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Pharmacology

Preliminary Phytochemical Screening and Evaluation of Anti-Arthritic Potential of *Ledum Pal* Mother Tincture using Macrophage Raw 264.7 Cell Lines

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Original Research Article

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Abstract: Rheumatoid arthritis is a chronic inflammatory disease affecting joints manifesting as pain, stiffness, and synovitis leading to articular destruction. Current medical treatments do not consistently halt the long-term progression of this disease, and surgery may still be needed to restore mechanical function in large joint. Patients with rheumatic syndromes often seek alternative therapies, with homeopathy being one of the most frequent. So the necessity of this study gains more interest to prove the efficacy of alternative system of medicine. Objective of the present study is to carry out preliminary phytochemical screening followed by the evaluation of anti-arthritic potential of *Ledum Pal* mother tincture (LPMT) in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophage cell lines. Phytochemical screening was done to find the presence of various primary and secondary metabolites of the plant. *In-vitro* anti-arthritic activity was evaluated by assessing the inhibitory property of LPMT on LPS stimulated cell lines using several assays like cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), inducable nitric oxide synthase (iNOS), and estimation of cellular nitrite level, myeloperoxidase (MPO) activity using diclofenac as standard. Phytochemical tests of LPMT indicated the presence of flavonoids, alkaloids, steroids, terpenoids, carbohydrates, proteins, tannins, saponins, and glycosides. Macrophages produce COX-2, 5-LOX, iNOS, cellular nitrite and myeloperoxidase etc., on inflammatory insult by LPS stimulation. Results showed the ability of mother tincture to reduce inflammatory response associated with RA by inhibiting COX-2, 5-LOX, iNOS along with reduction in myeloperoxidase and cellular nitrite levels due to the presence of flavonoids and terpenoids etc. Thus the study provides evidences to prove the effectiveness of alternative system of medicine like homeopathy in the treatment of arthritis.

Keywords: *Ledum pal*, RAW 264.7 cells, cyclooxygenase, lipooxygenase-5, inducable nitric oxide, cellular nitrite, myeloperoxidase.

INTRODUCTION

World plant biodiversity is the largest source of herbal medicine and still about 60 – 80 % world population rely on plant based medicines which are being used since the ancient ages as traditional health care system. Plants and their metabolites constituents have a long history of use in modern medicine and in certain systems of traditional medicine. Constituents of plants which are the natural bioactive compounds found in plants work with nutrients and fibers to form an integrated part of defence system against various diseases and stress conditions. Based on their functions in plant metabolism phytochemicals are basically divided into two groups, *i.e*. primary and secondary constituents. Primary constituents comprise common sugars, amino acid, proteins and chlorophyll while secondary constituents consist of alkaloids, terpenoids, steroids and flavonoids, so on. These bioactive compounds are having therapeutic potential, of which many eventually have been developed into drugs that are consumed worldwide for diverse disorders, including inflammatory and autoimmune diseases, infectious diseases, and cancer[1,2].

Various herbal products belonging to the alternative systems of medicine are either already being used by patients with autoimmune diseases including RA, with or without the primary physician's knowledge, or are under investigation for their therapeutic potential. Here this work mainly aims at one of the complementary and alternative medicinal system of therapy, "*homeopathic medicine"* especially *mother tincture* in the treatment of autoimmune disease rheumatoid arthritis. Complementary and alternative medicine (CAM) has witnessed an increase in use in recent times not only in North America, Europe and Australia but also in Asian countries including India. CAM is defined as a 'diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine'. In India, alternative systems such as Ayurveda, homoeopathy, Siddha and Unani medicine are supported by the Government of

India. CAM practices and modern, allopathic medicine run parallel to each other and may cater to the rural and urban populations, respectively, though not mutually exclusively[2,3].

Rheumatoid arthritis (RA) is an autoimmune diseases result from deregulated immune responses that attack the body's own tissues contrary to their traditional role in protecting the host against external infectious agents. Complex interplays among genetic and environmental factors are involved in the pathogenesis of autoimmunity. In other words the human immune system work in association with other physiologic systems in order to provide stable interior environment essential for the survival and reproduction of the host. Cell-mediated and/or antibody-mediated effector responses contribute to autoimmune inflammation and tissue damage. These processes can either affect multiple organs (systemic autoimmunity) or be limited primarily to one organ (organ-specific autoimmunity). These diseases are characterized by systemic inflammation, in which a dysregulated immune system causes damage or dysfunction to target organs. Rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), and type 1 diabetes (T1D) are examples of the major human autoimmune diseases. In general, the prevalence of these diseases is relatively higher in the developed countries compared to that in the developing countries[2,4].

