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EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF THE ROOTS OF ICHNOCARPUS FRUTESCENS

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ABSTRACT: The objective of the present study was to investigate the immunomodulatory activity of the roots of *Ichnocarpus frutescents* R.Br. In the present study Pet. Ether, Chloroform and Methanolic fractions of the ethanolic extract of the roots of *Ichnocarpus frutescens*, in the doses of 10mg/kg and 30mg/kg, were administered orally to evaluate the immunomodulatory activity. The Methanolic fraction in the dose of 30mg/kg showed most significant effect when compared with control group, in the *E.Coli* induced abdominal sepsis and Carbon Clearence Test as models for non-specific immune response. In the models of the Specific immune response, in cell mediated immune response to SRBC - delayed type of hypersensitivity (DTH) the Methanolic fraction in the dose 30 mg/kg showed most significant effect on decrease in footpad edema after treatment when compared with control group. Humoral immune response was assessed by Sheep erythrocyte agglutination test, in which the Methanolic fraction of IF 30mg/kg showed most significant (p<0.05) increase in antibody titer after treatment when compared with control group. The present investigation reveals that the Methanolic fraction of the ethanolic extract of the roots of *Ichnocarpus frutescens*, in the dose of 30mg/kg possesses most significant immunomodulatory activity.

INTRODUCTION: Immune system is the defense system in vertebrates, just to protect them from invading agents. It can produce variety of cells which have the capacity to recognize and eliminate foreign undesirable substances. Immunomodulation means any type of change in the immune response involving expression, induction, amplification, inhibition of any phase of immune response. So immunomodulator is an agent used for its effect on the immune system [1]. The immune system is very well known to have involvement in etiology and pathopysiologic mechanism of many diseases. Immunomodulatory drugs of natural & synthetic origin can be used to boost the immune system and overcome the disease.

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Very few synthetic immunopotentiators are used today and many of them are cytotoxic and have various side effects. So, there is increasing demand to investigate natural drugs having immunomodulatory activity [2]. Ayurveda, an indian traditional system of medicines, plays a significant role in the prevention of disease and promoting health towards longevity of life. In Ayurveda, in the concept of "rasayana" many drugs may influence the host immunity and resistance against infections. Many medicinal plants listed as "Rasayana" drugs are believed to improve defense mechanisms of the body, promote physical & mental health and enhance longevity of life.

Thus, Indian medicinal plants are a good source of therapeutic compounds which are reported to potentiate immune responses by modulating the functions of lymphocytes, granulocytes, macrophages, natural killer cells[3]. In this regard, the objective of the present study is to Evaluate Immunomodulatory Activity of the Roots of *Ichnocarpus frutescents* R.Br. was undertaken.

Ichnocarpus frutescens R.Br. is a large, evergreen, laticiferous, woody creeper with a rusty red appearance, it is found almost throughout India, upto an altitude of 4000 ft. In the local language called as Kali Dudhi. The root of the plant are traditionally used in medicine as a substitute for Indian sarsaparilla (*Hemidesmus indicus*). Roots are reported to have demulcent, tonic, diaphoretic and diuretic properties. Root powder is administered with milk used as blood purifier[4]. *Ichnocarpus frutescens* leaves and roots are used as a substitute for Indian Sarasparilla (Hemidesmus indicus), so considered to be an important drug in the traditional system of medicine. Chloroform and methanolic extracts of the whole plant are reported to have the hepatoprotective and antioxidant activity.

Methanolic extract of the roots are reported to have anti inflammatory and analgesic activities. Aqueous extract of roots reported to have antidiabetic activity. Hydroalcoholic extract of leaves showed anti inflammatory activity and also posses α-Glucosidase inhibitory and in-vitro antioxidant activities. Polyphenolic extract of the leaves is reported to have activity against tumors [5]. Drugs having anti-oxidant property can be used as an immunomodulators [6, 7], anti-inflammatory drugs [8] and hepatoprotective drugs [9] are also used as potential immunomodulators, thats why the roots of the plant *Ichnocarpus frutescens* R. Br. were selected to investigate its immunomodulatory activity.

MATERIALS & METHODS:

Plant material and it's Authentication:

The roots of the selected plant *Ichnocarpus frutescens* (L.) R.Br. were collected in the month of August from the local area of Tirupati, Dist. Chitoor (A.P.) India. It was then shade dried. The roots of the selected plants *Ichnocarpus frutescens* (L.) R.Br. were authenticated from the Botanist and Taxonomist, Dr. K. Madhava Chetty, Asst. Prof., Dept. of Botany, Sri Venkateswara University, Tirupati-517502. (Reference no. 2008-09/135).

