STUDIES ON IMMUNOMODULATORY EFFECTS OF MEDICINAL PLANT EXTRACTS ON FIFTH LARVAL INSTAR OF TASAR SILKWORM

R. K. Verma* and P.K. Roy

Post Graduate Department of Biotechnology, T. M. Bhagalpur University, Bhagalpur-812007

ABSTRACT

Immunomodulatory effect of medicinal plants may enhance the survival of many organisms including silkworm by stimulating the immunity against different microbial pathogens. Tasar silkworm is a wild silk producing insect and cannot be domesticated yet for their controlled rearing. Direct exposure with the open environment makes them highly prone towards microbial infection causing crop loss and reduces the economy related to silk farming. Active phytochemicals present in various medicinal plants may provide passive immunity and work along with the insects own immune system. This synergistic approach may become beneficial to get rid of various microbial diseases associated with tasar silk rearing. In thes present study extracts of Neem and Tulsi was tested against bacterial pathogens and produces promising results. Different aspects as reductions in larval mortality, Increase in nodulation and Phagocytosis were noticed against *E. coli* and *Streptococcus* spp.

Keywords: Immunomodulatoratory Effect, Microbial Infection, Phytochemicals, Tasar, Silkworm

Introduction:

Immune functional modulation with the help of medicinal plants and their products has become an accepted therapeutic approach against several pathological conditions. Use of plant based material for treatment of many diseases and ailments were in practice since the start of civilization. Plant having antimicrobial and immunomodulatory activity is regarded as medicinal plant. Many ancient literatures are available regarding the use of plant materials and production for therapeutic activity. Chinese and Indian literatures have a detailed view regarding the use of a particular plant against a particular ailment (Kaminski *et al.,* 2008).

Immunostimulation comprise a prophylactic and therapeutic method that aims at the simulation of non specific immune system. It causes the activation of immunomodulatory cells and may enhance the process of innate immunity seaparately or synergistically (Chessa *et al.,* 2020).

Plants that have therapeutic values is regarded as medicinal plant. Earlier literatures such as Rigveda and Charak-Samhita have a detailed view of therapeutic activity of many plants. Plants and their products were early medicines and were used against many physical and physiological disorders. Immunomodulation by plant products could provide an alternative to chemotherapy for a variety of diseased condition, especially with compromised host (Wagner 1985; Jantan *et al.,* 2015). Medicinal plants having immunomodulatory function can serve as a sourse of alternative source of chemical treatment.

There effect of medicinal plant on silkworm is basically confined with their antimicrobial properties. The medicinal plants have antimicrobial compounds and thus being used as pesticides for number of pests and pathogens.

Medicinal plants:

According to ancient literature about 80,000 different species of plants can be utilized in different medicinal practices. Due to rapid advancement in allopathic treatments the use of traditional medicinal gets reduced sequentially with time. There are number of medicinal plants present around us but due to lack of knowledge, their benefits remain unnoticed.

Materials and methods:

Silkworm samples:

One day old fifth larval instars were taken as biological test model to evaluate the efficacy of medicinal plant extract against bacterial and fungal pathogens. Disease free and asymptomatic larval samples were collected from a batch of winter crop.

Selection of medicinal plants:

Out of number of medicinal plants available, Neem and Tulsi were selected randomly and in the light of their common occurrence. This makes their use feasible for the reares and farmers.

Preparation of plant extracts samples:

Leaves were selected for the preparation of medicinal plant extracts. Collected leaf samples were first washed thoroughly under the running tap water to wash out dust and other adhered things. Leaves were air dried in shade till they become brittle. The dried leaves were then grinded by using grinder in fine powder. 60 gram of prepared fine powder was suspended in

160 ml of aqueous solvent respectively for each plant samples. Solutions were placed on rotator shaker and maintained with a rotation speed of 60 rpm per minute for 48 hours. After that the samples were filtered out and dried in dissicator to evaporate all the liquid content used during extaction. The dried power samples were used to prepare 2%, 5% and 10% concentration samples in 10ml of distilled water for further use.

