

# Generation and Characterization of chicken egg yolk antibodies against *Shigella* sp and comparison studies with *Rhododendron nilagiricum*.

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**Abstract:** *Shigella flexneri* is the main agents of bacillary dysentery. Shigellosis is a common bacterial disease is mainly transmitted through the faecal–oral route. Chicken IgY has been identified as an alternative for the treatment of Shigellosis. 21 weeks old white leghorn chicken which lays eggs was immunized with *Shigella flexneri*. The anti-Candida albicans IgY was purified and their protein content was estimated. The specificity of produced Serum IgY and egg yolk IgY was tested with Rapid Agglutination test. SDS-PAGE analysis of IgY showed a protein profile of the IgY, antibody titre potency of each serum IgY and egg yolk IgY was determined by Indirect ELISA. Collection and Preparation of leaf extract of *Rhododendron nilagiricum*. To evaluate the Antibacterial and Phytochemical analysis of leaf extract. The present study was undertaken to generate antibodies against *Shigella flexneri* which cause Diarrheal infection and to examine the growth inhibitory activity of the *Shigella flexneri* by antibodies produced at different concentration for both IgY antibody in Egg Yolk and serum. Then also as a comparative counterpart the methanol extract of the desired plant is taken. This extract was taken in different concentration and was also assayed for the growth inhibitory activity.

**Keywords:** *Shigella flexneri*, IgY, ELISA, *Rhododendron nilagiricum*, Growth inhibitory Assay.

## I. INTRODUCTION:

*Shigella* is the main agents of bacillary dysentery. It is a common bacterial disease is mainly transmitted through the faecal–oral route. The genus *Shigella* is differentiated into 4 species: *S. dysenteriae* (serogroup A, together with 12 serotypes) *S. flexneri* (serogroup B, together with 6 serotypes); *S. boydii* (serogroup C, together with 18 serotypes); and *S. sonnei* (serogroup D, together with an unmarried serotype). The global spread of *Shigella* clones or the emergence of new *S. flexneri* serotypes is associated with multidrug resistance. In contrast to the other enteropathogens, *Shigella* is highly contagious; as few as 200 *S. flexneri* organisms are sufficient to induce diarrhea and fever. Shigellosis is common in developing countries where poverty, overcrowding, poor hygiene, and malnutrition prevail. The World Health Organization (WHO) estimates that shigellosis causes 160 million cases of diarrhea each year and 1.1 million deaths worldwide (Thomas L. Hale *et al* Medical Microbiology. 4th edition 1996).

The yolk of eggs laid by immunized chickens has been widely recognized as an excellent source of polyclonal antibodies (pAb). Production of IgY antibodies from hen's egg yolk is an alternative method to cure the disease and also used to neutralize the pathogenic organisms in humans.

Herbal medicines are useful for healing and curing a variety of diseases from the ancient period. The phytochemicals present in the medicinal plants possess a defence mechanism against various diseases. The Nilgiris Rhododendron (*Rhododendron Nilagiricum* (Zenker) (Tagg)) is an interesting species of the genus *Rhododendron* which is a member of the plant family Ericaceae. The dried flowers and leaves of *R. arboreum* Sm sp *R. nilagiricum* are used to treat Diarrheal diseases. There are substances present in this species that inhibit the growth of organisms that causes diarrheal diseases. Herbal medicines are useful for healing and curing a variety of diseases from the ancient period. The phytochemicals present in the medicinal plants possess a defence mechanism against various diseases (Yao Xue Xue Bao 1989).

## II. MATERIALS AND METHODS

### 2.1 Experimental Animal:

Twenty one weeks old White Leghorn chickens in good health condition were obtained from Chandran poultry, Karadivaavi, Palladam. The chickens were maintained in our animal facility with normal feeding and used for the study and the production of anti-*Shigella flexneri* antibodies (IgY) without sacrificing it.

