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Studies of the Immuno-stimulant properties of *Tinospora Cordifolia*

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ABSTRACT

The present project is aimed at finding effective immunostimulant herbal drug. Tinosporacordifolia immunostimulant activity can be evaluated by Neutrophil adhesion test. Three groups of animals each of either sex at a weight of about 2kg was selected for the study. They were provided with standard diet. Aqueous extract of plant was obtained from leaves, stems and roots, 10 gram of each part taken with 100ml of water then stirred well and boiled for 10min and filtered. The aqueous extract was given orally at a dose of 5ml/day for 14 days rabbits for assessment of immune stimulant effect. Neutrophil adhesion test, Total count of white blood corpuscles, differential leukocyte counts carried out. Complement activity alternate pathway studied by erythrocyte was antigen to rabbit serum. The blood sample was taken alternate pathway and chemotaxy were evaluated. The study reveals that Tinosporacordifolia has significant alternate pathway activity and chemotaxy.

Keywords: Immunostimulant, Tinospora cordiofolia Neutrophil adhesion, altertnate pathway, Chemotaxy, Neutrophil adhesion, complement activity.

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INTRODUCTION

Management of infectious disease is still a major problem for health care administration throughout the world.

Infection

Infection is the entry and development or multiplication of an infecting agent in the body of man or animals. It also implies that the body responds in some way to defend itself against the invader, either in the form of an immune response or disease.[1]

Infectious disease management

Infectious diseases are mainly managed by chemotherapeutic, immunization and by making environment more hygienic.[2]

Development of newer antimicrobial agent also be limited use for the reasons that in due course that would become less effective due to development of microbial resistance as far as their mode of action is microbial. There is a large number of mechanisms by which the immune system works. Alternative pathway is considered as first line defense against infections.[3]

Complement system[4]

Complement system major effector of humoral branch of the immune system. It protects the body against infecting organism and may play a role in destruction of tumour cells.[5]

Complement system contains more than 30 proteins. When the system is activated various complements interact in a highly regulated cascade to form membrane attack complex.[6]

- Lysis of cell, bacteria, and viruses.
- Opsonization, which promotes phagocytosis of particulate antig
- Binding of specific complement receptors on cells of the immune system, triggering activation of immune response such as inflammation and secreation of immunoregulatory molecules that amplify of alter specific immune responses.
- Immune clearance, which remove immune complex from the
- Circulation and deposits them in spleen and liver.
  Formation of membrane attack complex can occur by alternative pathway.

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Alternative pathway:[7]

Alternative pathway is an antibody-independent, which generates bound C5b; this major pathway of complement activation involves four serum proteins: C3, factor B, factor D, and properdin. For example both gram negative and gram-positive bacteria have cell-wall constituents that activate the alternative pathway.

In alternative pathway, serum C3, which contains an unstable thioester bond, subject to slow hydrolysis to yield C3a and C3b bind to surface antigens such as bacterial cells or viral or even to host's own cells. Foreign cells can bind another serum protein called factors B by way of mg2+dependent bond. Factor D cleaves the C3b bound factor B releasing a small fragment Ba which diffuses away, and generates C3bBb. The C3 convertase activity of C3bBb has a half life of only 5minutes unlike serum proteins.

The enzymatic C3b component binds C5, and the Bb compounds subsequently hydrolyzes the bound, C5 to generate C5a and C5b the latter binds to the antigenic surface.

OBJECTIVE

Treatment of infectious disease is a major challenge for health care providers due to development of resistant microbial strain and making potent and costly anti-infective agents less effective in course of time. Recently there is a radical change in the concept, approach and experimental design for the development of anti-infective agents.

Long lasting solution could be the development of immunostimulants which can strengthen the body defense mechanism there by cure infectious disease. In this line the present project is aimed at finding effective immunostimulant herbal drug.[8]

The search is based on the assumption that "Fever if mostly due to microbial infection and a large number of herbal drugs are being used for fever. Further the mechanism of action may not by anti infective property but by immunostimulant property". Hence the herbal drugs used for the treatment of fever are the potential source for immuno stimulants.[9]

"Tinospora cordiofolia" for its immuostimulant acivity can be evaluated by neutrophils adhesion test and alternate pathway.