A growing line of evidences have indicated that inflammation is involved in the pathogenesis of many diseases including arthritis and other life- threatening and debilitating disorders. Among the various causes, oxidative stress is the primary cause of inflammation. It can induce inflammatory cells to produce inflammatory mediators, such as cytokines and chemokines, which enhance tissue damage from the recruitment of more inflammatory cells, eventually resulting in more oxidative stress. Induction of pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) can induce nitric oxide (NO) production, 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) which contribute in inflammation. Several reports have demonstrated the involvement of COX-2, 5-LOX, NO and myeloperoxidase (MPO) in inflammation of rheumatoid arthritis[5].

Uncontrolled autoimmune pathology may result in severe disabilities and/or deformities, and loss of organ function. Due to their chronic nature, autoimmune diseases impose a heavy economical, psychological and social burden on the society. Therefore, effective safe therapeutic agents, and treatment regimen are critical to the management of patients with autoimmunity [2]. Miserably, there is no permanent cure for RA, but it might be kept controlled by NSAIDs, DMARDs and corticosteroids, arthroplastic surgeries like partial or total replacement of joints are followed during aggressive conditions of RA. To overcome the issues aroused from these therapies, active phytocompounds and some alternative therapies have been investigating their feasibility along with commercial drugs[6].

In Homoeopathy, mother tinctures are routinely prescribed for the treatment of wide variety of ailments such as high blood pressure, fever, sedation, stomach pain, skin problems, and muscular problems. The use of homoeopathic medicines as complements or alternatives to conventional medicines has been increasing worldwide. The reasons, which have given rise to this trend, include the availability, and accessibility of these medicines. These mother tinctures contain a number of compounds in complex matrices in which no single active constituent is responsible for overall therapeutic effect[7].

This study deals with the preliminary phytochemical screening and *in-vitro* anti-arthritic activity of *Ledum pal* mother tincture (LPMT) using 264.7 RAW macrophage cell lines. *Ledum pal* mother tincture is obtained from the plant *Rhododendron tomentosum Harmaja*, a small, woody evergreen shrub, growing widely in peaty soils in northern and central Europe, northern part of Asia and North America. Plant is rich in several phytoconstituents like terpenoids, flavonoids, phenols, alkaloids etc. Hence the mother tincture also can be used for the treatment of rheumatoid arthritis due to the presence of phytoconstituents. Since there is no scientific evidence of the present study, it is considered worthwhile to evaluate the anti-arthritic activity of this mother tinctures in experimental animal models in order to provide scientific basis for its use in Homoeopathy[8,9].

MATERIALS AND METHODS

Drugs and chemicals

Homeopathic mother tincture was collected from Central Research Institute for Homeopathy. Cell lines for *in-vitro* study: RAW 264.7 cell line was purchased from NCCS, Pune; Dulbecco's modified eagles media (Himedia, India), 10% fetal bovine serum (Invitrogen, USA), and all other chemicals, reagents and solvents used in this study were of commercial or analytical grade.

METHODOLOGY

Qualitative phytochemical screening

The preliminary qualitative phytochemical screening of the extracts were carried out to determine the presence of saponins, flavonoids, alkaloids, phenols, tannins, volatile oils, glycoside and steroids as described.

Chemical tests for alkaloids

- Mayer's test: To 2-3 ml of mother tincture, add few drops of Mayer's reagent. The formation of white or creamy precipitate indicates the presence of alkaloids.
- Wagners test: To 2-3ml of mother tincture, added few drops of Wagner's reagent. The formation of reddish brown precipitate indicates the presence of alkaloids.
- Hagners test: To 2-3ml of mother tincture added few drops of Hagner's reagent. The formation of prominent yellow precipitate indicates the presence of alkaloids.
- Dragandorff's test: To 2-3ml of mother tincture, conc. Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendorff's reagent was added. Formation of orange or orange red precipitate indicates the presence of alkaloids.

Chemical test for glycosides

- Legal's test: To 1-2ml of mother tincture, add 1ml of pyridine and 1ml of sodium nitroprusside. The formation of pink to red colour indicates the presence of glycoside.
- Baljet's test: To 1-2ml of mother tincture, add 1ml of sodium picrate solution. A yellow to orange colour confirms the presence of cardiac glycoside.
- Keller-Killiani test: A mixture of Acetic acid glacial (2 ml) with 2 drops of 2% $FeCl_3$ solution was added to the mother tincture and H_2SO_4 concentrated. A brown ring produced between the layers which indicated the entity of cardiac steroidal glycosides.

Chemical tests for flavonoids

- Aqueous sodium hydroxide test: To the mother tincture, addition of increasing amount of sodium hydroxide shows yellow colouration, which decolourises after the addition of acid indicates the presence of flavonoids.
- Shinoda test: To the mother tincture, added 95% ethanol, few drops of concentrated hydrochloric acid and 0.5g of magnesium turnings. The formation of pink colour confirms the presence of flavonoids.