### 2.2 Sample Collection:

Bacterial strain used for the present study is *Shigella* sp. The sample was collected from PSG Institution of Science and Medical Research, Coimbatore. The collected sample was cultured on SS AGAR plates and incubated at 37°C temperature for 24 hours. The pure culture was maintained in SS AGAR plates and slants. Sub-culturing was done periodically. For all these experiments, standard aseptic techniques were followed; including sterilization of media, glass wares etc.

The Clinical Pathogens such as *Salmonella* sp culture was collected from the Bio line Laboratory, R S Puram, Coimbatore.

### 2.3 Characteristics of Organism:

To check for purity of the culture, microscopic and biochemical characters of the strains were carried out by the following tests.

#### a. Gram Staining Test:

The prepared smear was air dried and heat fixed. Crystal violet was flooded over the smear for one minute and drained. After washing Gram's iodine was added and left for one minute. The smear was then washed in 95% (V/V) ethanol for 30 seconds. It was counter stained with safranin for two minutes. After air drying, the smear was examined under oil immersion objective in a light microscope.

#### b. Cultural Characteristics of Organism:

The *Shigella flexneri* was cultured on Salmonella Shigella Agar, Hektoen enteric agar, XLD Agar and Nutrient agar plates. Plates were incubated at 37°C overnight and cultural characteristics of the colonies were studied after incubation. Cultural characteristics of the plates were studied after the incubation.

#### c. Biochemical Test for *Shigella flexneri*:

The biochemical test's for the confirmation of *Shigella flexneri* were carried out under sterile condition such as Indole, Methyl red, Voges proskauer, Citrate, Catalase test, TSI, Urease and Carbohydrate fermentation test. In IMVIC test the organism was inoculated into respective tube which contains media. Then the tubes were incubated at 37°C for 24 hours. After incubation the reagents were added and result was observed. For catalase test the test organism was mixed with hydrogen peroxide. In Carbohydrate fermentation test Glucose, Lactose, Sucrose, Mannitol sugar was taken and test was carried.

#### d. Antibiotic Susceptibility Test:

Muller Hinton agar is most commonly used agar for the antimicrobial activity test. So the Muller Hinton Agar media was prepared under sterile condition then the media was poured into a petri plate and allowed for solidification. After that agar plate was then inoculated with *Shigella flexneri* by making lawn culture using sterile swab. Then the Antibiotic discs (Ciprofloxacin, Cefatoxime, Amoxicillin, Azithromycin) were placed on the agar and incubated at 37°C for 24 hours.

### 2.4 Preparation of Whole Cell Antigen:

Pure isolated colonies *Shigella* sp were grown in 5ml of Tryptone broth at 37°C for overnight. They were then transferred into 250ml of Tryptone broth incubated overnight at 37°C. Then the culture broth was centrifuged at 7000 rpm for 15 mins. Remove the supernatant collect the pellet. Repeat this process at least three times and wash it with PBS until get a clear pellet. Formalin was added to it and kept overnight at room temperature Again the pellets were washed with PBS and stored under refrigeration.

#### a. Purity & Sterility Testing of Antigen:

For the sterility checking, complete killing of the bacteria was tested by resuspending an aliquot of the cell pellet in PBS saline and plating of this suspension into nutrient agar and SS agar. The plates were incubated overnight at 37°C and examined for the presence of bacterial growth. In purity checking, the in the absence of bacterial growth in agar plates the immunization of chicken was carried out in aseptic condition.

### 2.5 Immunization of Chickens:

For first immunization, the five month old white leghorn chickens were intra muscularly injected at multiple sites of the breast muscles with prepared bacterial antigens. Booster doses were given with two weeks intervals. (Table.1) Eggs were collected at the intervals of three weeks from the initiation of immunization and checked for the presence of antibodies. Further, eggs laid by the chicken under the test were collected regularly and stored at 4°C

