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Research Article: Pharmacological Screening for Anti-Arthritic and Anti-Inflammatory Activity of Nyctanthes Arbor-Tristis Linn Leaves Extract by Novel Drug Delivery System.

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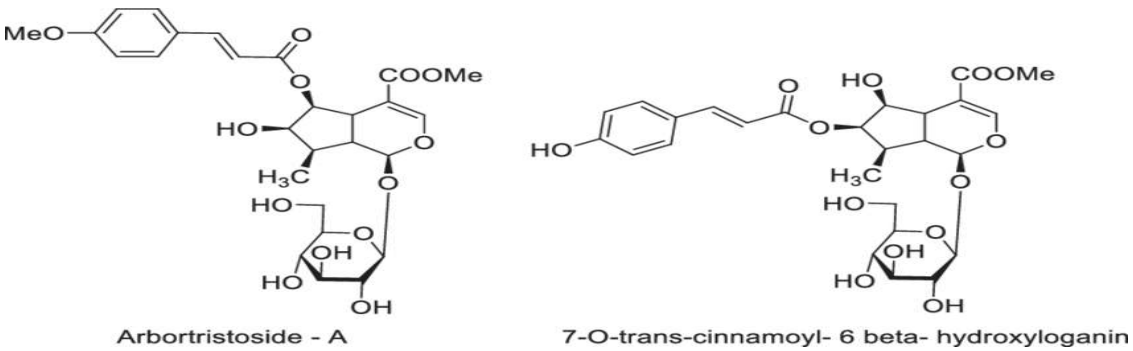
ABSTRACT:
Objective: - To evaluate anti-inflammatory and anti-arthritic activity of liposomes of Nyctanthes arbor-tristis Linn. Plant leaf extract.
Research Methodology: - The leaf extract was prepared by soxhlet apparatus with ethyl acetate, filtered and the filtrate evaporated to dryness. The liposomes of extract were prepared by fusion method. Anti-inflammatory and anti-arthritic activity of the liposomal extract at doses 500mg/kg was evaluated in rats using carrageenan induced paw edema and bovine serum albumin (BSA) assay was performed.
Result: - The liposome of leaf extract of Nyctanthes arbor-tristis Linn produced significant anti- inflammatory and anti- arthritic activity at 500mg/kg dose. When compared to the standard drug Diclofenac sodium 10mg/kg. The plain extract and liposomes was shows significant effect.
Conclusion: - This study demonstrates the potential anti-inflammatory and anti-arthritic effect of Nyctanthes arbor- tristis Linn leaves which supports the claim of traditional medicine is used with combination of novel drug delivery system like liposomes helps to overcome the conventional drugs side-effects and shows significant effect at very low dose.
Key words- Nyctanthes arbor-tristis Linn; iridoid glycoside, Liposomes, Rheumatoid arthritis.

I. INTRODUCTION

Nyctanthes arbor-tristis Linn is one of the well-known medicinal plants. Different parts of this plant are used in Indian systems of medicine for various pharmacological actions like anti-viral, anti-fungal, anti- malarial, anti-inflammatory& anti-arthritic & many more activities. NAT contain chemical constituents like iridoid glycosides, nyctanthic acid, ascorbic acid, methyl salicylate, friedeline, benzoic acid, etc. Quite recently, in research 7-o-ethyl-morroniside display potent in-vitro & in-vivo anti-inflammatory activity & that this iridoid can be used to treat various inflammations.

Iridoid glycoside with the general formula potently suppresses the effect of nuclear factor kappa B (NF-Kb), & thus the mRNA level. In addition, this compound has been shown significantly reduce the expression of relevant inflammatory factors.



Arbortristoside - A 7-O-trans-cinnamoyl- 6 beta- hydroxyloganin

Fig 01. Chemical structure of active constituents (Iridoid Glycoside)

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ARTHRITIS:-

Arthritis is joint disorder featuring inflammation. A joint is an area of the body where two different bones meet. A joint function moves the body parts connected by its bones. Arthritis literally means inflammation of one or more joints. It is frequently accompanied by joint pain; it is referred to as arthralgia. When four or more joints are involved, the arthritis is referred to as polyarthritis. When two or three joints are involved, it is referred to as oligoarthritis. When only a single joint is involved, it is referred to as mono-arthritis. [Research gate]

Types of Arthritis: - There are many types of arthritis (over 100 identified). The types of arthritis range from those related to wear And tear of cartilage (such as osteoarthritis) to those associated with inflammation resulting from a misdirected immune system (such as Rheumatoid arthritis) [Medicine net]

Rheumatoid Arthritis

Rheumatoid arthritis is a chronic multisystem disease. Though the most prominent manifestation of RA is inflammatory arthritis of the peripheral joints. Usually with symmetrical distribution, its systemic manifestation includes hematologic, pulmonary, neurological and cardiovascular abnormalities. The condition has high association with HLA-DR4 and HLA-DR1 and familial aggregation.