Preparation of inoculums:

Two bacterial samples of *E.coli* and *Streptococcus* were used to induce pathogenesis in fifth larval instar. *E.coli* bacterial samples were prepared by using log stage *E. coli* culture cells diluted with normal saline to make bacterial inoculums of $1x10^4$ cells/ml. Similarly, Steptococcus samples were prepared having a cell density of $5x\ 10^4$ cells/ml. The cell density of bacterial inoculums was adjusted to below $LD_{50.}$

Induction of pathogenesis in larvae:

Live bacterial inocula were injected in a volume of 3µl within abdominal region using Hamilton hypodermal syringe (Santiago *et al.,* 2015).

Application of prepared plant extracts:

Prepared extracts in different dilutions were separately sprayed directly upon feeding leaves of experimental test groups of *E.coli* and *Streptococccus spp*. 10ml of prepared plant extact sample was sprayed over 100 g of Arjuna leaves.

Assessment of larval mortality:

Numbers of deaths were recorded in each experimental set with respect of time in order to estimate the enhanced survival of infected larval population.

Assessment of THCs:

Change in total hemocyte count was observed by adopting the methods of Auber and Yeager (1935) and El-Bassyouni *et al.,* (2017). The hemolymph sample taken after 12 hours and 24 hours post inoculation was diluted by using thoma pipette to achieve a dilution of 20 times. Improved nubaur haemocytometer was used for counting hemocytes present in haemolymph samples of control and test groups according to formula given by Jones (1962).

Assessment of Phagocytosis:

By adopting the method of Rowley and Ratcliffe (1980), giemsa stained microscopic slides were observed under light microscope to calculate the percentage phagocytosis.

Assessment of nodulation:

Estimation of percentage nodulation in 5th larval instar was done according to the method of Gunnarsson and Lackie (1985). Larval instars were dissected under light microscope with the help hypodermal needle at 6, 12, 18 and 24 hours of post inoculation to observe the presence of nodules. The number of nodules observed was counted and percentage nodulation was calculated accordingly.

Results:

Percent mortality change in 5th larval instar:

For the period of 6 hours post inoculation showed minimum death percent while maximum death percentages were recorded at 48 hours of post inoculation in neem and tulsi extract treatment groups. For *E.coli* positive control group the 78.6%, 62.4%, 36.7% and 18.1% mortality were observed for 48, 24, 12 and 6 hours of post inoculation respectively. Percentage reduction in mortality of infected larvae were recorded for each treatment groups of 2%, 5% and 10% concentration of medicinal plant aquous extracts for both Neem and Tulsi. The maximum decrease in % mortality was observed in 10% treatment groups of both Neem and Tulsi extacts.

Table 1.0: % Mortality observed in 5th instar larval groups infected with *E coli.*

% 5 Mortality in *E. coli* treated Groups							
Time (hr)	Positive Control	Conc. of Neem Extract			Conc. of Tulsi Extract		
		2%	5%	10%	2%	5%	10%
6	18.1	15.4	12.5	8.2	20.4	16.5	8.3
12	36.7	34.3	28.6	15.8	26.2	20.8	12.6
24	62.4	58.2	46.7	34.2	45.7	36.4	30.4
48	78.6	72.7	64.3	41.3	65.3	54.7	40.5

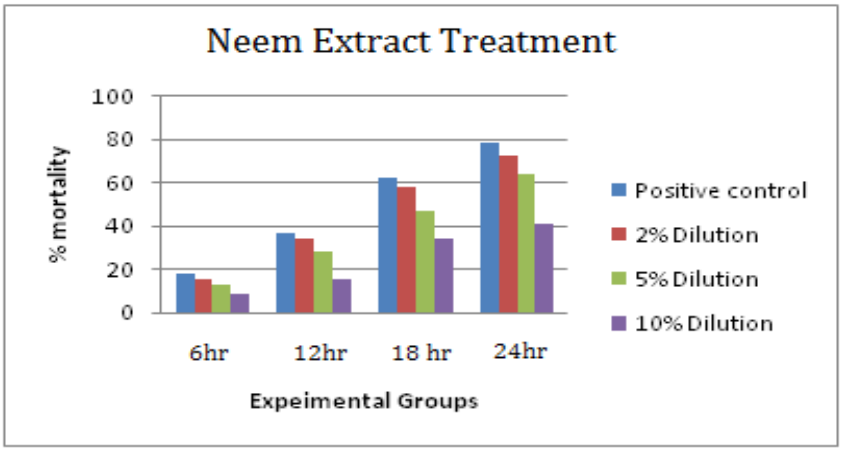

Fig 1.0: % Mortality in control and Neem etract treated 5th instar larval groups infected with *E.coli*

