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IN VITRO AND *IN VIVO* STUDIES OF THE IMMUNOMODULATORY EFFECT OF *CINNAMOMUM MALABATRUM* ON FEMALE WISTAR RATS

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ABSTRACT

Background: For thousands of years, cinnamon has been known as one of the most common spices, with multiple culinary usages. They have become very familiar immunomodulatory herbal medicine. However, the specific immunomodulatory effect of this particular species remains to be elucidated. **Objective:** The objectives of the study was to evaluate the immunomodulatory effect of ethanolic extract of *Cinnamomum malabatrum* in cell mediated immunity and to analyze its *invito* efficacy. **Methods:** The assessment of cell mediated immunity was carried out by neutrophil adhesion test order to induce immunostimulation, Levamisole (50mg/kg/day, p.o.) was used. The *invitro* effects of the extract was measured by cell proliferation method in followed by MTT assay using murine macrophage cell line stimulated by lipopolysaccharide (LPS). **Results:** Ethanolic extract of *Cinnamomum malabatrum* evoked a significant increase in percentage neutrophil adhesion to nylon fibers Both doses of the extract produced significant results in neutrophil adhesion tests when compared to control. Also in *invitro* tests, the extract produced dose dependent increase in cell proliferation effect in LPS stimulated macrophage cell line.(264.7 cell l ine). **Conclusion:** From the results of *invivo* test and *invitro* test, using the ethanolic extract, it can be concluded that ethanolic extract of *Cinnamomum malabatrum* has a significant immunomodulatory effect on experimental animals and cell lines which may be attributed to the terpenoid and flavonoid content of the plant extract.

KEYWORDS: *Cinnamomum malabatrum*, immunomodulatory, Levamisole, Neutrophil adhesion, Cell proliferation, MTT.

1. INTRODUCTION

Environmental pollutants and dietary habits cause a disturbance in immune activities and diet containing micronutrients and antioxidants are known to prevent these alterations.[1] Modulation of the immune system denotes any change in the immune response that can involve induction, expression, amplification, or inhibition of any part or phase of the immune response. The potential use of immunomodulators in clinical medicine include the replacement of immune deficiency(eg. AIDS) and suppression of normal or excessive immune function (eg. graft rejection prevention or management of autoimmune diseases**.**[2]

A diverse array of synthetic, natural and recombinant compounds are available for immunomodulation. Among the synthetic immunomodulators, levamisole, isoprinosine, pentoxyphilline, BCG are some of the most significant ones. Cyclosporine, Cyclophosphamide are some of the widely used immune-suppressants. These synthetic immune-modulating drugs have numerous benefits, their adverse side effect profile and generalized effect throughout the immune system poses a major limitation to the general deliberate use of these drugs and warrants the search for a more effective and safer agents exerting immune-modulatory activity. This research focus is becoming a field of major interest all over the world.[3]

The use of herbs as immune-modulator in the indigenous system of medicine, indeed, can modulate the body's defence mechanism.[4] A number of medicinal plants have been claimed to possess immune-modulatory activity, e.g. *Withania somnifera*, *Tinospora cordifolia*, and *Mangifera indica*.[5] The phytoactive constituents of plant derivatives such as polysaccharides, lectin, peptide, flavanoids, and tannins have been reported to modulate the immune system in different models.[4] Cinnamon, the eternal tree of tropical medicine, belongs to the Lauraceae family. Cinnamon is one of the most important spices used daily by people all over the world. Cinnamon primarily contains vital oils and other derivatives, such as cinnamaldehyde, cinnamic acid, and cinnamate.[6] An interesting factor about cinnamon is that it can act both as an immunostimulant and a suppressant depending on species and doses. Macromolecules isolated from cinnamon, such as glycoproteins and water soluble polysaccharides, were found to stimulate the immunological system, whereas smaller molecules such as cincassiol and their glycosides suppress the system.[7]

Cinnamomum malabatrum, commonly known as tejpatta, has proved its efficacy in inflammation, cancer, hepatotoxicity etc. However, there is paucity of scientific data on the *in vivo* and *invitro* immunomodulatory activity of bark of *Cinnamomum malabatrum*. The objective of the present investigation was to study the immunomodulatory activity of the ethanolic extract of the bark of *Cinnamomum malabatrum* in animal models.

2. MATERIALS AND METHODS

Collection and Identification Of Plant Material

The fresh bark of *Cinnamomum malabatrum* was gathered in the month of October from Poonjar, Kottayam District, Kerala state, India. The plant was identified and authenticated by Rojimon Thomas, Associate Professor, Department of botany, C.M.S College Kottayam, and a voucher specimen was deposited with a voucher specimen sample No CMS 1245.