Present concept on etiology and pathogenesis proposes that RA occurs in an immune genetically predisposed individual to the effect of microbial agents acting as trigger antigen. The role of super antigen which is produced by several micro-organisms with capacity to bind to HLADR molecules (MHC-II region) has also emerged.

A. Im-munologic Derangements:- A number of observations in patients and experimental animals indicate the role of immune processes, particularly autoimmune phenomenon, in the development of RA. These include

1. Detection of circulating autoantibody called rheumatoid factor against Fc portion of autologous IgG in about 80% cases of RA. RF antibodies are heterogeneous and consist of IgM and IgG class. The presence of antigen- antibody complexes in the circulation as well as in the synovial fluid.
2. The presence of other autoantibodies such as antinuclear factor (ANF), antibodies to collagen type II, and antibodies to cytoskeleton.
3. Antigenicity of proteoglycans of human articular cartilage.
4. The presence of γ- globulin, particularly IgG and IgM, in the synovial fluid.
5. Association of RA with amyloidosis.
6. Activation of cell-mediated immunity as observed by presence of numerous inflammatory cells in the synovial, chiefly CD4+ T lymphocytes and some macrophages.

B. Trigger Event: - Though the above hypothesis of a possible role of autoimmunity in the etiology and pathogenesis of RA is generally widely accepted, controversy continues as the trigger events which initiate the destruction of articular cartilage. Various possibilities which have been suggested are as:

1. The existence of an infectious agent such as mycoplasma, Epstein-Barr virus (EBV), cytomegalovirus (CMV), either locally in the synovial fluid or systemic infection some time prior to the attack of RA.
2. The possible role of HLA- DR4 and HLA-DR1 in initiation of immunologic damage.
3. In response to antigenic exposure in a genetically predisposed individual, CD4+ T cells are activated.
4. These cells elaborate cytokines, the important ones being tumor necrosis factor (TNF)-α, interferon (IF)-γ, interleukin (IL)-1 and IL-6.
5. These cytokines activate endothelial cells, B lymphocytes & macrophages.
6. Activation of B-cells release IgM antibody against IgG, this molecule is termed RF.
7. IgG and IgM immune complexes trigger inflammatory damage to the synovial, small blood vessels and collagen.
8. Activated endothelial cells express adhesion molecules which stimulate collection of inflammatory cells.
9. Activation of macrophages releases more cytokines which cause damage to joint tissues and vascularization of cartilage termed pannus formation.
10. Eventually damage and destruction of bone and cartilage are followed by fibrosis and ankylosis producing joint deformities.

ADVANCES IN DRUG DELIVERY SYSTEM FOR RA THERAPEUTICS.

Numerous treatments are existed to combat this disease; however they are not very efficient and possess severe side effects, higher doses, and frequent administration. Therefore, newer therapies are developed to overcome all

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these limitations. Despite the presence of wide variety of therapeutics for RA, the major challenges lie in their successful delivery to the affected area. Along with this, most of this therapeutics often causes side effect and drug resistance. The most important reason behind this is the nonspecific delivery of drug molecule. So nowadays the research is turned towards the development of targeted delivery strategies to the inflamed joints. Advancements of this delivery system and their efficacy in carrying the therapeutic molecule to the target site are listed below:

a) Liposomes
b) Nanoparticles
c) Polymeric micelles
d) Nano emulsion /micro emulsion
e) Nanogel

Liposomes:-

Liposomes are spherical vesicle made of phospholipid bilayer comparable to mammalian cell membrane. Liposomes contain an aqueous compartment which can carry molecule that is protected from external environment. Liposomes can be hence loaded with hydrophilic or hydrophobic molecule. To deliver the molecule to a site of action, the lipid bilayer can fuse with other bilayer such as the cell membrane, thus delivering the liposomes content; this is a complex & non- spontaneous event, however, that does not apply to nutrients & drug delivery.