Chemical tests for carbohydrates

- Molisch's test: To 2-3ml of mother tincture, add few drops of α-napthol solution in alcohol, shaken well and added conc. sulphuric acid from sides of the test tube. A violet ring at junction of the two liquid indicates the presence of carbohydrates.
- Fehling's test: 1ml of Fehling's A and 1ml of Fehling's solution B were mixed and boiled for one minute. Added equal volumes of mother tincture and reagent mixture in a test tube and heated in a boiling water bath for 5-10 minutes. At first, a yellow and then brick red colour precipitate is observed, indicate the presence of reducing sugars.
- Benedict's test: Mix equal volumes of Benedict's reagent and mother tincture in a test tube, heat it in a boiling water bath for 5 minutes. Solution appears green, yellow and red depending on the amount of reducing sugars present in the test solution.

Chemical tests for amino acid and protein

- Biuret test: To 3ml of the mother tincture added 4% sodium hydroxide and few drops of 1% copper sulphate solution. Appearance of violet to pink colour indicates the presence of proteins.
- Millon's test: 3ml of mother tincture was mixed with 5ml of Millon's reagent. White precipitate forms and upon warming precipitate turns brick red or the precipitate dissolves giving red coloured solution indicate the presence of proteins.
- Ninhydrin test: 3ml of the mother tincture was heated with 3drops of 5% ninhydrin solution in a boiling water bath for 10 minutes. Appearance of purple or bluish colour indicates the presence of amino acids.

Chemical tests for steroids

- Salkowski test: 2ml of the mother tincture was mixed with 2ml of chloroform and 2ml of concentrated sulphuric acid, shaken well. The appearance of red colour on chloroform and greenish yellow fluorescence on acid layer indicate the presence of steroids.
- Liebermann-Burchard test: To the mother tincture, 3-4 drops of acetic anhydride was added, the solution was boiled cooled and conc. Sulphuric acid (3 drops) was added. A brown ring appears at the junction of the two layers. The upper layer turns green showing the presence of steroids.

Chemical test for terpenoids

To 2-3ml of the mother tincture, 2ml of chloroform was added. Then 3ml of concentrated sulphuric acid was added carefully to form a layer. A reddish brown colouration at the interface indicates the presence of terpenoids.

Test for phenols and tannins

2ml of 2% solution of Ferric chloride was mixed with 2ml of mother tincture. Black or blue-green colour indicated the presence of tannins and phenols.

Lead acetate test: To 5ml of mother tincture 1ml of lead acetate solution was added. Flocculent white precipitate indicated the presence of flavonoids.

Chemical tests for saponins

- Foam test: 1ml of the tincture were diluted with water to 20ml and shaken in a graduated cylinder for 15minutes. One centimetre layer of foam indicates the presence of saponins.

***IN-VITRO* METHODS FOR ASSESSING ANTI-ARTHRITIC POTENTIAL**

For the *in-vitro* study, first evaporation of alcohol content from the mother tincture under reduced pressure in a rotary evaporator to obtain a semisolid mass that weighed 2.52 g[10].

Cell culture

The RAW 264.7 mouse macrophage cell line were cultured in DMEM media, supplemented with 10% heat-inactivated FBS, 3mM Glutamine, antibiotics (100U/mL penicillin and 100U/mL streptomycin) and 1.5% sodium bicarbonate at 37 °C under a humidified atmosphere of 5% CO_2. The media was filtered using 0.2µm pore sized cellulose acetate filter (Sartorius) in completely aseptic conditions. RAW 264.7 cells were grown to 60% confluency followed by activation with 1 µL lipopolysaccharide (LPS) (1µg/mL). LPS stimulated RAW cells were exposed with different concentration (25, 50, 75, 100 µg/mL) of mother tincture residue and Diclofenac sodium, a standard anti-inflammatory drug in similar concentration corresponding to the sample was added and incubated for 24 hours. After incubation the anti-inflammatory assays were performed using the cell lysate. In all experiments, cells were allowed to acclimate for 24 h before any treatments [11–14].

CYCLOOXYGENASE-2 INHIBITORY ASSAY

The COX activity was assayed by the method of Walker and Gierse. 100µl cell lysate was incubated in Tris-HCl buffer (pH 8), glutathione 5mM/L, and haemoglobin 5mM/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200mM/L and terminated after 20 minutes incubation at 37°C, by the addition 200µL of 10% trichloroacetic acid in 1N hydrochloric acid. After the centrifugal separation and the addition of 200µL of 1% thiobarbiturate, the tubes were boiled for 20 minutes. After cooling, the tubes were centrifuged for three minutes. COX activity was determined by reading absorbance at 632 nm.

