Remove the cloth after pouring of *Gandhaka*. *Gandhaka* in solid slab form was obtained. The slab is converted into multiple small pieces and subjected to dry in open air for overnight. This procedure is repeated for seven times.

Immunomodulatory Effect of *Shodhit Gandhak*

Materials & Methods

1. Sample-1: *Godugdha Shodhita Gandhak*
2. Sample-2: *Bhringraj Swaras Shodhita Gandhak*

Godugdha Shodhit Gandhak and *Bhrungraj Swaras Shodhit Gandhak* subjected for screening of Immunomodulatory activity by Neutrophil Function Assay Test.

Sample solution

- *Godugdha Shodhit Gandhaka* Solution (Sample-1) is prepared in chloroform and methanol. 10mg of *Godugdha Shodhit Gandhak* is mixed in 500µl chloroform and 500µl Methanol. Five concentrations made 100µl, 50µl, 25µl, 12.5µl, 5µl.
- *Bhringraj Swaras Shodhit Gandhak* solution (Sample-2) is prepared in chloroform and methanol. 10mg of *Bhringraj Swaras Shodhit Gandhak* is mixed in 500µl chloroform and 500µl Methanol. Five concentrations made 100µl, 50µl, 25µl, 12.5µl, 5µl.

Neutrophil Function Assay Test [12, 13-17]

- **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. The blood sample is collected either with heparin or EDTA as anticoagulant. Most of the isolation protocols use differences in cell density as the basis for separation. A 3% solution of gelatin or high molecular weight dextran in saline is most preferred method as it gives a

relatively pure population of WBCs with minimal contamination with RBCs. To obtain granulocytes, use of Ficoll- Hypaque is most commonly used. For separation of WBCs, the blood sample is diluted with saline and equal amount of 3% gelatin or dextran is added. The tubes are made to stand upright for about 45 minutes till all the RBCs settle down. The supernatant is collected in a fresh tube, centrifuged and washed with saline and the WBCs are counted in Neubauer's chamber and then diluted with Hanks balanced salt solution to get a specific concentration depending on the type of test to be performed.

- **WBC-Separation:** The EDTA blood is mixed with equal quantities of MEM (minimum essential medium) and 6% dextran solution of molecular weight of 150,000. The tube is kept upright without disturbing for about 45 min. All the RBCs settle down at the bottom whereas the WBCs and the plasma will be in the upper layer. This upper layer is collected in a centrifuge tube and spun for about 10 minutes at 3000rpm. The deposit contains the cells. This deposit is washed three times with phosphate buffered saline and the cell concentration is adjusted to 1000000/ml with MEM.
- HBSS (Hanks Blood Salt Solutions) used for almost all the functional methods of immune-modulatory activity for the viability of WBCs.
- For NBT - test whole blood was taken while for others only separated neutrophils (by dextran sedimentation process) were used to assess the phagocytic and chemotaxis activity.

Procedures

1. Nitrobluetetrazolium Test

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.

2. Phagocytosis

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 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).

3. Candidacidal Assay

Essentially, the basic procedure is same as for the phagocytosis. Only the dead Candida 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 percentage of dead cells i.e., those which have taken up methylene blue and appear blue in colour.

4. Neutrophil Locomotion And Chemotaxis Test

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 chemoattractant 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 travelled from the edge of the peripheral wells towards wells containing FMLP.

RESULTS

A) *Gandhaka Shodhana*

1. **By *Godugdha* (sample-1)**
2. **By *Bhringraj Swaras* (sample-2)**

Table 1: Weight loss & time to melt of sample -1 & 2

Samples	Stage of *Shodhana*	*Gandhaka* obtained	Loss	Time to melt
Sample 1	Initial Wt of *Gandhaka*	487 gm		
	Wt after 1st *Shodhana*	470 gm	17 gm	12 minute
	Wt after 2nd *Shodhana*	460 gm	10 gm	11 minute
	Wt after 3rd *Shodhana*	452 gm	8 gm	9 minute
	Initial wt. of *Gandhaka*	247 gm		
	1st *Shodhana*	238 gm	9 gm	7 minute
	2nd *Shodhana*	222 gm	16 gm	8 minute