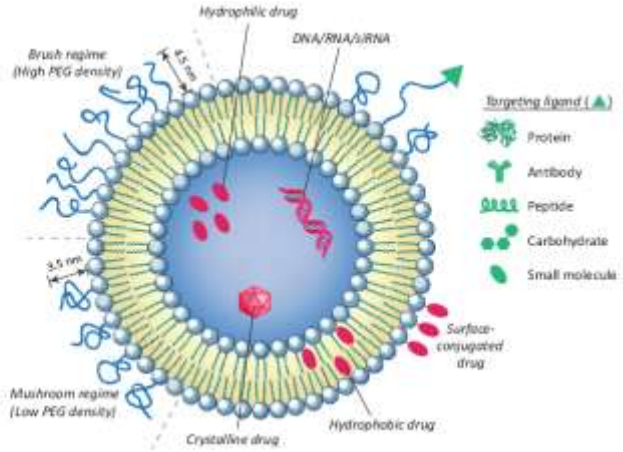

Fig 02.Structure of Liposomes.

SIGNIFICANT USE OF LIPOSOMES IN THE DELIVERY OF RHEUMATOID ARTHRITIS.

Till date, oral administration of anti-rheumatoid for treatment of arthritis has been a consistent challenge for the clinician, as there are severe clinical complications attached to their long-term oral use. The long-term administration of NSAIDS for the treatment of RA is associated with gastro destructive effects that may be manifested as ulcer and intra-abdominal bleeding. Oral or intra-muscular administration of steroidal drugs is generally associated with irreversible suppression of the immune system. DMARDs given by oral or intravenous or intramuscular route are known to be toxic to the immune system. In order to overcome the systemic effects of these drugs can be directly targeted to the synovial capsule of the affected joints through IV route, especially when the disease manifest only in limited number of joint. However, the rapid clearance of drug from the synovial cavity into the blood stream defeats the purpose of their intra- articular administration. In this regard, liposomes have proven to be the most suitable delivery system for retaining the drug in the synovial cavity by virtue of their size and chemical composition. The clearance of intra-synovial administered drug can be overcome through liposomes by virtue of the size of multi-lamellar vesicle (MLVs). This facilitates the uptake of drug by synovial cells and reduces the exposure to non-target sites, eliminating the undesirable side-effects.

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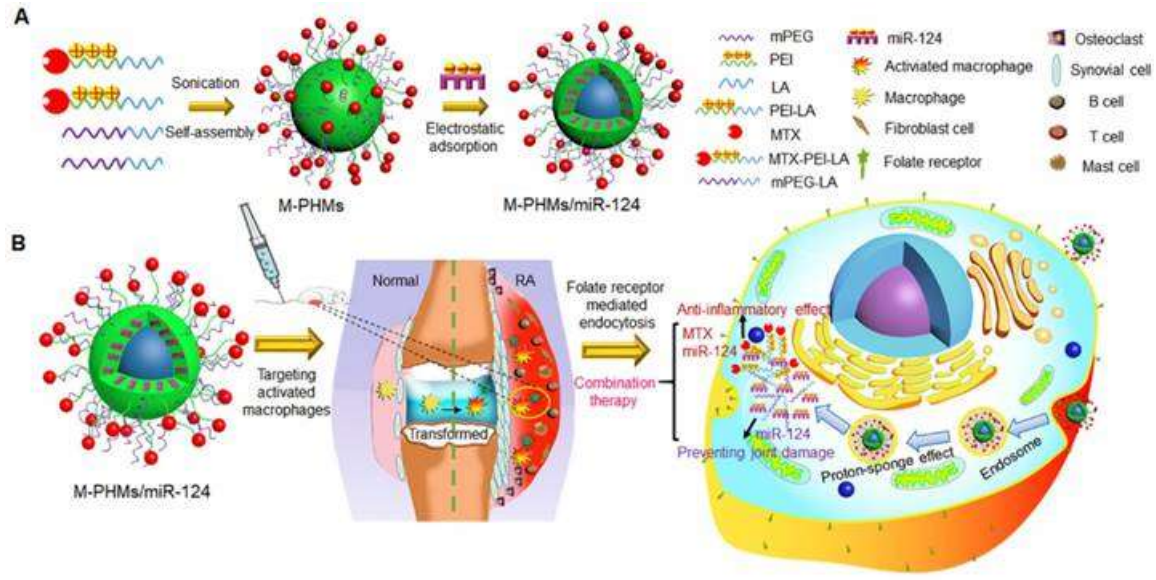

Fig 03.Significant delivery of liposomes at target in RA.

II. MATERIALS & METHOD

Selection of solvent.

The solvent used for the extraction of NAT leaves was ethyl acetate because the major constituent found in ethyl acetate extract was iridoid glycosides which is responsible for anti-inflammatory & anti-arthritic activity in NAT. in comparative analysis ethyl acetate of NAT produced the highest inhibition of paw edema & in ameliorated experimental rheumatoid arthritis ethyl acetate extract of NAT possessed the highest inhibitory activity against arthritis. **[MalihaUroos, et.al 2017]**

Preparation of crude extract.