Percentage inhibition of the enzyme was calculated as,

$$\%\boldsymbol{inhibition} = \frac{(\boldsymbol{Absorbance\ of\ control - Absorbance\ of\ test})}{\boldsymbol{Absorbance\ of\ control}} \times \mathbf{100}$$

5-LIPOXYGENASE INHIBITORY ACTIVITY

The determination of LOX activity was as per Axelrod *et al*. Briefly, the reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Percentage inhibition of the enzyme was calculated using the formula:

$$\%\boldsymbol{inhibition} = \frac{(\boldsymbol{Absorbance\ of\ control - Absorbance\ of\ test})}{\boldsymbol{Absorbance\ of\ control}} \times \mathbf{100}$$

ESTIMATION OF INDUCIBLE NITRIC OXIDE LEVEL

Nitric oxide synthase was determined by the method described by Salter *et al*. 1997. Cell lysate was homogenized in 2ml of HEPES buffer. The assay system contained substrate 0.1ml L-Arginine, 0.1ml manganese chloride, 0.1ml 30µg dithiothreitol (DTT), 0.1ml NADPH, 0.1ml tetrahydropterin, 0.1 ml oxygenated haemoglobin and 0.1ml enzyme (sample). Increase in absorbance was recorded at 401nm.

$$\%\boldsymbol{inhibition} = \frac{(\boldsymbol{Absorbance\ of\ control - Absorbance\ of\ test})}{\boldsymbol{Absorbance\ of\ control}} \times \mathbf{100}$$

ESTIMATION OF CELLULAR NITRITE LEVEL

Cell culture

RAW 264.7 cell lines were cultured in DMEM media, supplemented with 2mM glutamine, 10% heat inactivated FBS, antibiotics (Penicillin and streptomycin) and 1.5% sodium bicarbonate. The media was filtered using 0.2µm pore sized cellulose acetate filter (Sartorius) in completely aseptic technique. The cells were then grown till 60% confluency followed by activation with 1µl LPS (1µl/ml). LPS stimulated RAW cells were exposed with different concentrations mother tincture such as 25, 50, 75, 100µg/ml and different concentration of standard drug Diclofenac from a stock of 1mg/ml dissolved in DMSO and incubated for 24 hours.

The level of nitrite level was estimated by the method of Lepoivre et al. (Lepoivre et. al. 1990) To 0.5 mL of cell lysate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200µL of the supernatant, 30µL of 10% NaOH was added, followed by 300µL of Tris-HCl buffer and mixed well. To this, 530µL of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

MYELO PEROXIDASE ASSAY

Cell lysate was homogenized in a solution containing 50mM potassium phosphate buffer and 0.57% hexadecyl trimethyl ammonium bromide (HTAB) the samples were centrifuged at 2000 g for 30 minutes at 4°C, and supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% H 2 O. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C.

$$\boldsymbol{Enzyme\ \ units\ per\ ml} = \frac{\boldsymbol{OD \times 4 \times V_t \times DF}}{\boldsymbol{L \times \varepsilon 460 \times t \times V_s}}$$

OD is optical density, V_t is total volume in ml, L is light path in cm, €460 is extinction coefficient of tetraguaiacol, t is the time of measurement in minutes and V_s is sample volume in ml, ε460 = extinction coefficient for tetraguaiacol (26.6 mM-1·cm-1,)

RESULTS

Preliminary Phytochemical Analysis

Preliminary phytochemical studies revealed the presence of various constituents which is depicted in Table 1. The anti-arthritic potential of *Ledum Pal* mother tincture might be due to the presence of flavonoids, terpenoids, alkaloids, glycosides, steroids, phenols etc., which can also contribute to inhibition of mediators responsible for inflammatory responses rheumatoid arthritis.

Table-1: Preliminary Phytochemical Screening

Phytochemical constituents	*Ledum pal* mother tincture
Carbohydrates	++
Proteins	+
Alkaloids	++
Flavonoids	++
Glycosides	+
Terpenoids	++
Steroids	+
Lipids	++
Phenols	+
Saponins	++

(++) indicate active constituents in high amount, (+) indicate active constituents in low amount.

IN-VITRO METHODS FOR ASSESSING ANTI-ARTHRITIC ACTIVITY

Effect of Cyclooxygenase-2 Activity in Raw 264.7 Cell Lines

RAW 264.7 cells were pre-treated with lipopolysaccharides (LPS) (1µg/ml) concentration for 1hour and then incubated with *Ledum Pal* mother tincture and Diclofenac sodium standard at various concentrations (µg/ml) for 24 hours. Data were represented as Mean ± S.D of triplicate determination. The percentage inhibitions obtained from different concentration were tabulated in Table 2 IC_{50} value of mother tincture and standard found to be 79.16µg/ml and 72.21µg/ml respectively.