2.1. Preparation of extract[8]

The dried bark was crushed to moderately coarse powder. The bark powder passing through a 40 mesh sieve was used for the extraction procedure. Bark powder (500 g) was fed into a Soxhlet extractor and extracted with ethanol at 35-45°C temperature for 5 days. After ensuring entire extraction, the extract was collected,filtered and dried .The percentage yield was calculated

2.2. Drugs and chemicals

Levamisole was procured from local market, and all other chemicals and reagents used were of analytical grade.

2.3 Selection of Animals

Fourty five healthy wistar albino rats weighing between 150-170gm were obtained from animal house, Department of Pharmaceutical sciences, Cheruvandoor. The animals were housed in polypropylene cages in room where the congenital temperature 27±1, 30-60% relative humidity and 12 hours light and dark cycles were maintained. The animals were allowed to acclimize to the environment. They were fed on standard pellet diet collected from Hindustan lever limited, Bangalore and water given ad libitum. The experiment was approved by Institutional Animal Ethical Committee, Department Of Pharmaceutical Sciences, Cheruvandoor with no IAEC/MPharm/DPS/2018-14

2.6 METHODOLOGY

2.6.1 *Invitro tests*[9]

2.6.1.1 Cell proliferation assay: Raw 264.7 (Macrophage) cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO2 incubator (NBS Eppendorf, Germany).

2.6.1.2 Cells seeding in 96 well plate: Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension (5x104 cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO2 incubator.

2.6.1.3 Preparation of compound stock: 1mg of sample was weighed and dissolved in 1mL 5% DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

2.6.1.4 Immunomodulatory Evaluation: After 24 hours the growth medium was removed, freshly prepared each compounds in 5% DMEM were five times serially diluted by two fold dilution and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO2 incubator

2.6.1.3 Immunomodulatory Assay by MTT Method: Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm

The percentage of proliferation was calculated using the formula

$$\% \text{ of proliferation} = \frac{\text{OD Sample-OD of control X 100}}{\text{OD of control}}$$

2.6.2 *INVIVO TEST*

2.6.2.1 Neutrophil Adhesion Test[10]

Rats were divided into four groups of six animals each.The controle group I received vehicle, while animals of treatment group II and II were given EECM 100mg/kg/day/p.o and 200mg/kg/day/p.o for 14 days and group IV received Levamisole(50 mg/kg/p.o) oraly. After 14 days of treatment to all four groups, blood samples were collected in heparinised vials by retro-orbital puncture, and total as well as differential leukocyte count was determined. After initial counts, the blood samples were incubated with 80 mg per mL of nylon fibers at 37°C for 15 min. The incubated samples were further analyzed for total and differential leukocyte count. The product of total leukocyte count and percentage neutrophil, known as neutrophil index, was determined for each animal of each respective group using the formula,

$$\text{Neutrophil adhesion \%} = \frac{NIU-NIT}{NIU} \times 100$$

Where

NIU=Neutrophil index of untreated, NIT= Neutrophil index of treated sample.

Statistical analysis

Statistical analysis was performed using Graph pad prism software. All the results were expressed as mean±SEM. Data were analyzed using one way Analysis of Variance(ANOVA) followed by Tukey multiple comparison test. P values<0.05 were considered as statistically significant.

3. RESULTS

3.1 Extraction

The percentage yield obtained after the extraction of the dried powdered bark of *Cinnamomum malabatrum* (Burm. f.) .J. Presl.was found to be 19%.

3.2 Preliminary Phytochemical Screening

The preliminary phytochemical screening showed the presence of carbohydrates, glycosides, flavonoids, phenolics, proteins, amino acids, steroids and tannins.

3.3 In-Vitro Studies

Cell proliferation assay

In this study *Cinnamomum malabatrum* showed dose dependent increase in cell proliferation. Maximum proliferation was observed in 100µg/ml.

Table 3.2: Effect of ethanolic extract of *Cinnamomum Malabatrum* on cell proliferation assay.