Table 1 – Immunization Schedule

IMMUNIZATION	ANTIGEN VOLUME
First dose	1 ml
Second dose	0.5 ml
Third dose	0.5 ml

## 2.6 Purification and concentration of anti-*Shigella* sp., antibodies from Egg Yolk:

### a. Separation of Egg-Yolk:

Under strict aseptic techniques the egg yolk was separated from white and was washed with water to remove as much albumin possible. The yolk was rolled on tissue paper and the yolk membrane was punctured using an applicator sticks. Allow the yolk without membrane was allowed to flow into a graduated cylinder. The yolk membrane and any remaining egg white will stick to the tissue paper. The yolk sac was discarded. The amount of yolk obtained was measured. Approximately 10-15ml of yolk obtained from an average sized egg. Then a continuous constant flow of 25mM phosphate buffer was maintained until all the unretained protein came out. IgY was eluted with 250mM phosphate buffer pH 8.0.

### b. Purification of IgY:

The egg yolk antibodies were purified by the method of Polson *et al.*, (1980). To the 20ml of egg yolk, an equal amount of buffer "S" (10mM phosphate, 100mM NaCl, pH 7.4 containing 0.01% sodium azide) was added to the yolk and stirred. To this mixture 10.5% PEG 8000 in buffer "S" was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature. The stirred mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was filtered through Whatman filter paper. The PEG was added to the supernatant for final concentration of 8.5%. The mixture was stirred and centrifuged at 10,000 rpm for 20 minutes. The pellet was collected and the 10ml buffer-"S" was added to make final concentration of 12.5% PEG. The mixture was stirred thoroughly for 30 minutes at room temperature and centrifuged at 10,000 rpm for 20 minutes. The pellet was taken and 800µl of PBS was added and mixed it well.

### c. Purification of IgY Fraction by Dialysis:

The egg yolk antibodies were collected and mixed well immediately the antibodies were packed in dialysis bags for further purification process. The activation of cellulose membrane was facilitated by the membrane was cut into pieces of required and convenient length and allowed to boil for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1mM of EDTA (pH 8.0). The tubing was rinsed thoroughly in distilled water. Again it was allowed to boil in 1mM EDTA (pH 8.0). The tubing was cooled down and stored at 4°C. Care was taken that the tubing was thoroughly submerged. After this step, the tubing was handled with gloves. Before use, the tubing was washed inside and outside with distilled water. The pooled IgY fraction obtained from egg yolk was transferred to an active dialysis bag. The contents were pooled into the dialysis bag and clipped with dialysis bag clips after including some air and twisting the open end of the dialysis bag. The bags containing pooled contents were dispensed into the respective NaCl solution and kept overnight for dialysis process after the bags were transferred into phosphate buffer solution.

## 2.7 Packing of Chromatography Column:

For purification of immunoglobulin, the column size 2x30cm was used. The burette was first cleaned well and it was packed first with glass wool to form an even bed and a rubber tube with pinch-cock was attached to the tip of the burette. The column was fixed to stand in vertical position. The silica gel was poured into the column along the sides to avoid air bubbles and was allowed to settle. Once the column was set, it was equilibrated with 25mM phosphate buffer (till the out flow of buffer showed pH 8.0).

## 2.8 Sample Application and Elution:

Once the column was equilibrated (25mM phosphate buffer, pH 8.0) the level of buffer in column was allowed to run down to the matrix and the outlet was closed. The immunoglobulin IgY sample layered on the top of the column and was allowed to run till all the sample had entered the bed. Then a continuous constant flow of 25mM phosphate buffer was maintained until all the un retained protein came out. IgY was eluted with 250mM phosphate buffer pH 8.0.

## 2.9 Collection of blood:

The blood collection was performed after the second dosage of antigen was given. By using sterile syringe, 2ml of blood was collected from the hen for the comparison of IgY antibody concentration in Egg yolk and serum. Before collecting the blood breast area of the hen was wiped by using alcohol to avoid the contamination and infection. The blood was collected in tube which contains anticoagulant to prevent the blood from clotting and stored at 4°C.

## 2.10 Separation of serum:

The collected blood was undergone centrifugation. In a centrifuge tube blood was taken and allowed for centrifugation at 4500 rpm for 10 mins. The serum was present in the top of the layer and other cells were settled in a tube. Then the serum alone transferred into new Eppendorf tube by using micropipette.