The fresh leaves of NAT were thoroughly rinsed with water and shade dried at room temperature in hygienic condition. The leaves of NAT was powdered manually and extracted with ethyl acetate using soxhlet extraction technique. Extraction was performed until the solvent become colorless.

Preparation of Liposomes.

For novel drug delivery system liposomes are used. In present study liposomes are prepared by fusion method.

First components o lipid phase (Table no 01) and propylene glycol were kept 60 0 C water bath to form uniform lipid phase. Then, herbal extract dissolved in suitable amount of acetone was added to lipid phase. To evaporate acetone, the mixture was kept at 60 0 C. Subsequently, the aqueous phase (phosphate buffer saline) was warmed up to 60 0 C and added to lipid phase. Liposomes were formed after 15 min vortexing. [Jaafari et.al. 2005]

SN	Ingredient's	Percent
1	Eggphosphatidylcholine	15%
2	Cholesterol	2%
3	αtocopherol	0.3%
4	Methylparaben	0.1%
5	Propylparaben	0.02%
6	Propyleneglycol	7%
7	Ethylacetateextract	2%
8	Aqueousphase	Upto 100

Table No.01.Component of lipidphase.

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Evaluation of Liposomes:-

Optical microscopy:-

One drop of liposomal formulation was homogeneously spread onto a glass slide & left to dry overnight. The samples were observed under a Scanning Camera microscope. Photographs were taken at 100X magnification wherever necessary.

Stability study:-

Many physiological parameters are investigated as major reasons for liposomes instability. Oxidation & hydrolysis are the main reason for chemical instability, as they occur in phospholipid unsaturated chains. Stored at 4^0C temperature for one month & light preservation can avoid such reaction. Proper entrapment, pH adjustment, temperature, ionic interaction & application of cholesterol in bilayer structure can successfully cause liposomes stability. In this study liposomes were stored at 4^0C temperature for one month.

Anti-inflammatory Activity:-

(Carrageenan induced paw edema test)

Anti-inflammatory effect of NAT was screened by carrageenan induced paw edema model. The paw volume by carrageenan was measured by using plethysmometer. Indomethacin was used as std drug. Experimental animals, wistar albino rats of either sex (weighing between 180-200gm) where divided into 4 groups with 4 animals in each group. [Arruanchakam et.al] [kosala et.al]

Group 1: Animal received 0.5% carboxyl methyl cellulose (10mg/kg p.o)

Group 2: Animal received indomethacin (8mg/kg p.o)

Group 3: Animal received NAT extract 500mg/kg + 0.1 ml carrageenan of 1% w/v solution.

Group 4: Animal received liposomes of NAT extract 500mg/kg + carrageenan.

Wistar rats were subjected to fasting condition for 18 hours before treatment with only access to water and libitum. Prior to the induction of carrageenan, the right hind paw volume of all rats was measured an animals were exposed with 1.0 ml of test extract and standard drug before 1hr. afterword edema was induced by injecting 0.1 ml carrageenan (1% in 0.9 sodium chloride) in to the sub planter region f right hind paw in rat. The paw volume was measured at the time interval of 30 min, 1hr and 2hr up to 5hrs. Repeat the same step for liposomes. [Yogesh S. Thorat, et.al 2019]

Formula for % inhibition

% inhibition = Tc-Te/Tc×100

Where, Tc= test control

Te= test extract

Anti-arthritic Activity

(Inhibition of protein denaturation using bovine serum albumin Method)

In this method, anti-arthritic activity of liposomes of NAT extract was evaluated by % inhibition of denaturation. In this method bovine serum albumin was used. The reference drug was Diclofenac sodium. The 0.5ml reaction mixture consist of 0.45 ml of bovine serum albumin (5% aq. Solution) and 0.05ml of NAT in different conc. (100-500 µg/ml) test control and product control consist of 0.5ml of reaction mixture. Test control (0.45ml of BSA + 0.05ml of distilled water) product control (0.45ml of distilled water + 0.05mlof test solution) the pH of solution was adjusted to 6.3 by using 1N Hcl. The samples was incubated at 37 0 C for 20 min and heated at 57 0 C for 30min. after cooling, add 2.5ml of phosphate buffer (pH 6.3) the absorbance was measured using UV spectrophotometer at 660nm. [Arya D. Vaijayanthimala P, Alamgeer et.al]

The percentage inhibition of protein denaturation was calculated using the formula.

$$100-\left[\frac{f\ Test\ Solution - Abs\ of\ product\ Control}{\text{Abs of Test Control}}\right] \times 100$$

III. RESULT:-

Preliminary phytochemical analysis:

The ethyl acetate extract of NAT when tested chemically, were found to show the presence of iridoid glycosides, alkaloids, tannin, flavonoids. In which iridoid glycoside is responsible for anti-inflammatory and anti-arthritic activity.