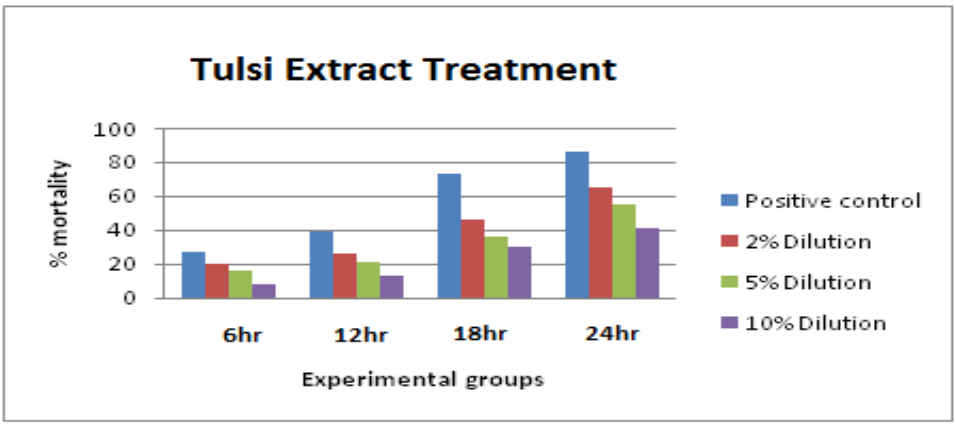

Fig. 2.0: % Mortality in control and Tulsi extract treated 5th instar larval groups infected with *E. coli*

It was revealed that the minimum and maximum deaths of 24.7% and 88.4% occurred at around 6 hr and 48 hours of post inoculation in positive control groups without any treatment. Both Neem and Tulsi treatment groups showed maximum decrease in % mortality in 10% extract groups. The decrease in % mortality was found higher in 10% treatment group followed by 5% and then 2% treatment groups of Tulsi extract for all concentrations.

Table 2.0: % Mortality observed in 5th instar larval groups infected with *Streptococcus Spp.*

% Mortality in *Streptococcus* Groups							
Time (hr)	Positive Control	Conc. of Neem Extract			Conc. of Tulsi Extract		
		2%	5%	10%	2%	5%	10%
6	31.4	24.7	17.5	11.5	21.4	19.8	10.5
12	46.3	32.5	27.2	21.6	31.7	24.3	20.1.
24	72.2	56.6	48.6	41.5	54.6	41.6	32.7
48	88.4	64.8	57.8	51.9	72.3	65.7	51.2

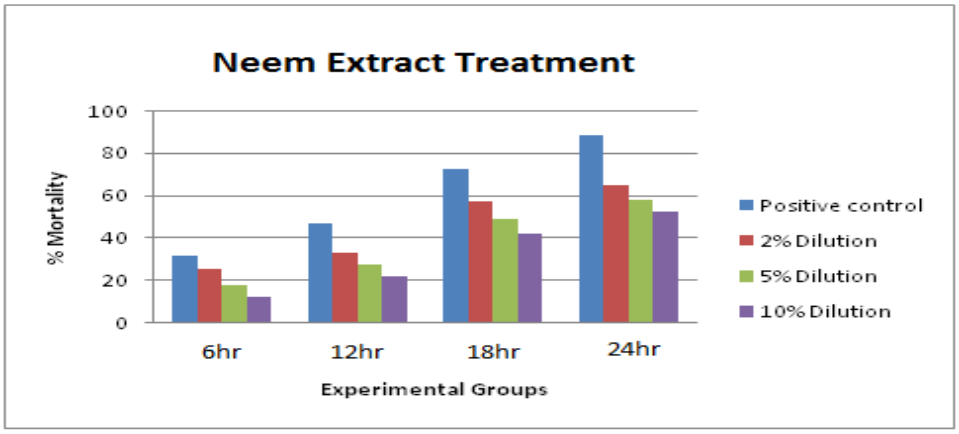

Fig. 3.0: % Mortality in control and Neem extract treated larval groups infected with *Streptococcus spp.*