Preparation of Extracts & Fractions:

Ethanolic extract of the dried roots of *Ichnocarpus frutescens* (IF) was prepared by maceration technique. Around 2kgs of the powdered crude drug was kept in contact with ethanol in a jar for around three weeks with daily vigorous intermittent shaking with the help of a mechanical shaker. It was then filtered. Filtrate was then evaporated to dryness with the help of Rota evaporators and Vacuum oven. The dried ethanolic extract of IF was then fractionated by using solvents like Pet. Ether (40-60), Chloroform and Methanol. All the fractions were dried.

Preparation of Drug Solution:

Accurately weighed quantities of all the powdered fractions of the ethanolic extract were dispersed in distilled water using a suspending agent TWEEN 80. The appropriate stock suspensions of the drugs were prepared. The doses were administered orally by selecting the appropriate concentration of the stock solution**.** The suspension of the distilled water with TWEEN 80 served as control.

Animals:

Swiss male albino mice (18-22 gm) and wistar rats of either sex (150-200 gm) were used. They were maintained at 25 ± 2° C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light: 12 h dark cycle). The animals had free access to food (Amrut feed, Chakan oil mills, India) and water *ad libitum* throughout study. Institutional Animal Ethical Committee approved the protocol (Proposal No. 03/IAECCOP/2009). All the experiments were carried out between 9:00-16:00 hrs.

Preliminary Phytochemical Screening of the Fractions:

All the fractions of the ethanolic extract were investigated for preliminary phytochemical analysis using various tests to determine the presence of different phytoconstituents in different fractions[10].

Acute Toxicity Studies:

In the Acute Toxicity Study, Oral administration of all the fractions of the ethanolic extract of crude drug at the doses of 175, 550 and 2000mg/kg in mice showed no adverse effect or mortality was observed in wistar rats up to 2000mg/kg, p.o of extracts during the 24hrs and 14days observation. From pilot study data and review of literature, two different doses 10mg/kg and 30mg/kg were selected for further study [11].

Pharmacological Screening of Different Fractions:
All the fractions of ethanolic extracts of drugs were screened for their immunomodulatory effect on using following pharmacological screening models.

Models for Non- specific Immune Response:
***E.Coli* Induced Abdominal Sepsis (Determination of Host Resistance):**
Seven groups each consisting of 06 animals were used. All animals were injected with 1.0 mg of *E.Coli* serotype 0111:B4 LPS in a volume of 500µl of sterile solution. Immediately after this, group I to IV received isotonic NaCl solution (10ml/kg), test drug dose I.

There after quantities of bacteria in the intra abdominal fluid (10ml of sterile saline was injected into the abdominal cavity and then sample of peritoneal levage fluid was collected under anaesthesia and subjected to quantitative evaluation of the bacteria.) and rate of lethality was observed at every 24 hour for the period of 72 hours[12].

Carbon Clearance Test (Phagocytic Response):
In this test seven groups of animals were used with 6 rats in each group. Group I served as a control and received vehicle i.e. plain suspension of sterile water with TWEEN 80 (10ml/kg) only. On the other hand, animals of group II received doses D1 mg/kg of particular fraction of drug extract orally daily for the period of 05 days. Carbon ink suspension was injected via the tail vein to each rat 48 hours after the five-days treatment. Blood samples (25 µl) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 minutes after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (03 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the following formula;

$$K = \log OD\ 1 - \log OD\ 2 / t_2 - t_1$$

Where, OD1 and OD2 are the optical densities at time t_1 and t_2 [13, 14].

Models for Specific Immune Response:
Cell-mediated Immune Response to SRBC (DTH): Cell mediated immune response was assessed by T - cell population test and delayed type hypersensitivity (DTH).

T - cell Population Test:
In this test, seven groups of rats were used with 6 rats in each group. Group I served as a control and received vehicle i. e. plain suspension of sterile water with TWEEN 80 (10 ml/Kg) only. Rats of group II received doses D1 mg/kg of particular fraction of drug extract orally daily for 10 days. On 11th day blood was collected from retro orbital plexus and anticoagulated with sodium citrate in separate test tubes. The test tube containing blood was placed in a left sloping position 45^0 at 37 ^{0}C for 01 hour. Supernatant fluid containing lymphocytes and leukocytes were removed using micropipette[15, 16].