PLANT INTRODUCTION:

In indigenous system many tinospora species are used for various ailments commonly used species are tinospora cordifolia, tinospora malabarica, tinospora crispa.[10]

Botanical name : Tinospora cordifolia
Family : Menispermaceae

Habitat : Found throughout tropical India, ascending to altitude of 1000 ft.
Chemical composition: Different constituents including glycosides, alkaloids, and Bitter principles identified as chasmanthin and palmarin, Alkaloid tinosporin was also identified. [11]
Pharmacology : studies on induced edema and arthritis and antipyretic action and is a potentiator. It induced reduction in blood sugar in Alloxan induced hyperglycemia rats and rabbits. [12]

Properties and Uses:

Tinosporacordifolia is mentioned in ayurvedic literature as a constituent of several compound preparations, used in general debility. Dyspepsia, fever and urinary diseases. The bitter principle of drug showed antiperiodic, antispasmodic, anti-inflammatory and antipyretic properties. [13]

Alcoholic extract shows activity against Eschrihchia coli. The acute and chronic effects of oral feeding of the plant extracts to rabbits and albino rats on fasting blood sugar, glucose tolerance against hypoglycemia studied.

The leaves are rich is protein and fairly rich in calcium and phosphorous, and used as fodder. They contain crude protein, a decoction of the leaves is used for the treatment of gout, and young leaves, bruised in milk, are used as liniments in erysipelas[14]

MATERIAL AND METHODS

Three groups of three animals each of either sex at a weight of about 2 kg was selected. They were provided with standard diet. Institutional animal ethical committee (IAEC) was approved.

Preparation of aqueous extract:[15]

Fresh leaves of Tinosporacordifolia was washed and cut in to small pieces. 10 gm of each part was taken with 100 ml of water then stirred well and boiled for 10 min and filtered. The filtrate was concentrated to 25 ml.

Drug administration:[16]

Albino rats of either sex weighing 2.5 kg were used for the study. The animals were divided in to three groups consisting of three animals each. Before administration of drug they act as control. The aqueous herbal extract was administered orally at a dose of 5ml/ day for 14 days to rabbits for assessment of immuno stimulant effect. The blood was drawn on 7th day and 14th day.

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Chemotaxy:

Chemotaxy was studied based on the principle that neutrophils tend to adhere to a foreign particle.

Neutrophil adhesion test:[17]

Before administration of the drug, 7th,14th of after administration of the drug treatment, blood samples were collected by puncturing the retro-orbital count plexus into heparinised vials and analysed total leukocyte counts (TLC) and differential counts (DLC) by fixing blood smears and staining after initial counts, blood samples were incubated by with 80 mg/ml of nylon fibers 15 min at 370c.

The incubated blood samples were again analysed for TLC & DLC. The product of TLC and % neutrophils index of blood sample. Perecent neutrophil adhesion was collected as shown below

$$\text{Neutrophil adhesion (\%)} = \frac{\text{NI}\mu - \text{Nt}}{\text{NI}\mu} \times 100$$

NIμ— Neutrophil index of untreated blood sample.
Nlt— Neutrophil index of treated blood sample.

TOTAL COUNT OF WHITE BLOOD CORPOSUCLES

Requirement:

1. **W.B.C Pipette:** [18]

It has got 3 graduations. 0.5 and 1, are present on the stem of the pipette and the third mark 11, is placed just above the bulb. Blood drawn, say, up to mark 1 and the rest of the bulb filled by diluting solution 11. The bulb of the pipette is constructed that it holds exactly 10 times the volume of fluid contained in the stem of the pipette up to mark 1. Although fluid is drawn up to 11, the dilation of the blood will be 10.

2. **The counting chamber:**

The ruling area consists of 9 square millimeters. The central of 10 small squares, each group separated by triple line.

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METHOD OF COUNTING W.B.C:

The white cells are counted in the fair corners of 1 square millimeter and in the central ruled are on both sides. The counting chambers of the haemocytometer 10 square millimeters in all.

$$\text{The number of leucocytes per cu.mm} = \frac{\text{Number of cells counted} \times \text{dilution} \times 10}{\text{number of 1square millimeter counted}}$$

Differential lecuocytes counts:[19]

A thin blood is made on a clean, dry, glass slide. It is dried stained to differentiate types of leucocytes. Leishman's stain is used.