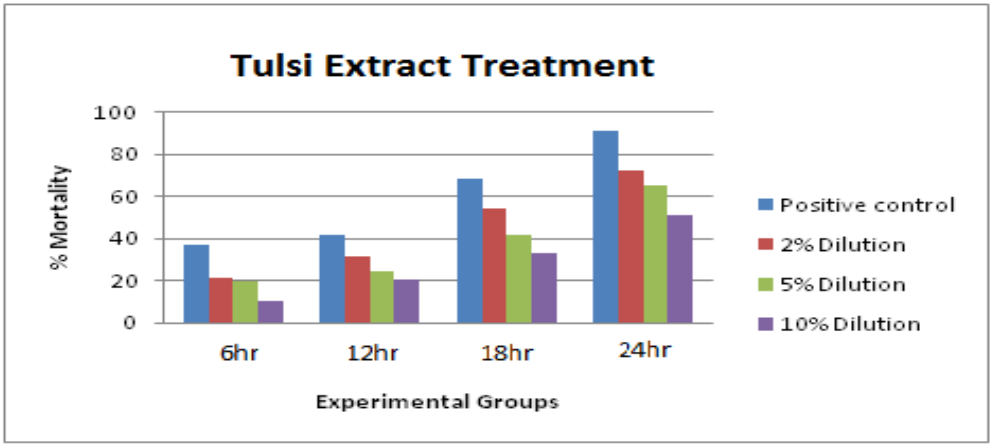

Fig. 4.0: % Mortality in control and Tulsi extract treated larval groups infected with *Streptococcus spp.*

Change in Toatal Hemocyte Counts (THCs):

It was revealed that the total hemocyte count increases in positive control groups and treatment groups of *E.coli* and *Streptococcus* experimental groups. The results the the number of THCs in positive control groups were 1487.58±187, and for 2%, 5% and 10% treatment group were have a average mean of 1493.47±182, 1501.45±147 and 1544.47±157 cells/mm3 respectively. *E. coli* experimental set treated with Neem leaves extract. The 5% experiment set of Neem treated experimental group the THCs were present in 15014.58 ± 147, 1578.75±186 and 1592.14±147 cell/mm3 respectively. The 10 % treatment group of Neem on *E. coli* treatment group reveals to have 1521.24 ±165, 1589.56 ±147 and 2042.25 ± 165 cell/mm^3. Similarly, within the Tulsi extract treatment *E.coli* groups the highest number of THCs was found in 10% treatment groups having a overall mean of 2011.24 ± 145 cell/mm^3.

In Streptococcus infected experimental groups, the 10% concentration of Neem and Tulsi extract were found to have the heighest average means value of THCs viz, 2147.45 ± 187 and 2217.26 ± 164 cells/cm^3 .

Estimation of % Phagocytosis:

Phagocytosis was observed within prepared slides for 4 different time intervals in control and treatment groups of Neem and Tulsi. It was observed that the % phagocytosis increases for 6 and 12 hours of post inoculation in both treatment groups. Results are presented in Table 5.03 and fig 5.05 & 5.06for *E. coli* treatment sets and in Table 5.05 and Fig 5.07 & 5.08 for *Streprococcus* treatment sets.

Table 3.0: % Phagocytosis observed in control and *E.coli* treated groups

% Phagocytosis in *E.coli* infected Larval Groups							
Time (hr)	Positive Control	Conc. of Neem Extract			Conc. of Tulsi Extract		
		2%	5%	10%	2%	5%	10%
6	4.2	6.7	6.8	7.2	13.1	14.3	14.9
12	41.6	55.1	57.2	59.4	60.2	62.2	64.5
24	14.8	16.4	18.1	19.8	26.3	27.5	29.6
48	3.4	4.5	4.8	4.8	10.1	11.6	12.4