### 2.11 Rapid agglutination test:

The specificity of anti-Salmonella antibodies of the chicken egg yolk was determined by Rapid Slide Agglutination Test (RSA). Test was done on a plastic strip; 20µl of antigen and 20µl of IgY were placed and mixed thoroughly by stirring with the help of applicator stick. Then the slide was observed for the appearance of agglutination within 2 minutes. The presence of clumping indicated the agglutination reaction, which confirms the presence of specific IgY antibodies against specific antigen.

### 2.12 Estimation of Protein Content in IgY and Serum fraction by Lowry et al., (1951):

The total protein content was estimated by the method described by Lowry *et al.*, (1951). [14] A quantity of 10mg Bovine Serum Albumin (BSA) was dissolved in 10ml of distilled water and used as Protein stock solution. To a series of clean test tubes 0.2 – 1.0 ml of BSA (Protein stock solution) was added and made up to final concentration of 5 ml with distilled water. From these dilutions 0.2ml was taken in to different test tubes and 2ml of Alkaline Copper Sulphate solution was added to each test tube and incubated for 10 minutes at room temperature. After incubation, 2ml of Folin-Ciocalteu reagent was added followed by 1 ml of test sample was poured in same each test tubes incubated under dark condition for 30 minutes at room temperature. Finally the optical density (OD) was measured at 660nm. The OD values of IgY were compared with standard graph. The same procedure is followed for serum.

### 2.13 UV-Vis Spectrophotometer:

The total protein content was estimated by UV-Vis spectrophotometer. An aliquot of 300 microliter of IgY samples were made up to 3ml with distilled water and the OD was read at 260nm against the reagent blank. The concentration of anti-shigella flexneri antibody and IgY was calculated using the formula,

$$\text{IgY concentration (mg/ml)} = \text{Absorbance } 660 \text{ nm} * 10 (\text{dilution factor}) / 1.33$$

### 2.14 Protein Profile of IgY:

Protein profile of IgY antibodies were analysed by Sodium Dodecyl Sulphate Poly Acryl amide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). According to Laemmli (1970) the proteins are resolved with 10% (W/V) polyacrylamide separating gel and 4% (W/V) poly acryl amide stacking gel at 250V and 10mA. Equal ratio of prepared bacterial antigens (30µl) and sample treating buffer (30µl) were mixed well and loaded into sample wells. A wide range molecular weight (6.5-205 KDa) marker was also run along with the proteins. The sample was run until they reach the bottom of the gel. The characteristic protein pattern for the IgY antibodies can be visualized after silver staining.

### 2.15 Concentration of Antibodies:

The eluted IgY fractions were pooled together and concentrated. The dilutions were made from 1:10 to 1:1,00,000. In 1:10 dilution 200µl of Crude IgY was mixed with 800µl of PBS buffer. From this mixture 200µl was taken and transferred into next dilution tube which contains 800µl of PBS. This is known as 1:100 dilution repeat this process up to 1,00,000 dilutions from that mixture 200µl was taken and discarded.

### 2.16 Titration of Antibody by ELISA:

The antibody titer potency of each serums and IgY fractions obtained above was determined by the following modified ELISA (Enzyme-Linked Immuno Sorbent Assay). The particulate *Shigella* antigen was dispensed at a concentration of 5µg/100µl in phosphate buffer (buffer pH 9.6). The resultant dispersion was coated into the individual wells of a 96-well plate (Polyvinyl ELISA plates), at a rate of 100µl per well and incubated at 4°C overnight. The solution was then discarded and unbound antigens in the wells were removed by washing with PBS-T (PBS containing 0.05% tween 20) for 3 times. The nonspecific binding sites were blocked by adding 200µl per well of 1% bovine serum albumin in PBS and incubating the plates at 37°C for 1 hour. Plates were subsequently washed with PBST and the individual wells of each plate were then added with 100µl aliquots of Egg yolk antibodies (IgY) at appropriate dilutions, followed by reaction at 37°C for 1 hour. For control wells PBS were served as respective control. After the reaction the plates were washed three times with PBS-T. As a secondary antibody biotininated rabbit anti chicken IgY coupled to horseradish peroxidase (Genei Pvt. Ltd, Bangalore) was added at the rate of 100µl per well and the plates were incubated for 1 hour at 37°C. The plates were then washed three times with PBST. The antibody titer were determined by adding 100µl of freshly prepared substrate solution (4mg of Phenylene diamine dissolved in 10ml of 50mM citrate buffer, pH 5.0 containing 10µl of hydrogen peroxide) to wells of each plate and followed by reaction at room temperature in dark for 15 min. The reaction was terminated by adding 50µl of terminating solution (4N H2SO4). The absorbance of the well was measured in an ELISA reader at OD490. The same protocol followed for Serum.