Sample 2	3rd *Shodhana*	199 gm	23 gm	10 minute
	4th *Shodhana*	178 gm	21 gm	9 minute
	5th *Shodhana*	165 gm	13 gm	10 minute
	6th *Shodhana*	140 gm	25 gm	9 minute
	7th *Shodhana*	122 gm	18 gm	10 minute

B) Immunomodulatory Effect of *Shodhit Gandhak*

Observation and Results

- For NBT - test and Candidacidal Assay 100 cells per slide were observed. Out of which only those stimulated were are counted for NBT- test, while in Candidacidal only dead cells were considered. These values were converted into percentage and recorded in the table.
- For Phagocytosis MPN (Mean Particle Number) was noted i.e., the number of candidas engulfed per neutrophil was noted and recorded in table.
- In Neutrophil Locomotion and Chemotaxis Test, the distance travelled by the cells towards the source of stimulus was noted and recorded in table.

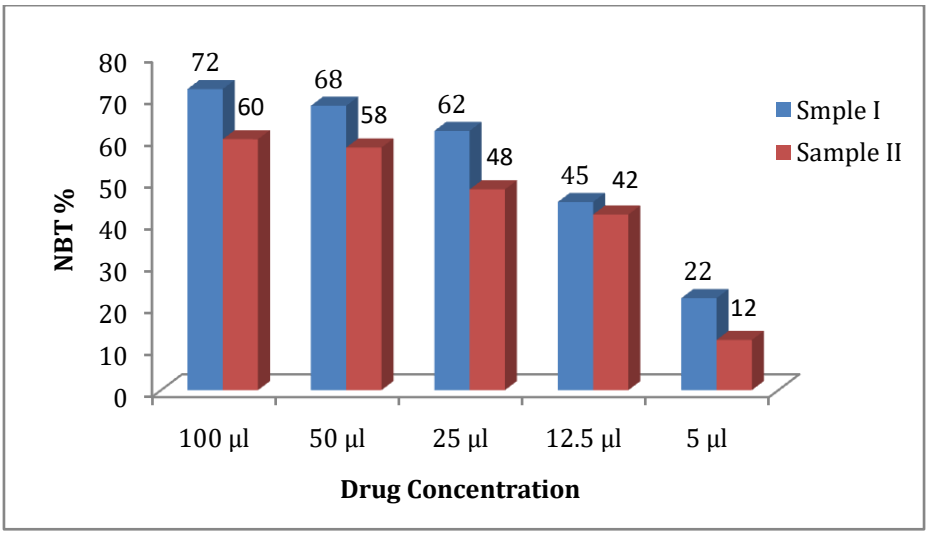
Table 2: Shows result of Immunomodulatory activity of sample 1 & 2

Drug concentration of sample 1 & 2	NBT %		Phagocytosis (MPN)		Candidacidal assay %		Neutrophil locomotion (Chemotaxis) in mm	
	Sample1	Sample2	Sample1	Sample2	Sample1	Sample 2	Sample1	Sample2
100 µl	72	60	5	2	32	38	2.1	1.0
50 µl	68	58	2	2	32	34	1.8	0.8
25 µl	62	48	2	2	30	34	0.8	0.6
12.5 µl	45	42	2	2	25	19	0.6	0.6
5 µl	22	12	2	2	21	19	0.6	0.6
Pc (positive control)	65	65	5	5	30	30	2.3	2.1
Nc (negative control)	20	20	2	2	16	18	0.5	0.5

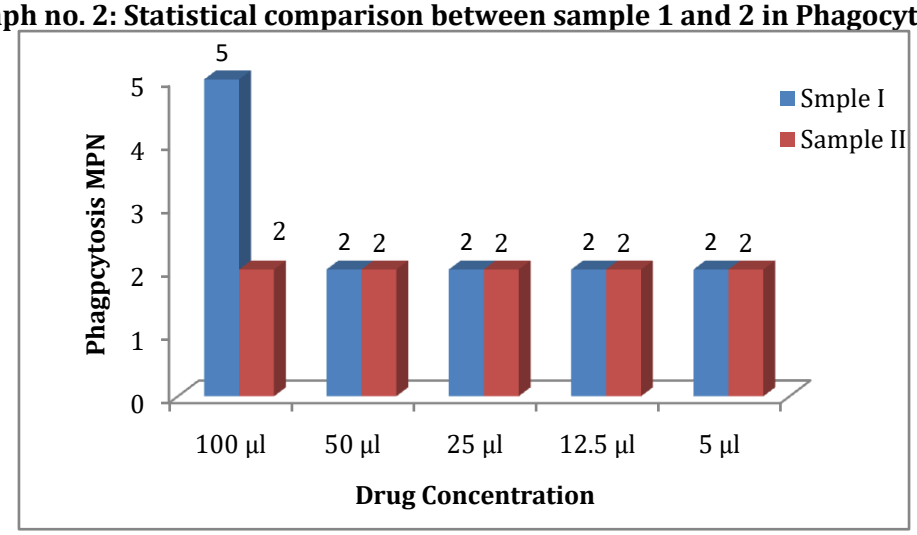
Table 3: Neutrophil Locomotion & Chemotaxis Test Difference