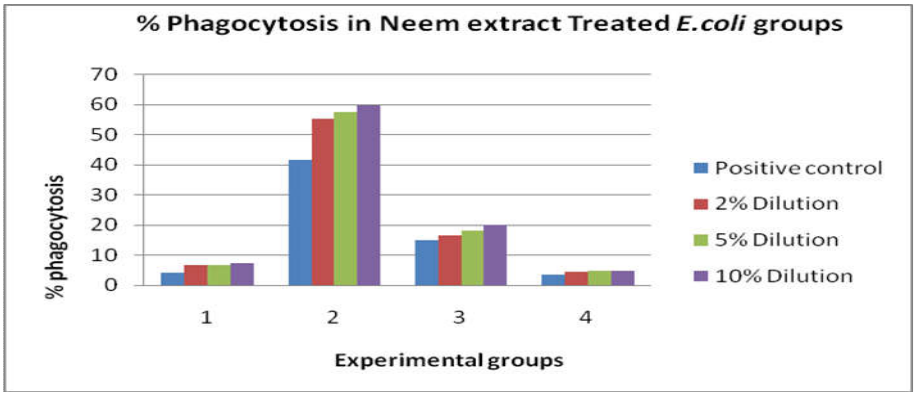

Fig 5.0: % Phagocytosis in Neem extract treated larval groups infected with *E. coli*

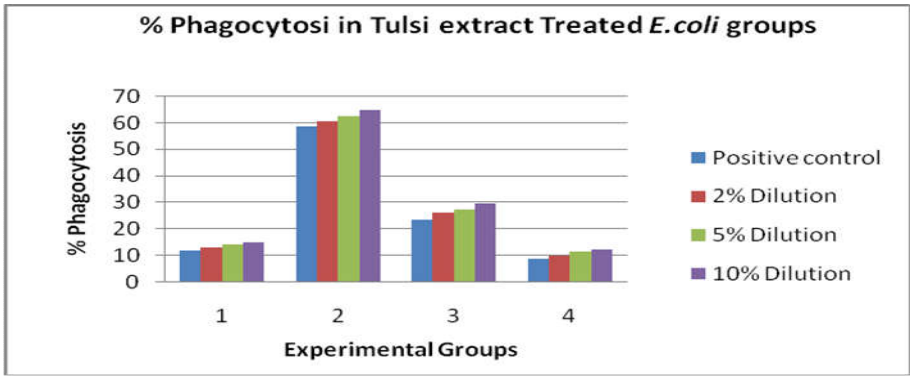

Fig. 6.0: % Phagocytosis in Tulsi extract treated larval groups infected with *E. coli*

Table 4.0: % Phagocytosis observed in control and Streptococcus treated groups.

% Phagocytosis in *Streptococcus* infected Larval Groups								
Time (hr)	Positive Control	Conc. of Neem Extract			Positive Control	Conc. of Tulsi Extract		
		2%	5%	10%		2%	5%	10%
6	11.5	12.8	14.2	15.3	13.1	14.4	15.7	16.8
12	60.2	61.4	63.4	64.7	62.5	63.4	65.8	66.1
24	25.1	27.3	29.4	31.2	27.6	29.5	33.4	34.5
48	10.1	12.2	14.1	15.3	11.8	12.7	13.8	15.4