Table-2: Effect of *Ledum Pal* and Diclofenac on COX-2 activity in RAW 264.7 cell lines

Sample	Concentration (µg/ml)	Absorbance (632nm)	% inhibition
Control	-	0.1058±0.0002	
Standard (diclofenac sodium)	25 50 75 100	0.0881±0.0016 0.0722±0.0010 0.0481±0.0017 0.0330±0.0011	16.82 31.94 54.63 68.80
Ledum pal mother tincture residue	25 50 75 100	0.0951±0.0005 0.0825±0.0005 0.0522±0.0005 0.0365±0.0005	11.53 27.53 50.66 65.50

Data's were represented as Mean±SD of triplicate values

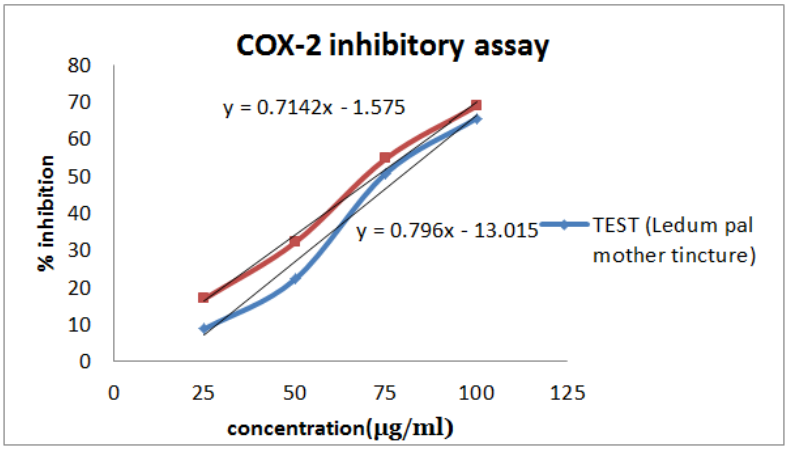

Fig-1: Percentage Inhibition of Cyclooxygenase-2 enzyme

5-Lipoxygenase Inhibition Assay

RAW 264.7 cells were pre-treated with lipopolysaccharides (LPS) (1µg/ml) concentration for 1 hour and then incubated with *Ledum Pal* mother tincture and Diclofenac sodium standard at various concentrations (µg/ml) for 24 hours. The percentage inhibitions obtained from different concentration were tabulated in Table 3. Data were represented as Mean ± S.D of triplicate determination. Both standard and mother tincture found to possess good inhibitory effect against lipoxygenase enzyme. Results are expressed as mean±SD (n=3). IC_{50} value of mother tincture and standard was found to be 69.46 µg/ml and 80.16 µg/ml respectively.

Table 3: Effect of Ledum pal and Diclofenac on 5-Lipoxygenase enzyme activity

Sample	Concentration (µg/ml)	Absorbance (234nm)	% inhibition
control	-	0.4781±0.0002	
Standard (diclofenac sodium)	25 50 75 100	0.3902±0.0001 0.3116±0.0002 0.2146±0.0001 0.1388±0.0001	18.38 34.82 55.9 40.96
Ledum pal mother tincture residue	25 50 75 100	0.4315±0.0001 0.3571±0.0001 0.2476±0.0001 0.1717±0.0001	9.74 25.30 48.21 64.15

Datas were represented as Mean±SD of triplicate values

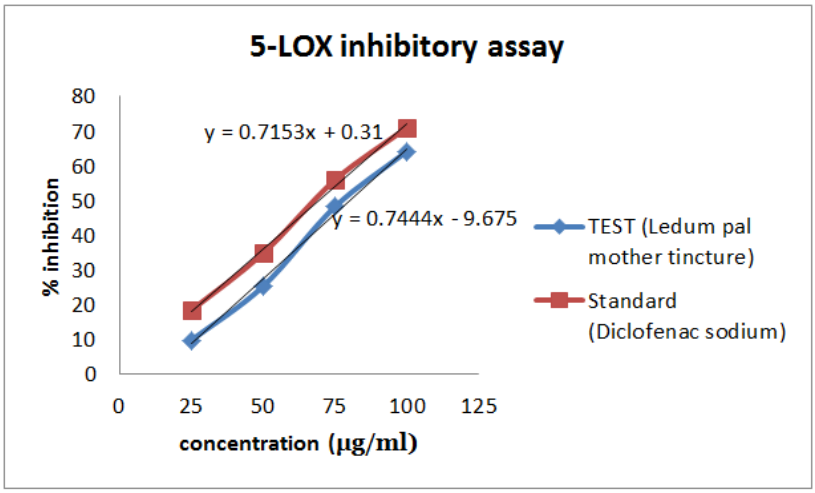

Fig-2: 5-Lipoxygenase Inhibitory assay

INDUCABLE NITRIC OXIDE SYNTHASE (iNOS) LEVEL ESTIMATION

RAW 264.7 cells were pre-treated with lipopolysaccharides (LPS) (1µg/ml) concentration for 1 hour and then incubated with *Ledum Pal* mother tincture and Diclofenac sodium standard at various concentrations (µg/ml) for 24 hours. The percentage inhibitions obtained from different concentration were tabulated in Table 3. Data were represented as Mean ± S.D of triplicate determination. Both standard and mother tincture found to exhibit a significant reduction in the inducible nitric oxide synthase level in a dose dependant. IC_{50} value of mother tincture and standard was found to be 57.02µg/ml and 66.88 µg/ml respectively.