Treatment	Absorbance	Proliferation(%)
6.25µg	0.6893±0.001	1.90
12.5 µg	0.6974±0.015	4.44
25 µg	0.7376±0.004	10.97
50 µg	0.7980±0.001	19.56
100 µg	0.9010±0.032	34.49
Control	0.6706±0.013	

Values were expressed as mean±SD of triplicate, n=3

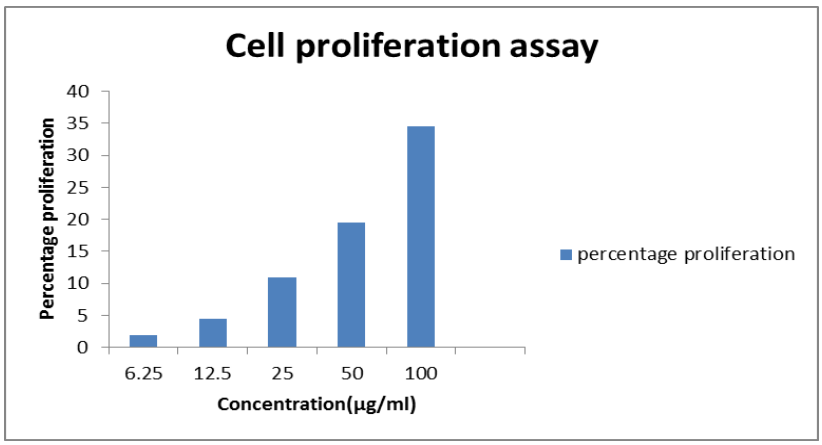

Fig 3.1 Effect of ethanolic extract of *Cinnamomum malabatrum* on cell proliferation

3.4 Acute Toxicity Studies.

The animals treated with the ethanolic extract of *Cinnamomum malabatrum* (Burm. f.) .J. Presl. at dose of 2000mg/kg/p.o exhibited normal behavior without any sign of passivity, stereotypy, and vocalization. Their motor activity and secretary status were of_normal. The animals did not exhibit any signs of depression at selected dose. The ethanolic extract upto the dose of 2000mg/kg body weight did not produce any behavioral abnormalities and mortality. Therfore two dose levels 100 mg/kg, 200mg/kg were selected to explore the immunomodulatory activity of *Cinnamomum malabatrum* (Burm. f.). J. Presl. bark exract.

3.5 Neutrophil Adhesion Tests

The percentage neutrophil adhesion was significantly increased ($p<0.001$) by both the doses of ethanolic extract of *Cinnamomum malabatrum* (EECM) when compared with the control group. Increase in the adhesion of neutrophil to nylon fibre which correlates to the process of margination of neutrophils to the site of infection.

Table 3.3: Effect of ethanolic extract of *Cinnamomum malabatrum* on neutrophil adhesion test.

Treatment groups	Total count		Neutrophil count		Neutrophil index		Neutrophil adhesion%
	UB	NFTB	UB	NFTB	UB	NFTB	
Control	7.26±50.57	6.74±65.09	16.67±0.42	13.50±0.619	121110±3176	91183±4612	24.91±2.140
EECM(100mg/kg)	5.69±65.44	4.52±22.90	17.50 ±0.76	12.17±0.60	99655±4716	55013±2655	*** ### 44.61±1.348
EECM(200mg/kg)	7.67±18.87	6.76±24.04	26.00±0.57	17.33±0.80	199503±4804	171172±5469	*** ### 41.18±2.359
Levamisole (50mg/kg)	8.28±107.0	7.09±121.7	28.17±0.70	10.17±0.54	232823±5080	71987±3603	*** 69.09±1.54454

EECM: Ethanolic extract of *Cinnamomum malabatrum*, TLC: Total leucocyte count, UB: Untreated blood, NFTB: Nylon fiber treated blood, Values plotted were the Mean±SEM(n=6), *P<0.05, **p<0.01, ***p<0.001, when compared with control, #P<0.05,## p<0.01, ###p<0.001, when compared with standard, Statistically analyzed by one way ANOVA followed by Tukey-multiple comparison test.

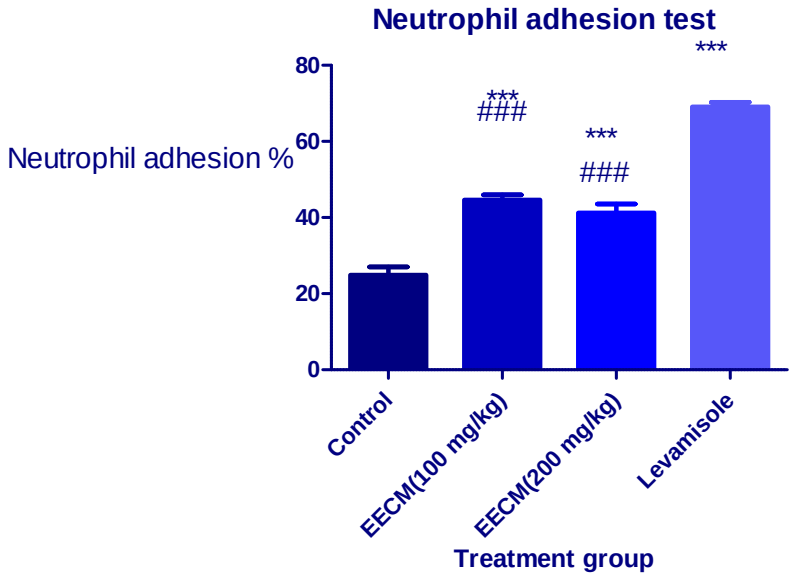

Fig.3.2 Effect of ethanolic extract of *Cinnamomum malabatrum* on neutrophil adhesion test.