Procedure:

A thin blood film is made on a clean dried glass slide. It is dried stained with Leishman's stain solution. During this time methyl alcohol fixes the blood films. Add double films. The acidic dye eosin stains various acidophilic structures and basic methylene blue stains basophilic structure like nucleus, basophilic granules cytoplasm of the lymphocyte and monocyte.

COMPLEMENT ACTIVITY ALTERNATIVE PATHWAY:[20]

Complement activity alternative pathway studied on rat erythrocyte will undergo haemolysis when rabbit serum is added, due to the antigen antibody reaction. Heamolysis leads to liberation of haemoglobin which imparts red colour to the solution.

The intensity of red colour can be determined as a measure the absorbance 450 nm using reagent blank.

Erythrocyte cell preparation:[21]

Collect the blood from the rat in to acid citrate dextrose solution and store blood at 410c. Wash the erythrocytes twice in 20 volumes PBS. After the final centrifugation in a graduate centrifuge tube resuspends the cells in phosphate buffered saline 10%.

Ingredients	Sample	100% hemolysis	Blank
RBC	0.25	0.25	0.25
Serum	0.1	—	—
Dilityfulid	—	—	1ml
Water	—	1ml	—

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Test procedure:[22]

Blank, sample and 100% lysis are incubated 370C for 1 hour then make up to 50ml using diluting fluid. Centrifuge and collected to supernatant solution. Measure the absorbance of B and C at 450nm using A is blank.

Complement hemolytic activity in serum is most often expressed in serum is CH50 unit produces 50% haemolysis. It is treated with the optimal concentration of serum and lysed at 370C in complement fixed diluents.

Table 6.1.1: NEUTROPHIL ADHESION TEST OF LEAVES TREATED ANIMALS—I

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	5.7	5.4	45%	40%	256.5	216.0	15.78	—
2	7thday	5.8	4.8	48%	43%	278.40	206.4	25.86	63.87
3	14thday	6.7	5.5	58%	44%	388.6	258.5	33.47	112.10

Table 6.1.2: NEUTROPHIL ADHESION TEST OF LEAVES TREATED ANIMALS—II

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	5.8	5.1	38%	33%	220.4	168.3	23.63	—
2	7thday	5.0	4.0	50%	47%	250.0	180.00	28.00	18.49
3	14thday	6.5	6.1	58%	49%	383.50	298.9	32.05	35.67

Table 6.1.3: NEUTROPHIL ADHESION TEST OF LEAVES TREATED ANIMALS—III

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	5.4	5.1	47%	45%	253.80	229.50	9.57	—
2	7thday	6.8	6.0	48%	40%	326.40	240.00	26.47	176.50
3	14thday	5.3	4.0	35%	33%	185.50	132.00	28.84	112.40

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CHEMOTAXY- NEUTROPIL ADHESION TEST

Figure no: 6.1.1(a) Leaves treated animals

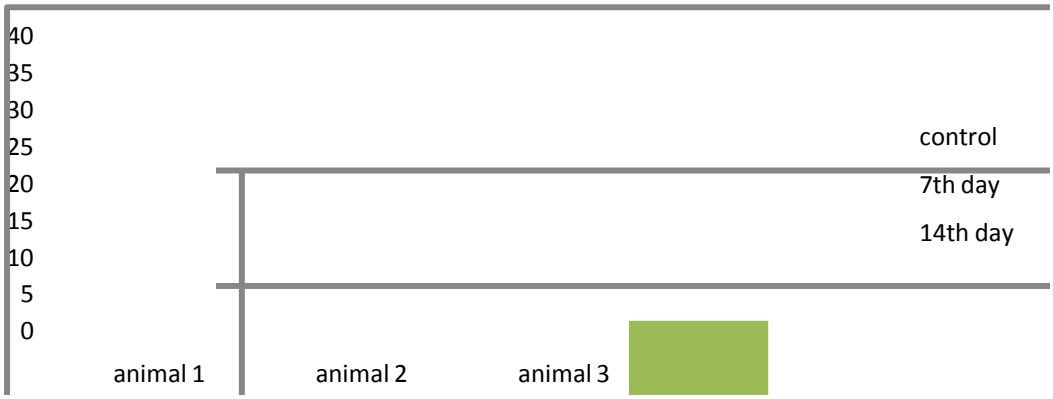

Table 6.1.4: NEUTROPHIL ADHESION TEST OF STEM TREATED ANIMALS—IV

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	5.1	4.2	48%	40%	244.8	168.00	31.37	—
2	7thday	5.8	4.2	43%	40%	249.4	168.0	32.63	4.01
3	14thday	6.2	5.1	50%	42%	310.0	214.2	36.90	17.62