Table-4: Effect of Ledum Pal and Diclofenac on iNOS level

Sample	Concentration (µg/ml)	Absorbance (401nm)	% inhibition
Control	-	0.083±0.0005	
Standard (diclofenac sodium)	25	0.057±0.0006	31.32
	50	0.042±0.0001	49.39
	75	0.036±0.0006	56.62
	100	0.021±0.0006	74.69
Ledum pal mother tincture residue	25	0.062±0.0005	25.3
	50	0.046±0.0005	44.57
	75	0.041±0.011	50.6
	100	0.025±0.0003	69.75

Datas were represented as Mean±SD of triplicate values

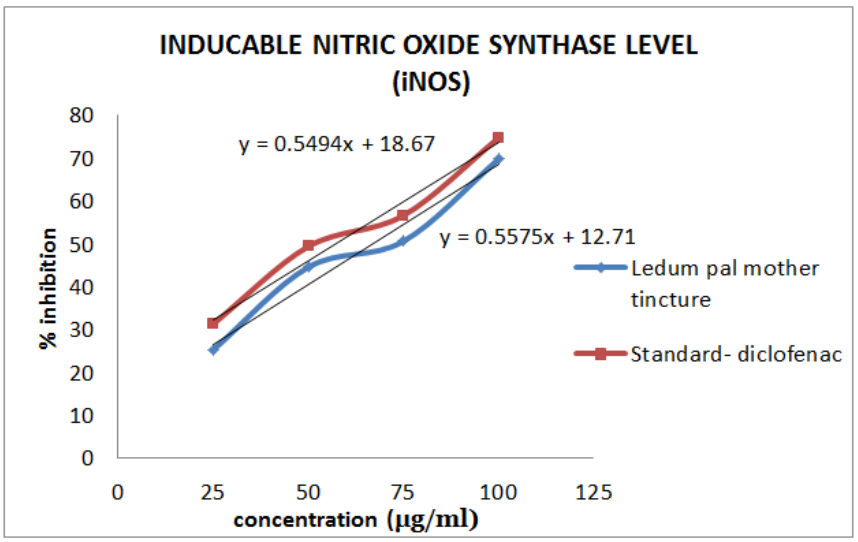

Fig-3: Percentage inhibition of iNOS activity

Cellular Nitrate Level Estimation

Standard graph was plotted for different concentration of sodium nitrite Vs absorbance. The assay analysis concentration of nitrite release. The graph was plotted with absorbance on y-axis and concentration on the x-axis in µg depicted in Table 2 and figure:4

Table-5: Standard Sodium Nitrite

CONCENTRATION (µg)	ABSORBANCE
100	0.021
200	0.042
300	0.063
400	0.080
500	0.110

Table-6: Cellular nitrite level estimation of *Ledum pal* and diclofenc on RAW cell line

Sample	Concentration (µg/ml)	Absorbance (540nm)	Nitrite concentration (µg)
Control	-	0.1467±0.0001	729.59
Standard(diclofenac sodium)	25	0.0980±0.0001	490.1
	50	0.0808±0.0001	400.21
	75	0.0747±0.0001	365.94
	100	0.0648±0.0001	320.41
Ledum pal mother tincture residue	25	0.1183±0.0002	588.44
	50	0.1041±0.0001	520.10
	75	0.0841±0.0002	420.21
	100	0.0684±0.0001	339.94

Datas were represented as Mean±SD of triplicate values

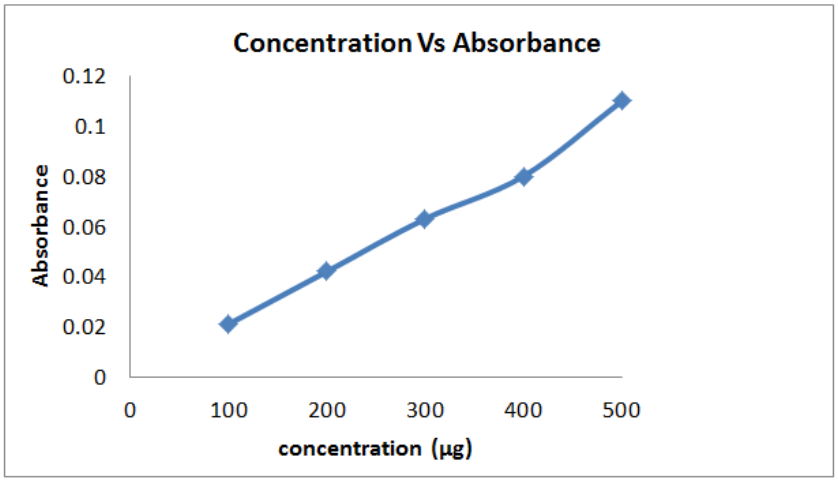

Fig-4: Standard graph of sodium nitrite

Estimation of nitrite level

It was observed that there was dose dependent decrease in the nitrite level in RAW 264.7 medium were observed at the concentration ranges from 25 to 100 µg/ml of the test mother tincture and standard diclofenac. Lipopolysaccharide (LPS) (1µg/mL) treated well was served as a control with maximum nitrite level of about 729.59 µg. Decreased cellular nitrite level is an indication of the capacity to inhibit nitric oxide synthase, thus inhibiting the production of nitric oxide. Results are expressed as mean±SD (n=3)