### 2.17 Collection of Leaf Extracts:

The leaf sample *Rhododendron nilgircum* was collected from The Western Ghats, The Nilgiris. It was collected freshly and dried at room temperature for 2 weeks. Then it is powdered and stored in a sterile bag. The antimicrobial analysis and phytochemical analysis were observed and used for further analysis.

## 2.18 ANTIBACTERIAL ANALYSIS

### a. Preparation of Leaf Extract; Solvent Extraction:

10 gram of leaf powder was dissolved in 100 ml of solvent (Methanol) in a conical flask, mixed and tightly plugged. It was kept in rotary shaker for 24 hours at a minimum rpm. Then it was filtered using Whatman filter paper, filtrate was evaporated in a petri dish at room temperature for 2-3 days until it evaporates. Then the dried samples was resuspended with sterile Deionized (DI) water and stored in vials at 4°C.

### b. Preparation of Broth Cultures:

All the five clinical pathogens (*Shigella flexneri*, *Salmonella* sp., *Staphylococcus aureus*, *Streptococcus* sp., *Candida albicans*) was cultured in Nutrient broth and grown overnight at 37°C for 24 hours, it was used in antibacterial analysis.

### c. Media Preparation:

#### Mueller Hinton agar (MHA) Composition (grams/ L)

HM infusion B form-	300.00
Acicase	-17.50
Starch	-1.50
Agar	-17.00
Final p <sup>H</sup>	-7.3±0.1

38 gram of MHA (Muller Hinton Agar) was dissolved in 1000 ml of distilled water and then autoclaved for 15 minutes at 121°C. Once the medium was about 45°-50°C, it was poured into sterile petri dishes. Then it was allowed to set completely.

### d. Antibacterial Activity:

Antibacterial activity was performed by Agar well diffusion method. The 20µl of overnight broth culture was swabbed on MHA plates with a sterile cotton swabs and allow the plates for 2-3 minutes. The well was punctured with a well cutter and then 30µl of leaf extracts was loaded in a well. Azithromycin (30µg/ disc) was used as a positive control for *S.flexneri*. The plates were incubated at 37°C for 24 hours. After incubation, the diameter of zone of inhibition (mm) was measured and recorded.

## 2.19 PHYTOCHEMICAL ANALYSIS

### a. Preparation of Leaf Extract: Solvent Extraction:

The 10 grams of powdered leaf sample was mixed with 100 ml of solvents (Methanol) and kept for 24 hours and filtered by whatman filter paper.

### b. Qualitative Phytochemical Analysis of the Leaf Extracts as follows:

#### 1. Test for Alkaloids (Hager's test):

2 ml of leaf extracts was mixed with few ml of dilute Hydrochloric (HCl) Acid and filtered. The filtrate was added with few drops of Hager's reagent (Aqueous solution of Picric acid). A yellow precipitate indicates the presence of Alkaloids.

#### 2. Test for Glycosides:

2 ml of leaf extract was added with glacial acetic acid, ferric chloride (FeCl<sub>3</sub>) and H<sub>2</sub>SO<sub>4</sub> of each 1 ml. A green blue colour indicates the presence of Glycosides.