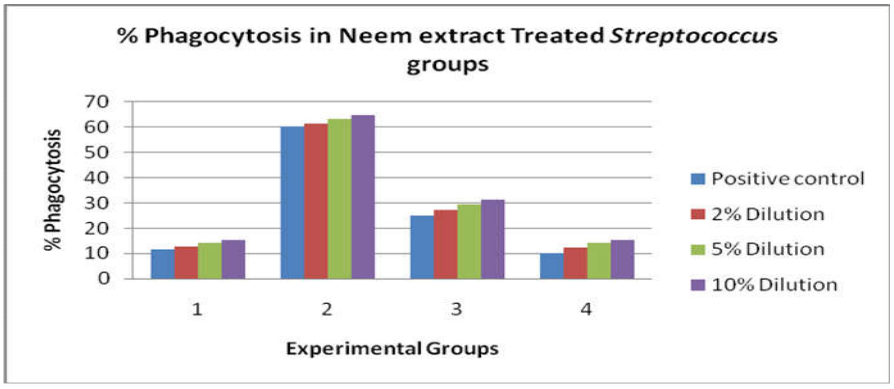

Fig. 7.0: % Phagocytosis in Neem extract treated *Streptococcus* sets

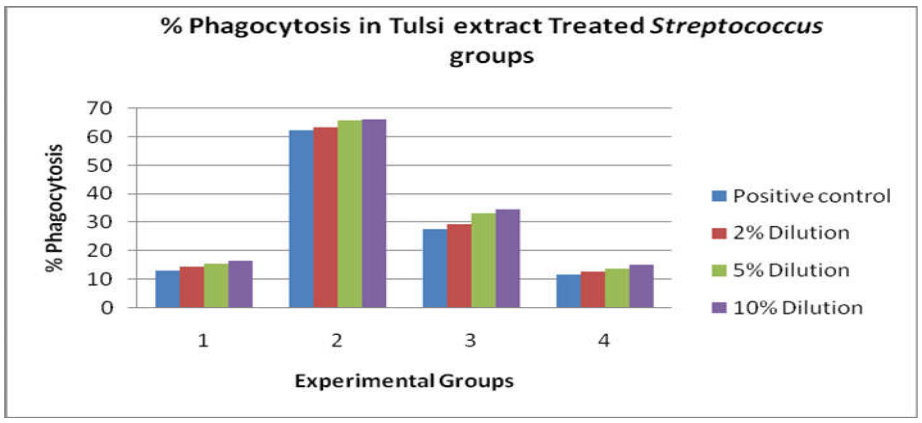

Fig. 8.0: % Phagocytosis in Tusli extract treated *Streptococcus* sets

Estimation of Nodulation:

The percentage occurrence of nodulation in positive control and treatment groups of *E. coli* experimental sets were estimated through light microscopic examinations of control and treated larval samples with 2%, 5% and 10% concentration of Neem and Tulsi extracts. The results are summarised in table 5.0 and fig. 9.0 & 10.0. The results shows that the percentage of nodulation increases linearly in both control and treated groups for both Neem and Tulsi. 10% concentration of Neem and Tulsi leaves extract were found to mount maximum % nodulation under similar set of conditions. Comparatively Tulsi leaves extracts showed higher nodulation percentage with respect to Neem leaves extracts prepared in distilled watern solvent.

Table 5.0: % Nodulation in *E. coli* infectedd groups with neem and tulsi extract treatment

% Nodulatin in *E.coli* experimental Groups								
Time (hr)	Positive Control	Conc. of Neem Extract			Positive Control	Conc. of Tulsi Extract		
		2%	5%	10%		2%	5%	10%
6	11.8	17.2	20.3	22.1	8.5	10.4	12.4	14.7
12	21.5	26.4	32.1	34.3	31.4	33.6	36.1	39.4
24	42.1	48.6	50.3	52.2	52.1	55.8	58.2	61.4
48	62.3	69.7	73.5	76.1	72.8	74.8	76.2	78.6