Sample 1	Sample 2	Difference
2.1 mm (26.25%)	1.0 mm (12.5)	13.75 %
1.8 mm (22.5%)	0.8 mm (10%)	12.5%
0.8 mm (10%)	0.6 mm (7.5%)	2.5%
0.6 mm (7.5%)	0.6 mm (7.5%)	0%
0.6 mm (7.5%)	0.6 m (7.5%)	0%

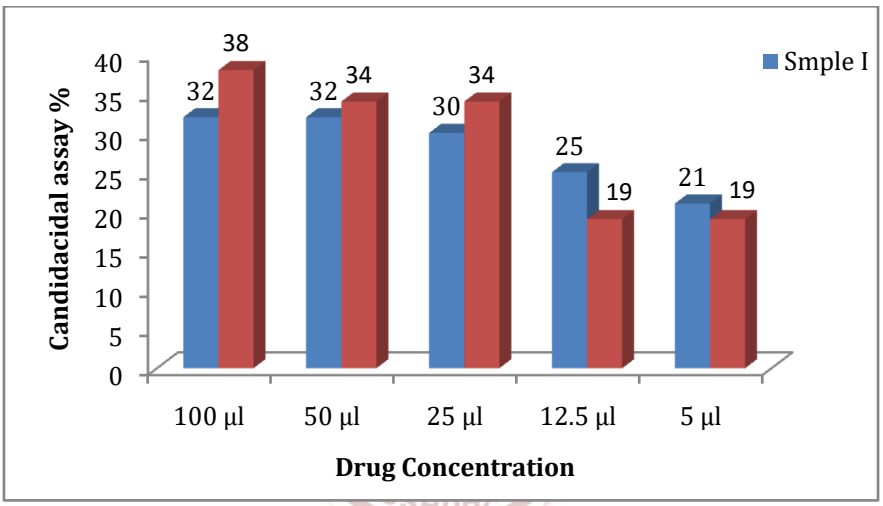
Graph no. 1: Statistical comparison between sample 1 and 2 in NBT Test



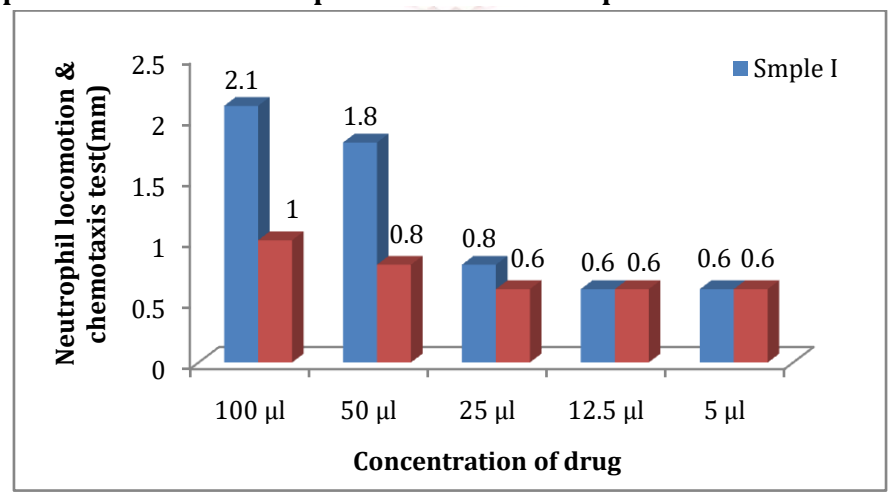
Graph no. 2: Statistical comparison between sample 1 and 2 in Phagocytosis



Graph no. 3: Statistical comparison between sample 1 and 2 in Candidacidal Assay



Graph no.4: Statistical comparison between sample 1 and 2 in Chemotaxis



C) Analytical results

pH of *Godugdha Shodhit Gandhaka* sample and *Bhringraj Swaras Shodhit Gandhaka* sample was 7.7 and 7.5 respectively.