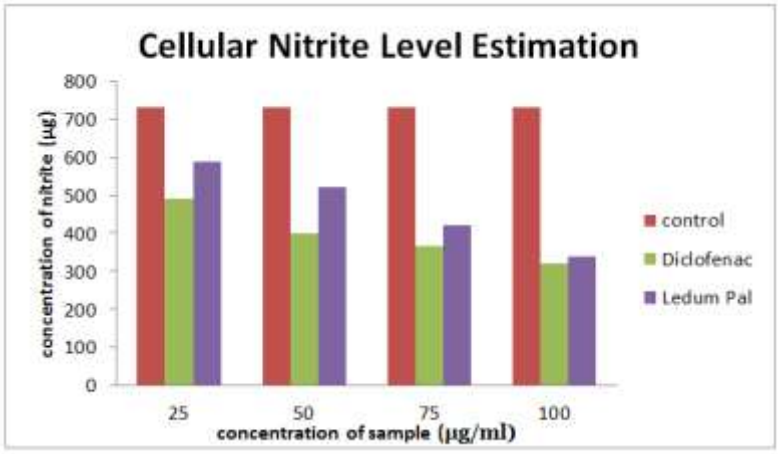

Fig-5: Effect of cellular nitrite level

Myeloperoxidase Enzyme Activity

The mother tincture was found to be effective in inhibiting Myeloperoxidase. A dose dependent increase in the inhibition of myeloperoxidase activity was exhibited by both standard and test. At higher concentrations inhibitory effect was very high. *Ledum pal* shows excellent MPO inhibition suggesting the good anti-inflammatory activity contributes to anti-arthritic property. The reduction in the myeloperoxidase activity by ledum pal and Diclofenac sodium on comparison with control are tabulated in Table 7.

Table-7: Estimation of Myeloperoxidase (MPO) activity

Sample concentration		Absorbance (460nm)	Enzyme activity (U/ml)
Control		0.0595±0.0002	
Diclofenac	25	0.0340±0.0002	0.0452
	50	0.0254±0.0001	0.0338
	75	0.0216±0.0002	0.0287
	100	0.0165±0.0001	0.0219
Ledum pal	25	0.0383±0.0001	0.0509
	50	0.0315±0.0002	0.0418
	75	0.0236±0.0001	0.0319
	100	0.0189±0.0001	0.0251

Datas were represented as Mean±SD of triplicate values

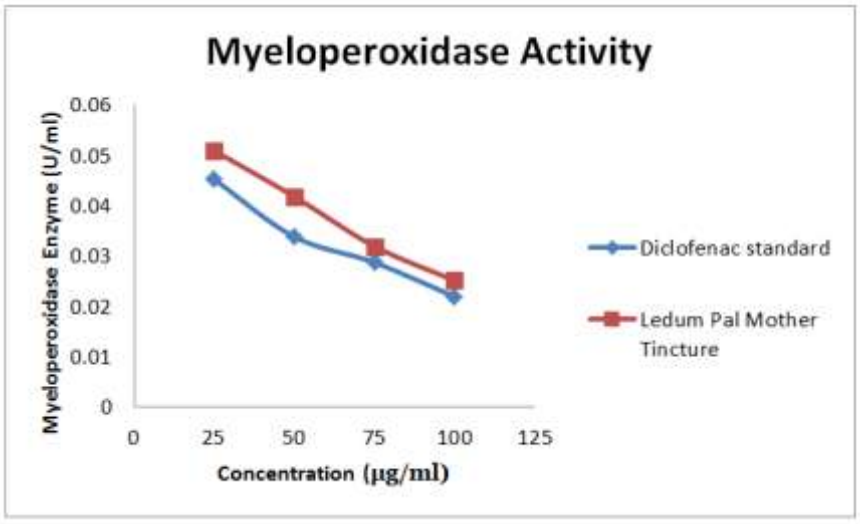

Fig-6: Myeloperoxidase level estimation

DISCUSSION

The preliminary phytochemical screening showed that the *Ledum pal* mother tincture is rich with the presence of terpenoids, flavonoids, steroids etc. are known anti-inflammatory products and this may contribute to its anti-arthritic

activity. Other chemical constituents present are alkaloids, glycosides, saponins, phenol, carbohydrates, protein etc. To determine the anti- arthritic activity of *Ledum Pal* mother tincture *in vitro*, the mouse macrophage-like cell line, RAW 264.7, was used. Here we were evaluating the ability to suppress various inflammatory responses.