#### 3. Test for Steroids:

2 ml of leaf extract was mixed with 5 ml of chloroform, 2 ml of acetic anhydride and 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> and the colour changes was observed. Reddish brown colour indicates the presence of Steroids.

#### 4. Test for Flavonoids: (Alkaline reagent test)

To a 2ml of leaf extracts, few drops of NaOH solution was added, a yellow colour solution was formed. Then add few ml of diluted Hydrochloric (HCl) Acid which turns yellow colour solution into a colourless solution, which indicates the presence of Flavonoids.

#### 5. Test for Tannins: (Braymer's test)

A small amount of leaf extract was mixed with 2ml of ferric chloride (FeCl<sub>3</sub>) and the colour change was recorded. The formation of green grey / dark blue colour indicates the presence of Tannins.

#### 6. Test for Saponins: (Foam test)

The leaf extract and the distilled water were mixed as same volume and the mixture was shaken vigorously. The formation of a layer of foam indicates the presence of Saponins.

#### 7. Test for Resins: (Precipitate test)

10 ml of distilled water was added to 5ml of leaf extract. A precipitate indicates the presence of Resins.

#### 8. Test for Phenols: (Ferric chloride test)

To a 2 ml of leaf extract, 1 ml of ferric chloride (FeCl<sub>3</sub>) solution was added. Deep blue black colour indicates the presence of Phenols.

### 2.20 Growth Inhibition Assay for *Shigella flexneri* Against IgY:

The fresh *Shigella flexneri* culture broth was prepared and kept for incubation at 37<sup>o</sup> c for 24 hours. Then the 24 hours culture was taken and 1 ml is poured into every 11 tubes. First tube is control tube without antibody. In second tube 0.1µl of IgY was added, in third tube 0.2µl of IgY was added. Continue this up to last tube. In last tube 1.0µl of IgY was poured. After the addition of antibodies the tubes were kept in incubation at 37<sup>o</sup> c for 24 hours. The OD value was taken at 470nm.

### 3.21 Growth Inhibition Assay for *Shigella flexneri* Against Serum:

The MIC value of the extract was determined as the lowest concentration that completely inhibited bacterial growth after 48hrs of incubation at 37C. The fresh *Shigella flexneri* culture broth was prepared and kept for incubation at 37<sup>o</sup> c for 24 hours. Then the 24 hours culture was taken and 1 ml is poured into every 11 tubes. First tube is control tube without serum. In second tube 0.1µl of serum was added, in third tube 0.2µl of extract was added. Continue this up to last tube. In last tube 1.0µl of serum was poured. After the addition of antibodies the tubes were kept in incubation at 37<sup>o</sup>c for 24 hours. The OD value was taken at 470nm.

### 3.22 Growth Inhibition Assay for *Shigella flexneri* Against Leaf Extract:

The MICs value of the extract was determined as the lowest concentration that completely inhibited bacterial growth after 48hrs of incubation at 37C. The fresh *Shigella flexneri* culture broth was prepared and kept for incubation at 37<sup>o</sup> c for 24 hours. Then the 24 hours culture was taken and 1 ml is poured into every 11 tubes. First tube is control tube without extract. In second tube 0.1µl of extract was added, in third tube 0.2µl of extract was added. Continue this up to last tube. In last tube 1.0µl of extract was poured. After the addition of antibodies the tubes were kept in incubation at 37<sup>o</sup>c for 24 hours. The OD value was taken at 470nm.

## III. RESULT AND DISCUSSION

### 3.1 Characterization of *Shigella flexneri*

The *Shigella flexneri* was grown on SS agar for 24 hours in 37°C temperature. The purity of the culture was checked by Gram staining. The cultural characteristics were rod, smooth, and opaque colonies. The microscopic appearance was observed and confirmed as *Shigella flexneri*.

### 3.2 Rapid Slide Agglutination Test:

Specificity of anti-*Shigella flexneri* antibodies in the egg yolk from immunized laying chickens was determined by Rapid slide agglutination Test (RSA). Appearance of agglutination within 2minutes, when the antigen was mixed with the corresponding IgY on plastic strip, revealed that the antibody generated in the immunized chicken which was purified as IgY-extracts from eggs which were specific against to their respective antigens.