Specific Gravity of *Godugdha Shodhit Gandhaka* sample and *Bhringraj Swaras Shodhit Gandhaka* sample was 0.9907 and 1.002 respectively.

Moisture content of *Godugdha Shodhit Gandhaka* sample and *Bhringraj Swaras Shodhit Gandhaka* sample was 0.4% and 0.6% respectively.

Both sample was non soluble in all solvents.

Analytical reports of *Godugdha Shodhit Gandhaka* sample was obtained by using XRF

contains Silicon <00.10%, Sulphur 99.17%, Zinc <1.0 ppm.

Analytical reports of *Bhringraj Swaras Shodhit Gandhaka* sample was obtained by using XRF contains Silicon <0.10%, Sulphur 99.82%, Iron <1.0 ppm and Zinc <1.0 ppm.

DISCUSSION

500 gm of *Ashudhha Gandhak* subjected for *Shodhana* procedure by *Goghrita-Godugdha* Method, after *Shodhan* procedure the weight found was 452gm. The total loss after three *Shodhana* procedures was 48gm. Another sample of 250gm of *Ashudhha Gandhak* subjected for *Shodhana* procedure by *Bhringraj Swaras*, after *Shodhan* procedure the weight found 122gm. The total loss after seven *Shodhan* procedures was 128gm.

Godugdha Shodhit Gandhaka (Sample-1) and *Bhringraj Swaras Shodhit Gandhaka* (Sample-2) and was subjected for screening for immunomodulatory activity on human polymorphoneutrophils with four parameters i.e., NBT test, Phagocytosis Assay, Candidacidal Assay, Neutrophil Locomotion and Chemotaxis test etc.

In NBT test of *Godugdha Shodhit Gandhaka*- 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations compared to positive control (standard drug) shown that there was no significant difference. 100µl and 50µl shown highly significance compared to negative control. 25µl, 12.5µl and 5µl concentrations shown no significance compared to negative control.

In NBT test of *Bhringraj Swaras Shodhit Gandhaka*- 100µl and 50µl concentrations compared to positive control (standard) shown that there was no significant difference. 25µl, 12.5µl and 5µl shown highly significance compared to positive control. 100µl, 50µl, 25µl, 12.5µl concentrations were compared to negative control shown that there was highly significant difference.

In Phagocytosis Assay of *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhaka* 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations compared to positive control (standard) shown no significant difference. 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations of *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhaka* shown no significance compared to negative control.

In Candidacidal Assay of *Godugdha Shodhit Gandhaka*- 100µl, 50µl, 25µl and 12.5µl concentrations compared to positive control (standard) shown no significant difference. 5µl shown highly significance compared to positive control. 12.5µl and 5µl concentrations shown no significance compared to negative control. 100µl, 50µl and 25µl concentration compared to negative control shown highly significant difference.

In Candidacidal assay of *Bhringraj Swaras Shodhit Gandhaka*- 100µl, 50µl, 25µl and 12.5µl concentrations compared to positive control (standard) shown no significant difference. 5µl concentration compared to positive control (standard) shown highly significance. 12.5µl and 5µl concentrations shown no significance compared to negative control. 100µl, 50µl and 25µl concentrations compared to negative control shown highly significance i.e., drug significantly stimulated for candidacidal activity.

In neutrophil locomotion and chemotaxis test of *Godugdha Shodhit Gandhaka*- 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations compared to positive control i.e., with standard shown highly significant difference. 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations compared to negative control shown highly significance.

In neutrophil locomotion and chemotaxis test of *Bhringraj Swaras Shodhit Gandhaka*- 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations were compared to positive control (standard) shown highly significance. 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations compared to negative control shown highly significance i.e., drug shown significant distance of migration by neutrophils.

Comparision between *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhaka*- when it was compared from 5µl concentration to 100µl concentration overall it was found that there is no significant difference between sample1 & 2 ($p>0.05$).