Inflammation is implicated in the pathogeneses of arthritis, cancer, stroke, neurodegenerative and cardiovascular disease. Long term use of NSAIDs can lead to various harmful effects. So it is necessary to explore plants to obtain traditional herbal medicines[15].

Pro-inflammatory cytokines, such as TNF-α and IL-6 are key mediators of these processes, however, it remains unclear which mechanisms are involved in the initiation and regulation of cytokine production and other tissue-destructive mediator in auto immune inflammatory diseases[16]. Free radicals like reactive oxygen and nitrogen species can damage the articular components of the joints may lead to inflammatory arthritis. Free radicals such as hydroxyl radicals can degrade proteoglycans in extracellular matrix, hydrogen peroxidase blocks the synthesis of proteoglycan, tissue inhibitor of metalloproteinases are inactivated by peroxinitrate radical through destroying the cartilage tissue. Hypochlorous acid (HOCl) is an oxidising, chlorinating compound; it activates neutrophil collagenase and gelatinase lead to fragmentation of collagen matrix. Myeloperoxidase enzyme presents in neutrophils and monocyte are responsible for the generation of HOCl. ROS is also functions as a signal mediator to activate NF kB, AP-1, etc[6].

Macrophage produce NO, pro inflammatory cytokines such as TNF-α, IL-6 etc and pro inflammatory markers such as COX-2, 5-LOX,iNOS, upon inflammatory stimulation by LPs. Overproduction of these mediators is present in macrophage of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and hepatitis. Detection of high levels of these pro inflammatory cytokines is considered to be essential for clinical diagnosis.

Cyclooxygenase catalysing the biosynthesis of inflammatory mediators such as prostaglandin, thromboxane and prostacyclin. Inhibition of COX was considered to be partly responsible for the anti-inflammatory activity. In the present stud, *Ledum Pal* mother tincture showed significant anti-COX-2 activity with IC_{50} value 79.16µg/ml.

Lipooxygenase are a family of non-heme iron containing dioxygenases catalysing the biosynthesis of leukotrienes. Leukotrienes as initiators of inflammation and their inhibition are considered to be partly responsible for the ant inflammatory activity[19,20]. In the present study the *Ledum Pal* mother tincture showed significant anti 5-LOX activity with IC_{50} value 80.16µg/ml.

Large amount of NO produced by iNOS is believed as one of the most important inflammatory reactions in activated macrophage. iNOS is one of three key enzymes generating NO from amino acid L-arginine. The inducible NOS (iNOS), is not expressed in resting cells In inflammation, bacterial end product and inflammatory cytokines induce the expression of iNOS, which produces high amount of NO over prolonged periods. NO has regulatory and pro inflammatory properties in inflammation. A number of inflammatory and infectious diseases are mediated by nitric oxide, by exhibiting its action as a direct effector and as a regulator of other pathways. We showed that the extract was able to significantly lower the levels of LPS induced NO as a result of down regulation of INOS. In the present study mother tincture showed significant reduction in iNOS activity with IC_{50} value of 66.88µg/ml.

The reactive free radical NO, which is synthesized by iNOS, is a major macrophage derived inflammatory mediator and has also been reported to be involved in the development of inflammatory disease. Nitric oxide free radical is generated from sodium nitroprusside in aqueous solution at physiological pH. This nitric oxide is spontaneous interacts with the oxygen to produce stable products nitrates, nitrites which can be determined using Griess reagent. The present study showed that tincture with different concentration (20, 50, 75, 100 µg/ml) and diclofenac 10 µg/ml reduces the nitrate level. The nitrite produced in the cell culture supernatant was measured at 24hr of treatment showing the sample induced nitrite production is significant reduced when compared to the positive control.

Myeloperoxidase (MPO), the most frequent protein in mature neutrophils, seems to play an important role in this scenario. MPO released by neutrophils binds to macrophages initiating a molecular cascade resulting in secretion of interleukin-1, interleukin-8, interferon α/β, and tumor necrosis factor-α (TNF-α). These events attract more neutrophils and cause further degranulation. Therefore, MPO has an important immune regulatory function, and its presence associated to the expression of ROS and cytokines give support to the chronic inflammatory state. Inhibition of MPO, which is a naturally occurring constituent of neutrophil, showed that the mother tincture is able to prevent the accumulation of neutrophils and thus inhibits inflammation during rheumatoid arthritis[19,20].

CONCLUSION

A variety of mechanisms are involved in rheumatoid arthritis. In the present study the *Ledum Pal* mother tincture exhibited COX-2, 5-LOX, iNOS, and cellular nitrite and MPO inhibition properties. This indicates the ability of

the sample to produce anti-arthritic activity by multiple actions on pro-inflammatory enzymes and cytokines including COX, iNOS etc in LPS stimulated RAW 264.7 macrophage in a similar manner to that of diclofenac sodium Taken together, these findings demonstrate that *Ledum Pal* mother tincture has potential anti-inflammatory parameters that could make it useful for the treatment of inflammatory disorders, especially in rheumatoid arthritis.

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