### 3.3 IgY Profiling by SDS PAGE:

In this SDS Page it forms ladder with ranges (Figure 1). Each band in the ladder is known molecular weight. The samples can be determined from these known weights.



- Lane 1-Marker
- Lane 2- IgY fraction by 14<sup>th</sup> day of *Shigella flexneri*
- Lane 3- IgY fraction by 28<sup>th</sup> day of *Shigella flexneri*

Fig.1 - SDS PAGE of IgY

### 3.4 Quantification of antibody titer in chicken egg yolk and serum using Indirect ELISA

The antibody titre potency of specific IgY fractions obtained above was determined by the following Indirect ELISA method. The antibodies showed good titer value at 3rd and 4<sup>th</sup> week after immunization, which indicated the positive response of specific IgY against target antigens. In ELISA the concentration 1:100 it gives 0.126 value at OD<sub>450</sub>. The titration of IgY antibodies was determined using ELISA where In the highest titer of 0.222 was observed at 1:1,00,000 at OD<sub>450</sub>(Figure 2).

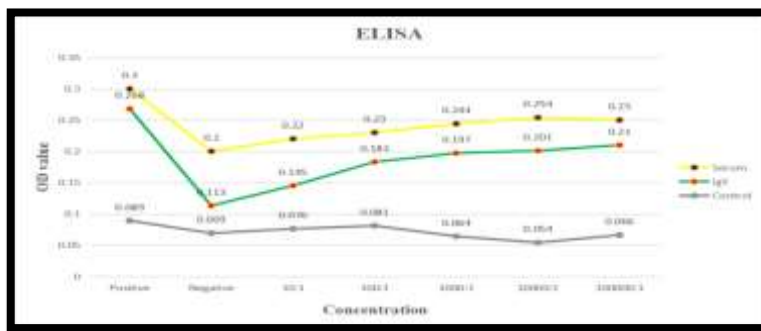


Fig.2 - ELISA Test using IgY and Serum

3.5 Antibacterial effect on leaf extract against clinical pathogens:

From the results obtained, the leaf extract inhibits the majority of clinical pathogens used in this study. The Methanol extract of Rhododendron shows inhibitory activity against *Shigella flexneri* and *Salmonella* sp., (Table-2).

The Azithromycin, which serves as a control shows intermediate inhibitory activity against three of the clinical pathogens and sensitive inhibitory activity against two of the clinical pathogens (Table-2).

3.6 Phytochemical analysis of leaf extract:

Majorly, all the phytochemicals like Alkaloids, Glycosides, Steroids, Flavonoids, Tannins, Saponins, Resins and Phenols were present in both the spice (*Rhododendron nilagiricum*) extracts. The only phytochemical, Glycosides were absent in Star anise extracts (Table-3)

ANTIBACTERIAL ACTIVITY OF LEAF EXTRACT:  
TABLE 2- *Rhododendron nilagiricum* EXTRACT

S. No.	ORGANISM	ZONE OF INHIBITION (mm)	
		METHANOL EXTRACT	CONTROL
1.	<i>Shigella</i>	12mm	25mm(azithromycin)
2.	<i>Salmonella</i> sp.,	14mm	24mm(amoxicillin)

PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACT  
TABLE 3 - *Rhododendron nilagiricum* EXTRACT

S. No.	PHYTOCHEMICALS	LEAF EXTRACT
1.	Alkaloids	-
2.	Glycosides	+
3.	Steroids	+
4.	Flavonoids	-
5.	Tannins	+
6.	Saponins	+
7.	Resins	-
8.	Phenols	+
PRESENT (+) ABSENT (-)		

3.7 Growth Inhibitory Assay using Egg Yolk IgY:

The Minimum inhibition concentration depends upon the level or concentration of antibody which we added to test sample. The smallest concentration of an antibody that inhibits the growth of *Shigella* antigen is 0.222. This value is known as Minimum inhibitory rate. It is useful for identifying inhibitory compounds present in it(Figure 3).