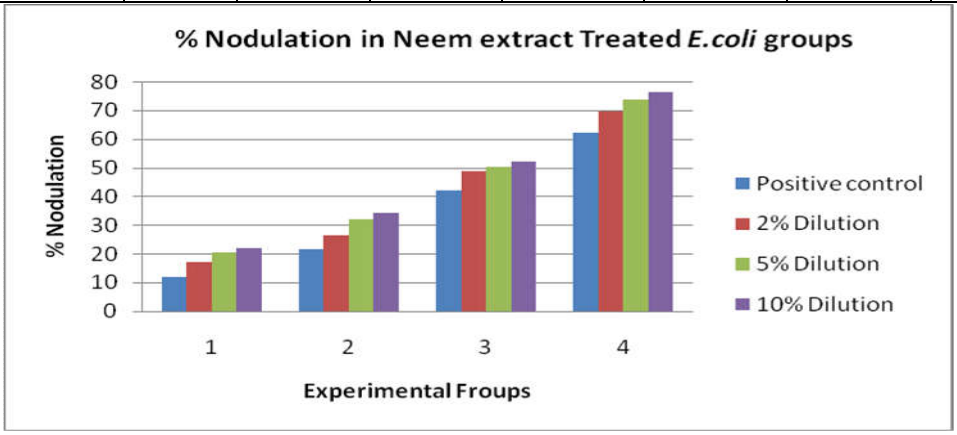

Fig 9.0: % Nodulation in Neem extract treated *E. coli* groups

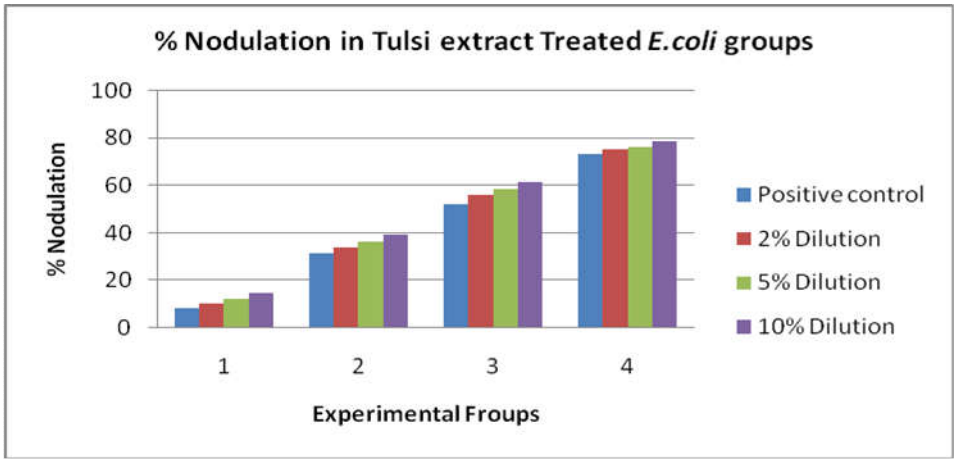

Fig. 10.0 : % Nodulation in Tulsi extract treated *E.coli* groups

It was revealed that Streptococcus bacterial pathogen elicit higher % of nodulation in both positive control and treatment groups. The percentage of nodulation increases linearly post inoculation, having a peak after 48 hours of post inoculation. the tree different concentration of leaves extracts shows similar kind of immunomodulation by increasing the percentage of nodulation. Table 6.0 and Fig. 11.0 & 12.0 show the result of nodulation pattern in *Streptococcus* experimental sets.

Table 6.0: % Nodulation in *Streptococcus spp.* infectedd groups with neem and tulsi extract treatment

% Nodulation in *Streptococcus* experimental Groups								
Time (hr)	Positive Control	Conc. of Neem Extract			Positive Control	Conc. of Tulsi Extract		
		2%	5%	10%		2%	5%	10%
6	11.5	13.5	14.2	15.1	13.4	14.8	15.2	15.8
12	42.3	44.1	46.1	48.7	44.1	45.7	46.7	47.1
24	64.7	65.2	66.2	68.6	65.4	66.4	68.4	69.3
48	88.4	88.7	89.1	91.3	86.3	87.5	88.3	89.2

The result shows that there is a linear trend of elevation of % nodulation in both positive control and treatment groups with slight variation among different experimental sets of a particular group.