Delayed Type Hypersensitivity (DTH):
The mice were divided into 07groups, each containing 06 animals. Group I served as normal control Group and received plain suspension of sterile water with TWEEN 80 orally (01 ml/Kg) for the period of 21 days. Group II served as negative control Group receives Cyclosporine 100 µg/mouse, i.p. on 14th day of study. Animal of group III were administered doses D1 mg/kg of particular fraction of drug extract orally daily for the period of 21 days. Mice were immunized with 0.1ml of 20% SRBC's in normal saline intraperitonially on 14th day of study.

On day 21st, animal from all group get challenged with 0.03 ml of 1% SRBC's in sub plantar region of right hind paw. Footpad reaction was assessed after 24 hours i.e. on 22nd day. Increase in foot paw edema was measured using digital plethysmometer – LE7500 (Panlab, USA) [16].

Humoral Immune Response (Haemagglutination Antibody Titer):
Humoral immune response was assessed by Sheep erythrocyte agglutination test.

Sheep Erythrocyte Agglutination Test:
Rats were divided into seven groups with 06 rats in each group. Group I served as a control and received vehicle i.e. plain suspension of sterile water with TWEEN 80 (10 ml/Kg) only. Rats in groups II were administered two doses D1 mg/kg

of particular fraction of drug extract orally daily for 10 days[16].

Preparation of Sheep Red Blood Cells (SRBC's): Sheep blood was collected from local slaughter house in sterile Alsever's solution in 1:1 proportion of Alsever's solution (freshly prepared). RBC's were counted microscopically (5 x 10^9/ml). Blood was kept in the refrigerator at 4 ^{0}C and processed, for the preparation of Sheep RBC (SRBC's) batch, by cetrifugating (3000rpm for 05minutes) and washing with physiological saline 4-5times followed by suspending into buffered saline for further use [14].

Formula of Alsever's Solution:

Sodium chloride	:	0.42 gms/100 ml
Sodium citrate	:	0.80 gms/100 ml
Glucose	:	2.05 gms/100 ml

All the rats were injected with 0.25ml of 5 x 10^9 SRBC/ml on 6th, 8th, and 10th days for achieving maximum titer of antibody. On day 11, blood was collected and serum was separated by centrifuging at 200 rpm for the period of 15 minutes. 100 µl of serum diluted serially with normal saline in separate test tubes. Dilutions were made i.e.20, 40, 60up to 1280. To this, 50µl of SRBC added and incubated at 37 ^{0}C for 18 hours. All the tubes were then subjected to examine under microscope for agglutination and compared with control. The highest dilution giving hemagglutination was taken as the antibody titer. The antibody titers were expressed in the graded manner, the minimum dilution (01/02) being ranked as 01, and mean ranks of different groups were compared for statistical significance[17].

Data Analysis:
Data obtained were subjected to statistical analysis using one way ANOVA followed by Dunnetts 't' test using graph pad software.

RESULTS & DISCUSSION:

Plant Extraction & Fractionation:
The dried roots of *Ichnocarpus frutescens* R.Br. was extracted with the help of ethanol by maceration method. The yield was 6.86%. The ethanolic extracts of the roots of *Ichnocarpus frutescens* R.Br. was fractionated by using different solvents like Pet. Ether (40-60), Chloroform and Methanol with increasing polarity. The yields of the fractions were calculated and have been shown in the **Table 1**.

TABLE 1: YIELD OF THE FRACTIONS OF THE ETHANOLIC EXTRACT OF CRUDE DRUG

Crude Drug	Pet. Ether Fraction % Yield	Chloroform Fraction % Yield	Methanol Fraction % Yield
Ichnocarpus frutescens	9.20	7.75	13.30

Preliminary Phytochemical Screening of the Fractions: The results of the Preliminary phytochemical screening of the Pet. Ether, Chloroform and Methanol fractions of the ethanolic extract of the roots of *Ichnocarpus frutescens* R.Br. have been shown in **Table 2**.

TABLE 2: PRELIMINARY PHYTOCHEMICAL SCREENING OF THE FRACTIONS OF THE ETHANOLIC EXTRACT OF *ICHNOCARPUS FRUTESCENS* R.BR.

S.N.	Phytoconstituents	Pet. Ether fraction	Chloroform fraction	Methanolic fraction
1.	Carbohydrates	-	-	+
2.	Glycosides	-	-	+
3.	Saponins	-	-	-
4.	Flavonoids	-	-	+
5.	Alkaloids	-	+	-
6.	Tannins	-	-	+
7.	Steroids	+	-	-
8.	Amino Acids	-	-	-
9.	Proteins	-	-	-

Models for Non- specific Immune Response:
***E. coli* Induced Abdominal Sepsis (Determination of Host Resistance):**
The Methanolic fraction of IF 10mg/kg showed significant and IF 30 mg/kg showed most significant (p<0.05) and (p<0.01) effect when

compared with control group for 24 hrs, 48 hrs and 72 hrs. Also IFC 30mg/kg and AMM 30mg/kg showed less significant (p<0.05) effect as compare to control group. The results have been shown graphically in the **Fig. 1**.