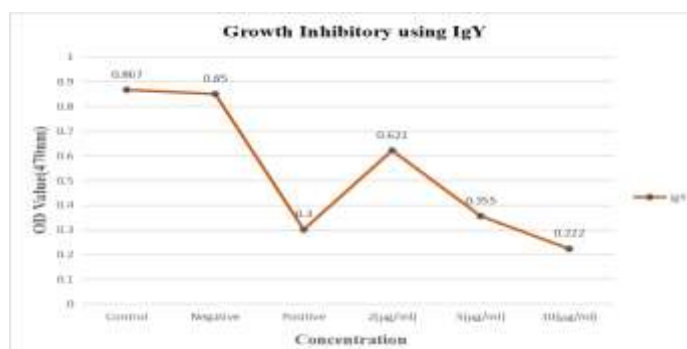


Fig.3 - Growth Inhibitory Assay using IgY



### 3.8 Growth Inhibitory Assay using Leaf Extract:

The Minimum inhibition concentration depends upon the level or concentration of leaf extract which we added to test sample. The smallest concentration of an extract that inhibits the growth of *Shigella flexneri* is 0.321. This value is known as Minimum inhibitory rate (Figure 4).

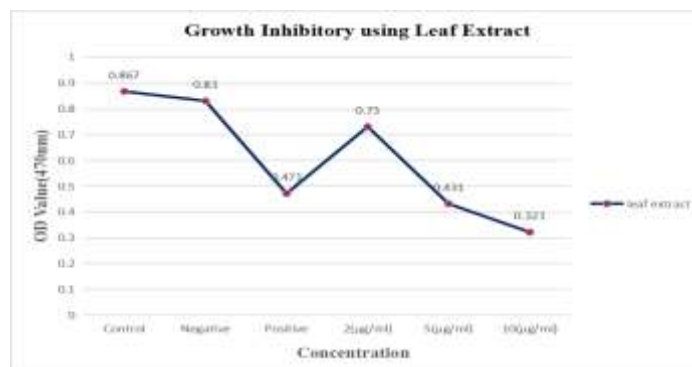


Fig.4 - Growth Inhibitory Assay using Leaf Extract

### 3.9 Growth Inhibitory Assay using Serum:

The Minimum inhibition concentration depends upon the level or concentration of Serum which we added to test sample. The smallest concentration of an antibody that inhibits the growth of *Shigella flexneri* is 0.09. This value is known as Minimum inhibitory rate (Figure 5).

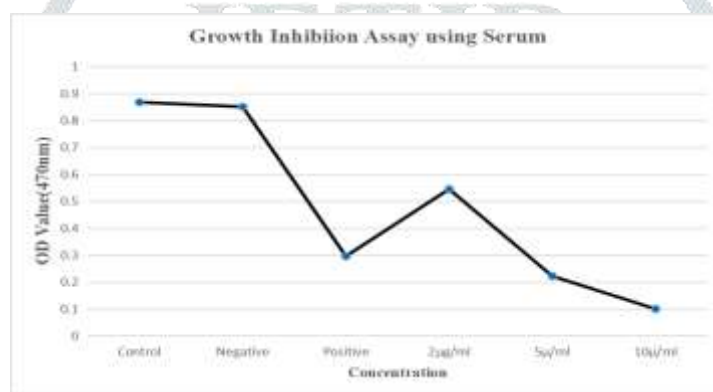


Fig.5 - Growth Inhibitory Assay using Serum

## IV. CONCLUSION

The present study was undertaken to generate antibodies against *Shigella flexneri* which cause Diarrheal infection and to examine the growth inhibitory activity of the *Shigella* antigen produced at different concentration for both IgY antibody in Egg Yolk and serum. Then also as a comparative counterpart the methanol extract of the desired plant is taken. This extract was taken in different concentration and was also assayed for the growth inhibitory activity.

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