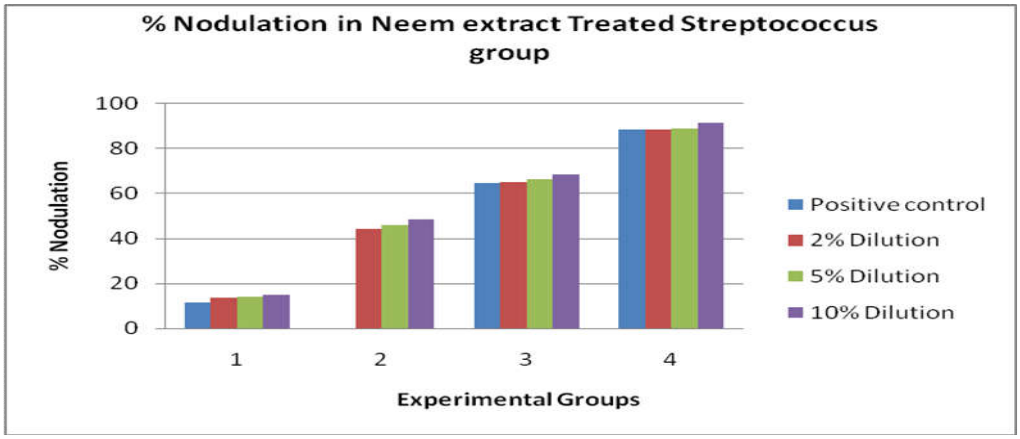

Fig. 11.0: % Nodulation in Neem extract treated streptococcus groups

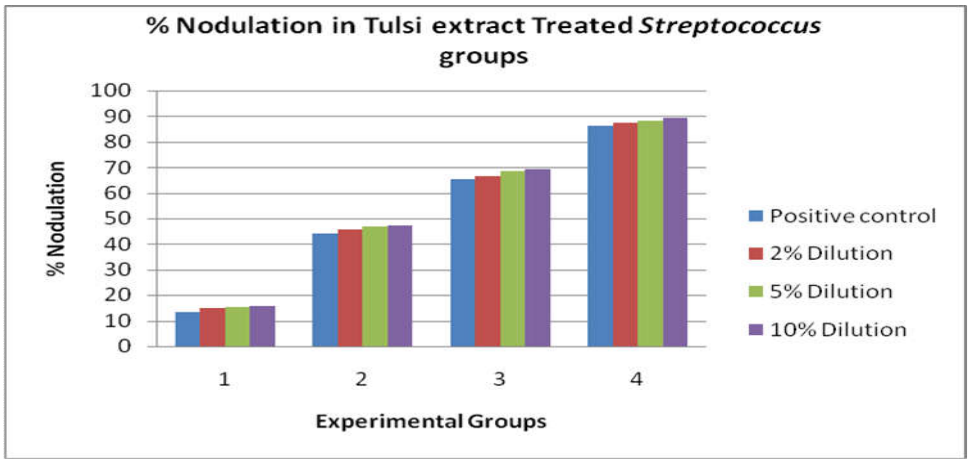

Fig 12.0: % Nodulation in Tulsi extract treated *Streptococcus* groups

Discussion:

The present study reveals that medicinal plant extract posses immunomodulatory effect on tasar silkworm, *Antheraea mylitta* Drury. As evident from different parameters tested in fifth larval instar of winter crop. Medicinal plants such as Neem and Tulsi has immunomodulatory effect as it contains number of active phytochemicals such as flavonoids, alkaloids. Terpenoids, saponins etc. these molecules are known to immunomodulatory effect on numbers of animals including humans.

The method of extraction and solvent used may change the profile of active phytochemicals in the extracts. Most commonly, phytochemicals were extracted with aqueous, ehanolic and methanolic solvents, according to the solubility in a particular solvent; a unique profile of phytochemicals gets leached out into the solvent according to their density separation. Tasar silkworm possesses innate immune system at both cellular and humoral level and efficient to

cope up number of microbial diseases along with the inert foreign molecules. The best way is to phagocytose them along with nodulation and melanisation. Present stuhy shows similarity with this theory, as it was observed that the process of phagocyticis is quick and occurs early than that of nodulation, nodulation process of innate immunity was found to operate optimally after 24 hours of post inoculation.

Immunomodulation technique by the application of medicinal plant extracts may become an alternative to the use of costlier antibiotics. As the regular use of antibiotics make the pathogen more tolerant against the regular dose generally prescribed. Medicinal plant extracts also possess antimicrobial properties against number of microbial agents such as bacteria and fungi. Thus its use on infected silkworm larvae may provide two folds effectiveness against pathological agents. This may be the case with the present study as evident from much higher survival percentage after successful established infection results. The use of such immunomodulatory compound are viable as it elevates the hemocyte counts of the host insect and help them to cope up the infection much efficiently, this is evident from the result as there wes an increase in the total hemocyte was noticed in both *E. coli* and Streptococcus bacterial pathogen infected experimental control and treated groups. The use of medicinal plants thus is encouraged among the rural, poor and small scale farmers to minimise the chances and severity of microbial infections.

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