Table 6.1.5: NEUTROPHIL ADHESION TEST OF STEM TREATED ANIMALS—V

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	4.5	3.8	45%	38%	202.5	144.4	30.17	—
2	7thday	5.5	4.2	48%	43%	264.0	176.4	33.18	9.97
3	14thday	6.5	5.1	50%	47%	325.0	239.7	36.24	20.11

Table 6.1.6: NEUTROPHIL ADHESION TEST OF STEM TREATED ANIMALS—VI

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	4.8	4.2	45%	48%	216.0	168.0	22.22	—
2	7thday	5.2	4.5	48%	40%	249.6	249.0	27.88	25.47
3	14thday	6.5	5.3	55%	47%	357.5	357.5	30.32	36.45

Mean % increase in neutrophil adhesion after 7thday —13.15
Mean% increase in neutrophil adhesion after 14thday —24.72

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CHEMOTAXY-NEUTROPHIL ADHESION TEST

Figure 6.1.1(b): Stem treated animals

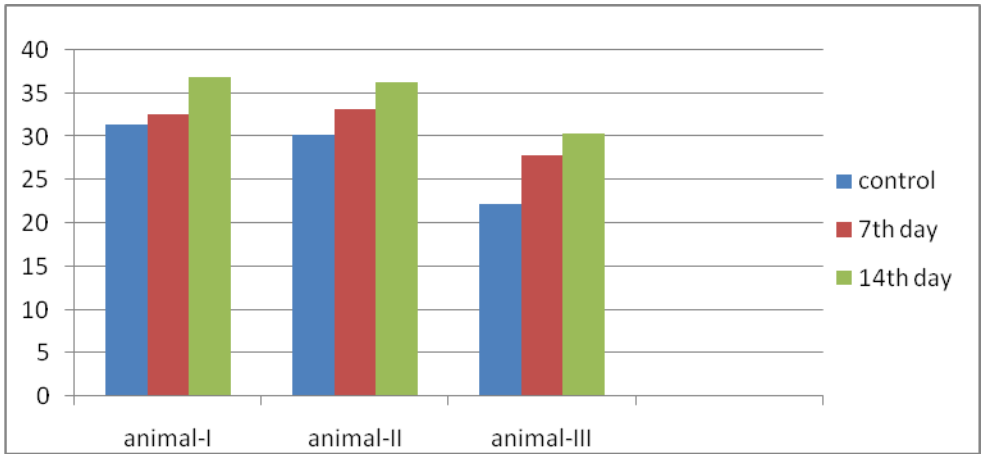

Table 6.1.7: NEUTROPHIL ADHESION TEST OF ROOT TREATED ANIMALS- VII

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	5.9	5.2	45%	43%	265.5	223.6	15.78	—
2	7thday	5.7	5.0	38%	33%	216.6	165.0	23.82	50.95
3	14thday	6.9	5.5	48%	42%	331.2	231.0	30.25	91.69

Table 6.1.8: NEUTROPHIL ADHESION TEST OF ROOT TREATED ANIMALS—VIII

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	5.3	5.0	43%	48%	227.9	190.0	16.99	—
2	7thday	5.2	4.8	39%	30%	202.8	144.0	28.59	68.27
3	14thday	6.3	5.7	48%	41%	304.8	233.7	30.16	77.51

Table 6.1.9: NEUTROPHIL ADHESION TEST OF ROOT TREATED ANIMALS—IX

S.No	Treatment	TLC (103/mm3) (A)		Neutrophil (%) (B)		Neutrophil index (A× B)		Neutrophil Adhesion (%)	(%)Increase Neutrophil Adhesion
		UB	**FTB**	**UB**	**FTB**	**UB**	**FTB**		
1	Control	4.9	4.7	40%	37%	196.0	173.9	11.27	—
2	7thday	5.5	5.0	43%	40%	236.5	200.0	15.43	36.91
3	14thday	5.9	5.3	45%	43%	265.5	227.9	17.16	52.26