CONCLUSION

Godugdha Shodhit Gandhaka shown more alkaline nature than *Bhringraj Swaras Shodhit Gandhaka*. *Godugdha Shodhit Gandhaka* shown less moisture content than *Bhringraj Swaras Shodhit Gandhaka*. Specific Gravity of *Bhringraj Shodhit Gandhaka* was greater than *Godugdha Shodhit Gandhaka*.

Immunomodulatory activity of *Shudhdha Gandhak* was conducted by neutrophils function tests. In NBT test, 100µl, 50µl, 25µl, 12.5µl and 5µl concentrations of *Godugdha Shodhit Gandhaka* and 100µl and 50µl concentrations of *Bhringraj Swaras Shodhit Gandhaka* drug stimulated neutrophils for phagocytosis similar to that of standard and 100µl and 50µl of *Godugdha Shodhit Gandhaka* and 100µl, 50µl, 25µl, 12.5µl concentrations of *Bhringraj Swaras Shodhit Gandhaka* drug stimulated neutrophils significantly for phagocytosis.

In Phagocytosis Assay, both drug promoted to the phagocytosis activity similar to that of standard. In Candidacidal Assay 100µl, 50µl, 25µl and 12.5µl concentrations of *Godugdha Shodhit Gandhaka* and 100µl, 50µl, 25µl and 12.5µl concentrations of *Bhringraj Swaras Shodhit Gandhaka* stimulated for candidacidal activity similar to that of standard and 100µl, 50µl and 25µl of *Godugdha Shodhit Gandhaka* and 100µl, 50µl and 25µl of *Bhringraj Swaras Shodhit Gandhaka* drug significantly stimulated for candidacidal activity.

In neutrophil locomotion and chemotaxis test, 100µl, 50µl, 25µl, 12.5µl and 5µl of *Godugdha Shodhit Gandhaka* and 100µl, 50µl, 25µl, 12.5µl and 5µl of *Bhringraj Swaras Shodhit Gandhaka* drug shown significant distance of migration by neutrophils.

Godugdha Shodhit Gandhaka and *Bhringraj Swaras Shodhit Gandhaka* these both drugs was shown significant Immunomodulatory Effect. When it was compared from 5µl concentration to 100µl concentration of drugs of both *Godugdha Shodhit Gandhaka* and *Bhringraj Swaras Shodhit Gandhak*, overall it was found that there is no significant difference between sample1 & 2 ($p>0.05$).

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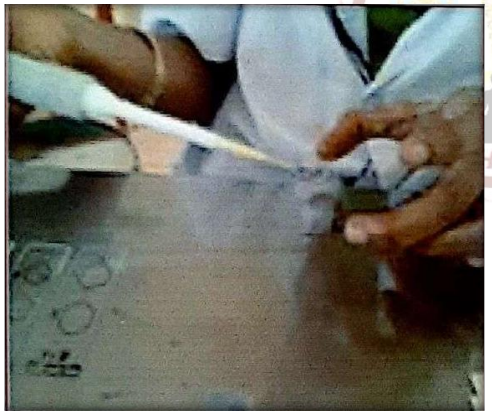
Gandhaka Shodhana* by *Godugdha