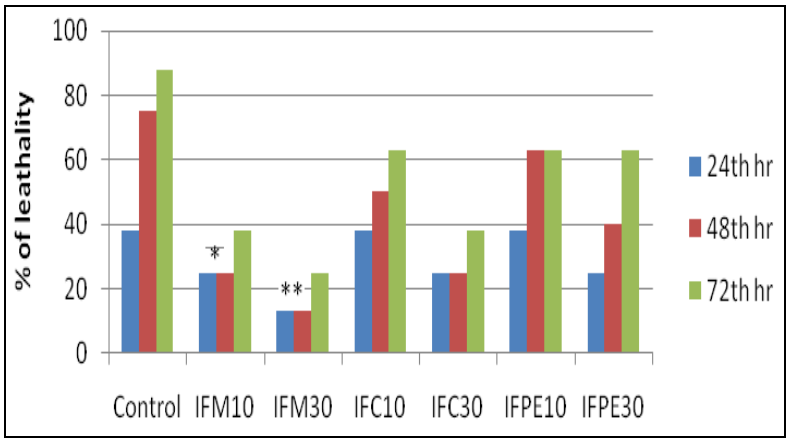

FIG. 1: EFFECT OF ICHNOCARPUS FRUTESCENS FRACTIONS TREATMENT ON *E. COLI* INDUCED ABDOMINAL SEPSIS

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by *Dunnett's t test*. *P<0.05, **P<0.01

Carbon Clearance Test (Phagocytic Response):

The Methanolic fraction of IF 30mg/kg showed most significant activity (p<0.05) and (p<0.01) dose dependent increase in carbon clearance respectively when compared with control group. The Methanolic fraction of IF 10mg/kg showed significant activity by increase in carbon clearance when compared with control group. Also IFPE 30mg/kg and AMM 30mg/kg showed significant increase in carbon clearance when compared with control group. Other fractions IFC 10mg/kg, IFC 30mg/kg and IFPE 10mg/kg also showed some good activity by increase in carbon clearance when compared with control group. The results have been shown graphically in the **Fig. 2.**

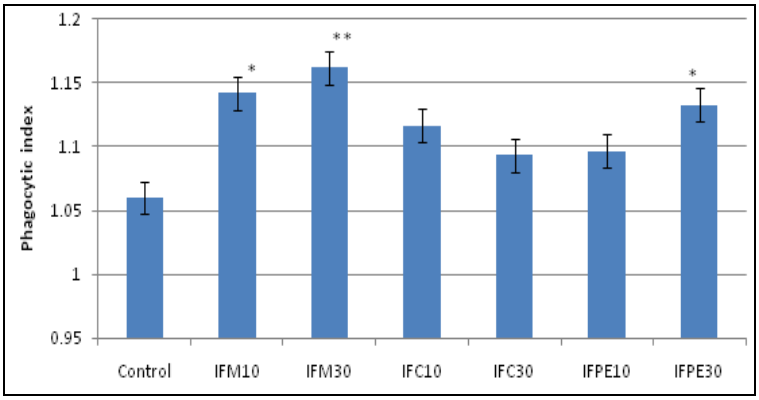

FIG. 2: EFFECT OF *ICHNOCARPUS FRUTESCENS* FRACTIONS TREATMENT ON CARBON CLEARANCE TEST

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by *Dunnett's t test*. *P<0.05, **P<0.01

Models for Specific Immune Response:

Cell-mediated Immune Response to SRBC (DTH): Cell mediated immune response was assessed by T- cell population test and delayed type hypersensitivity (DTH).

T-cell Population Test: None of the doses of fractions of the crude drug showed any significant change. As no significant changes were observed during the T-cell Population test so the data have not been Presented.

Delayed Type Hypersensitivity (DTH):

The Methanolic fraction of IF 30 mg/kg showed most significant effect (p<0.01) decrease in footpad edema after treatment when compared with control

group. The Methanolic fraction of IF 10 mg/kg showed significant effect (p<0.01) decrease in footpad edema after treatment when compared with control group. The Chloroform fraction of IF 30 mg/kg showed some effect in decrease in footpad edema after treatment when compared with control group. The other fractions and doses of IF did not show any significant activity in decrease in footpad edema after treatment when compared with control group. The results have been shown graphically in the **Fig.3**.