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CHEMOTAXT-NEUTROPHIL ADHESION TEST

Figure No.6.1.1: Root treated animals

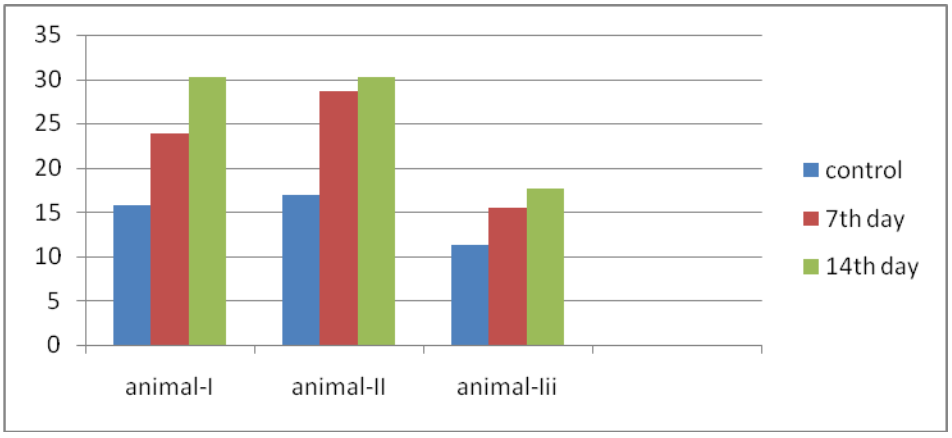

Table 6.2.1: COMPLEMENT ACTIVITY ALTERNATE PATHWAY LEAVES TREATED ANIMALS

S.No	% Haemolysis			% increasing activity after	
	Initial (A)	7thDay (B)	14thDay (C)	7th day (B-A)/BX100	14thday (C-A)/CX100
1	10.78	58.16	121.60	81.46	91.11
2	23.69	78.63	107.73	69.49	78.00
3	40.55	58.25	122.21	30.38	66.77
Mean	25.00	65.01	117.18	60.44	78.62

COMPLEMENT ACTIVITY ALTERNATE PATHWAY

Fig no 6.2.1 (a): Leaves treated animals

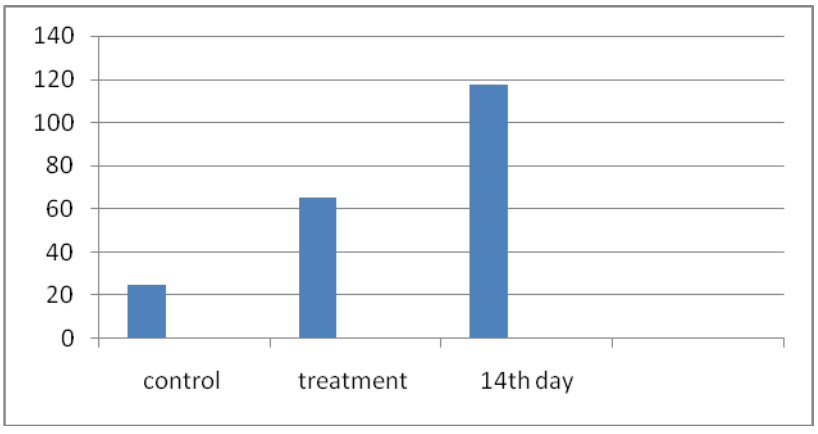

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Table 6.2.2: COMPLEMENT ACTIVITY ALTERNATE PATHWAY STEM TREATED ANIMALS

S.No	% Haemolysis			% increasing activity after	
	Initial (A)	7thDay (B)	14thDay (C)	7th day (B-A)/BX100	14thday (C-A)/CX100
1	18.74	10.49	126.50	78.64	85.18
2	45.02	94.63	110.93	52.42	59.41
3	37.65	119.19	105.92	57.27	76.98
Mean	33.80	74.77	114.45	62.77	73.85

COMPLEMENT ACTIVITY ALTERNATE ACTIVITY

Fig 6.2.1(b): Stem treated animals:

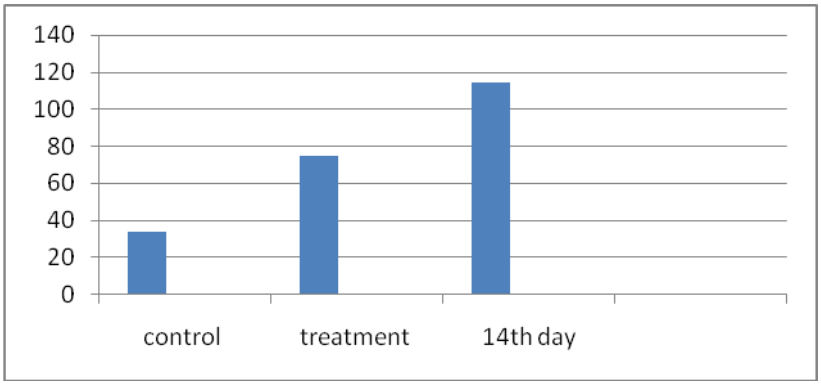

Table 6.2.3: COMPLEMENT ACTIVITY ALTERNATE PATHWAY ROOT TREATED ANIMALS

S.No	% Haemolysis			% increasing activity after	
	Initial (A)	7thDay (B)	14thDay (C)	7th day (B-A)/BX100	14thday (C-A)/CX100
1	27.55	92.50	119.30	70.16	76.90
2	26.26	100.90	119.60	73.93	78.04
3	38.49	63.90	113.5	39.76	66.08
Mean	30.76	85.76	117.4	61.28	73.67

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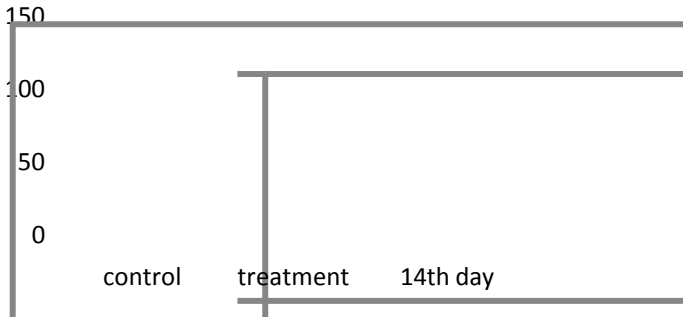
COMPLEMENT ACTIVITY ALTERNATE PATHWAY

Fig no.6.2.1 (c).Root treated animals



RESULTS AND DISCUSSION

Tinospora cardifolia was collected, and the fresh leaves stem and roots were washed and cut into pieces. 10gm each of the three parts were boiled for 10 min with water and filtered.

The filtrates were concentrated to 25ml. Albino rats of either sex weighing 2.5kgwere divided into three groups consisting of three animals each. Aqueous extract was administered the drug, on 7th& 14thday and immunostimulant activity was evaluated by chemotaxy and the alternative pathway was determined.

Drug was administered as aqueous extract of phytochemical techniques. Drug was administered as aqueous extract of fresh plant continuously for 14 days. The blood samples were taken first, 7th and 14thday. Complement activity alternate pathway chemotaxy were evaluated. The results were encouraging. There was mean increasing 60.44., 62.77., and 61.28%. After 7thday and 78.62., 73.85., and 73.67%., after 14thday for leaves, stem and roots respectively.

The increase was more first seven days than for next seven days. Similarly chemotaxy also exhibited a mean increase of neutrophil adhesion 86.28, 13.15 and 52.04% after 7thday and 110.72, 24.72, 73.48% after 14thdays for leaves, stems and roots.

SUMMARY AND CONCLUSION

The present work on studies on the immuno-stimulant properties of tinospora cardifolia was carried out to evaluate the immuno stimulant activity of the plant intended for use in fever. Immunostimulant activity was evaluated by complement activity alternate pathway and by chemotaxy. The selected drug was administered in the form of aqueous extract to albino rabbits of either six.

Complement activity alternate pathway evaluated based upon haemolysis of RBC as a immune response and chemotaxy was evaluated by ability of neutrophils adhesion adhere to foreign particles like nylon 7th day 60.44, 62.77, 61.28% and 14th day 110.72, 24.72 and 73.48% for leaves, stems and roots.

The study reveals that T.cardifolia has significant complement alternate pathway activity and chemotaxy. Hence phytochemical investigation may results promising immunostimulant drug.

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