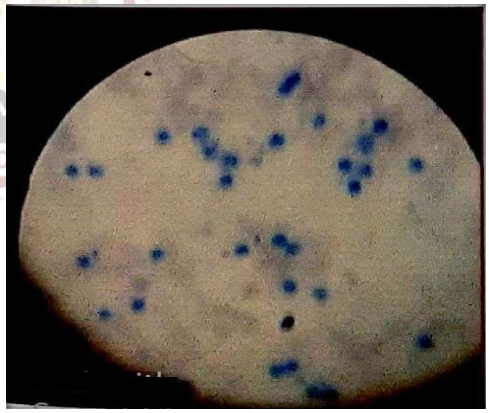
Gandhaka Shodhana* by *Bhringraj Swaras

Blood Samples

Preparation of Growth Media

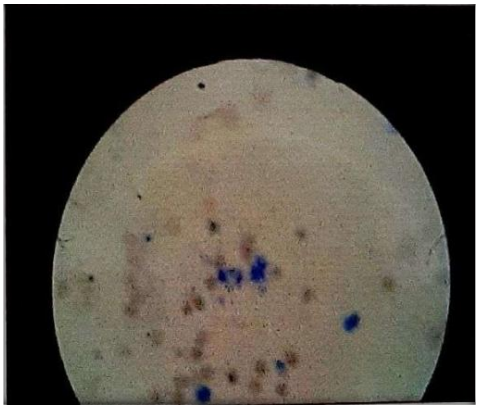


Preparing Smear



Stimulated Cells

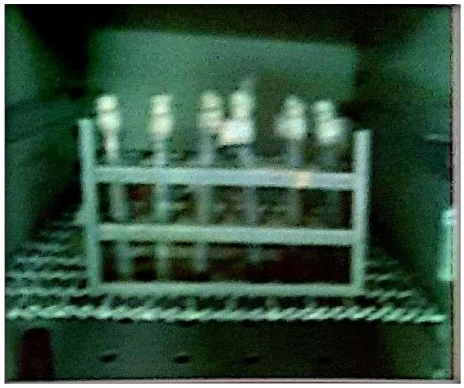
Slides-Staining



Unstimulated Cells

Phagocytosis And Candidacidal Assay

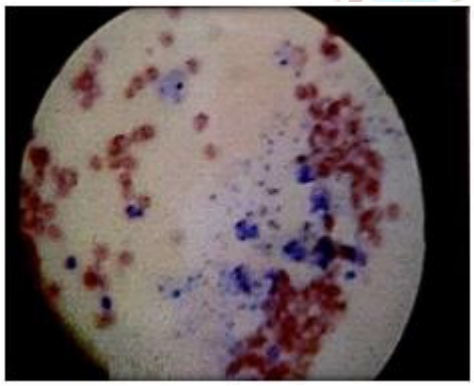
Candidacidal suspension

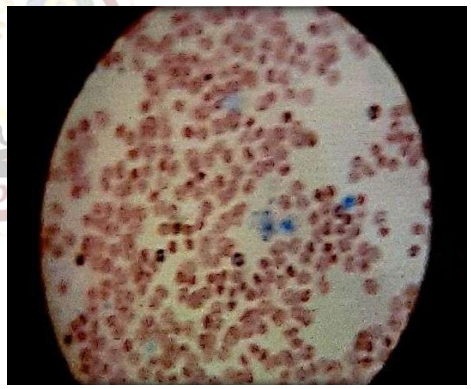


Incubation preparation

Giemsa Staining

Fixation

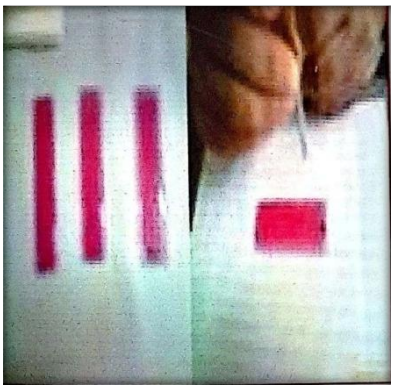




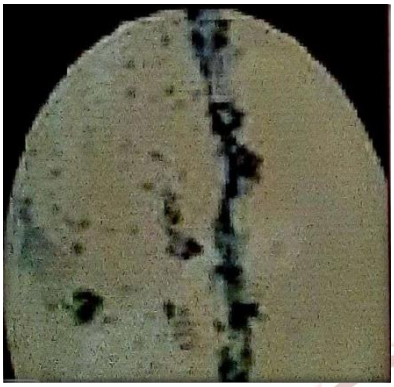
Candidas Engulfed By Neutrophils

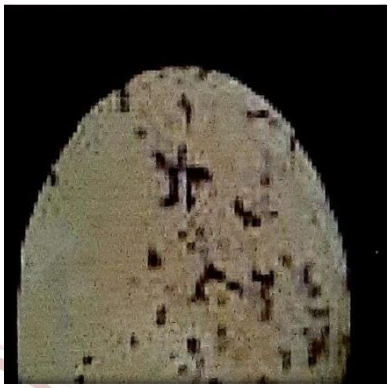
Neutrophil Locomotion and Chemotaxis Test

Test Samples Preparation Filling of Wells



Wells Preparation Separation of Neutrophils





Microscopic View of Neutrophils towards Concentration of Drug