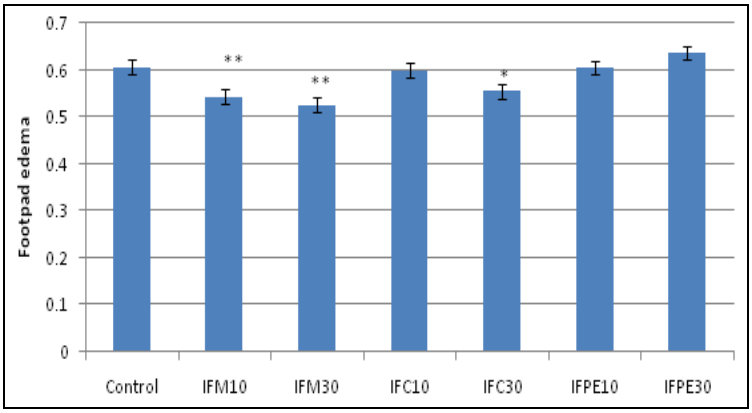

FIG.3: EFFECT OF *ICHNOCARPUS FRUTESCENS* FRACTIONS TREATMENT ON FOOT PAD EDEMA

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by *Dunnett's t test*. *P<0.05, **P<0.01

Humoral Immune Response (Haemagglutination Antibody Titer): Humoral immune response was assessed by Sheep erythrocyte agglutination test.

Sheep Erythrocyte Agglutination Test: The Methanolic fraction of IF 30mg/kg showed most significant (p<0.05) increase in antibody titer after treatment when compared with control group. The Methanolic fraction of IF 10mg/kg showed significant increase in antibody titer after treatment when compared with control group. The Chloroform fraction of IF 30mg/kg showed significant increase in antibody titer after treatment when compared with control group. The other fractions IFC 10mg/kg, IFPE10mg/kg, IFPE 30mg/kg did not showed any significant increase in antibody titer after treatment when compared with control group. The results have been shown graphically in the **Fig.4.**

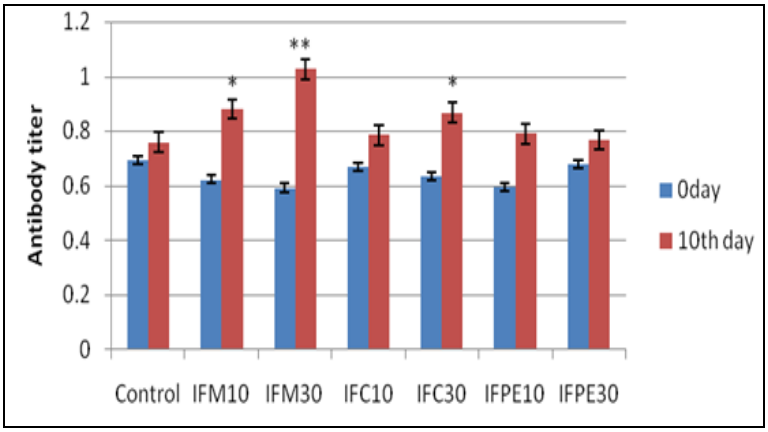

FIGURE 4: EFFECT OF *ICHNOCARPUS FRUTESCENS* FRACTIONS TREATMENT ON SHEEP RBC

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by *Dunnett's- test*. *P<0.05, **P<0.01

CONCLUSION: The roots of *Ichnocarpus frutescens* (L.) R.Br. were selected to investigate the immunomodulatory activity. Traditionally the crude drug was used in the treatment of chronic rheumatoid arthritis [18] and crude drug was scientifically reported for having anti-oxidant

property, Anti-inflammatory & Hepatoprotective activity [5]. As the anti-oxidant, anti-inflammatory properties and anti-rheumatism drugs can be used as immunomodulators [19]. The present investigation indicates that the methanolic fraction of the ethanolic extract of the roots of *Ichnocarpus frutescens*, exert a significant immunomodulatory activity by enhancing Specific Immune response as well as Non-specific immune response. The Preliminary phytochemical screening of the methanolic fraction of the crude drugs revealed the presence of flavonoids which are the phytoconstituents responsible for immunomodulatory activity [20].

Thus the crude drug shows significant immunostimulant property, and which may be due to the flavonoids present in it. Further investigation is underway to find out the pytoconstituents present in the fraction which is responsible for this action.

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CONFLICT OF INTEREST: None

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