EECM: Ethanolic extract of *Cinnamomum malabatrum*, Values plotted were the Mean±SEM(n=6), *P<0.05, **p<0.01, ***p<0.001, when compared with control, $^{\#}$P<0.05,$^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, when compared with standard, Statistically analyzed by one way ANOVA followed by Tukey-multiple comparison test.

4. DISCUSSION

Immunomodulation involves the modulation of the immune responses either by motivation or inhibition and help in maintaining a disease free state. If the improvement of immune reactions occurs, it is named as an immune stimulative which primarily implies stimulation of non-specific system, i.e. Macrophages, complement, granulocytes, certain T-lymphocytes and different effectors substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors.[11]

Immunomodulators are natural or synthetic agents, which by modifying the immune system achieve a therapeutic benefit. They may have the ability to augment, restore, inhibit or help to produce the desired immune response.[12] Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when host defence mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. In recent years there has been a renewed interest into the biological activity of

traditional plant medicines and of natural products in drug discovery.[13] The immunomodulating characteristics of plants are being examined widely to achieve the desirable effects on disease prevention. As a result, herbal remedies have been utilized for centuries for safety, effectiveness, minor side effect and cultural acceptability.[14] The present study evaluated the immunomodulatory activity of ethanolic extract *Cinnamomum malabatrum* (Burm. f.) .J. Presl bark. The invitro method included cell proliferation assay using LPS stimulated raw macrophage cell line and the invivo method include neutrophil adhesion.

The acute toxicity testing was performed with different doses of *Cinnamomum malabatrum* in female animals. The plant extract did not show any signs of serious toxicity. No animal was found to be moribund state and no animal died even after 14 days. So, it was confirmed that the plant *Cinnamomum malabatrum* was safe up to 2000 mg/kg body weight. The preliminary phytochemical investigation for ethanolic extract of *Cinnamomum malabatrum* showed the presence of carbohydrates, glycosides, flavonoids, phenolics, proteins, amino acids, steroids and tannins.

The most commonly used *in vitro* method for screening immunomodulatory activity involves the use of macrophages. This is because they play a key role in host protection against wide range of tumors and microbes. During the development of specific immunity, macrophages present antigen to lymphocytes and thus serve as supportive accesessory cells to T lymphocytes. The cell proliferation assay is used to determine macrophage activation and thus the cell mediated immune responses. The different concentration of extracts showed dose dependent increase in cell proliferation with LPS stimulated macrophage.

The ethanolic extract of *Cinnamomum malabatrum* exhibited significant effects on *in vivo* studies. Levamisole was used as the standard drug in the *invivo* study. Cell adherence property of neutrophils is one of the earliest responses of both immunological and physical injury. In the neutrophil adhesion test the cell adherence property of neutrophils was assessed in blood samples from different groups, by treating with nylon fibers to which the neutrophils adhere. The neutrophil adhesion to nylon fiber is associated with the up-regulation of the β_2 integrins.[15]

Both doses of EECM exhibited significantly augmented ($p<0.001$) neutrophil adhesion when compared to control which associates to the process of marginalization of cells in blood

vessels. Levamisole also showed significant effect on neutrophil adhesion. This may help in increasing immunity of body against microbial infections.

From the *invitro* and *invivo* results, it is evident that the ethanolic extract *Cinnamomum malabatrum* has immunomodulating property.

5. CONCLUSION

The *Cinnamomum malabatrum* is a moderate ever green tree. The preliminary phytochemical screening of *Cinnamomum malabatrum* stem bark ethanolic extract shown the presence of carbohydrates, phenolics, proteins, and flavanoids. The constituents responsible for the immunomodulating behavior may be flavanoids and terpenoids present in the extract. These constituents might stimulate the immunological cells as well. The invitro study of the extract has shown proliferation in Lipopolysaccharide stimulated cell line. The ethanolic extract has a significant role in cell mediated immunity and the same is confirmed by the increased percentage of neutrophil adhesion and associated migration of neutrophil to the site of foreign body in the extract treated group compared to the control group. From the above findings it can be concluded that Cinnamomum malabatrum The present investigations therefore reveals that ethanolic extract of *Cinnamomum malabatrum* certainly possesses immunomodulatory properties. However, further studies are warranted to elucidate the exact mechanism of action.

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