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Sr.no	Phytochemical	Testname	Ethylacetateextract
1	Alkaloids	Hager'stest	-
2	Carbohydrates	Benedict's test	+
3	Flavonoids	NaOHtest Jone'stest	- -
4	Glycosides	Foamtest	+
5	Tannins	Ferricchloride&$KMnO_4$ test	+
6	Terpenoids	Salkowski test	+
7	Acids	Bicarbonates	+
8	Coumarins	Testfor coumarins	-
9	Carotenoids	Testfor carotenoids	-
10	Phenols	$FeCL_3$test	+

Table no 02. Qualitative phytochemical analysis of NAT ethyl acetate extract

Evaluation of Liposomes:-

Sr. no	Batches	Avg. Globule Size (µm)
1.	F1	13.66
2.	F2	13.38
3.	F3	12.45
4.	F4	11.75
5.	F5	12.01
6.	F6	10.54
7.	F7	10.85
8.	F8	9.97
9.	F9	9.54

Table no 04. Avg particle size of liposomes.

Measurement of particle size:-

Particle size analysis was done by Backman Counter size Analyzer. Particle size analysis depict that the liposomes were ranging 190.6, 212.9 & 181.6 nm respectively.

Stability study:-

The satisfactory formulation was packed in the tube & stored at 4-8^{0}C temperature for one month. At the one month the samples were analyzed for their physical property. So there were no significant changes found in one-month stability.

Anti-inflammatory Activity:

Rats injected with carrageenan showed a significant increase in paw edema volume when compared to normal rats and standard. Ethyl acetate extract of NAT at the dose of 500mg/kg and liposomes of NAT extract at the dose of 500mg/kg showed a significant reduction in rat paw edema volume when compared with the inflammatory rats. The reduction in paw edema volume in liposome NAT extract is greater than normal NAT extract.

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SN	Group	Changes in paw thickness(mm)±SD(%inhibition)			
		0 hr	1 hr	2 hr	3 hr
1	Carrageenan control (0.1ml of1% w/v)	0.3575± 0.01548	1.480± 0.01780	1.545± 0.02661	1.543± 0.01931
2	Carrageenan (0.1ml of1% w/v)+ethyl extract	0.4425± 0.1876	1.035± 1269**	0.8025± 0.3473	0.7525± 0.04608
3	Carrageenan (0.1ml of1% w/v)+liposomes	0.9000± 0.1339	0.980 0±0.0 60**	0.9000± 0.1339**** 47.99%	0.9000 ±0.133 9**** 55.50%
4	Carrageenan (0.1ml of1% w/v)+Indomethacin	0.8950± 0.1973	1.093± 0.0429*	0.7883± 0.7126**** 48.99%	0.6975± 0.0110**** 54.85%

Table 05.Changes in paw thickness (mm) ± SD (%inhibition)

The value are expressed a mean±SEM (n=3)
Significance determined by ANOVA followed by Dunnett's multiple comparison tests.
* Statistically significant at ($p \leq 0.05$)
** Statistically significant at ($p \leq 0.01$)
*** Statistically significant at ($p \leq 0.001$)
**** Statistically significant at ($p \leq 0.0001$)

Anti-arthritic Activity:-

Investigation of anti-arthritic of liposomes of NAT in-vitro models for assessing the % inhibition of denaturation was done in 5 different concentrations (100-500µg/ml). The detailed results are tabulated above.

SN	Concinµg/ml	%inhibitionwith standarddrug Diclofenacsodium	% inhibitionwithextract
1	100	14.36±0.72	20.11±0.63
2	200	22.98±0.83	24.13±0.49
3	300	32.18±0.48	35.63±0.82
4	400	44.82±0.96	42.25±0.57
5	500	75.86±0.77	72.41±0.72

Table no 06. % inhibition with standard drug & with extract.

All the value are expressed as Mean±SEM (N=3)
All the value is significant when compared to control ($p \leq 0.05$)
The effect of liposomes of NAT and standard drug Diclofenac in concentration
100,200,300,400 and 500µg/ml was found to be significant ($p \leq 0.05$) when compared to control.

IV. DISCUSSION

Disease with inflammatory etiopathology is rising worldwide. The synthetic drug used as anti-inflammatory agents are having deleterious side effects besides being costly & rarely available at all place. Some latest therapy are available for the treatment of chronic inflammation like RA these include different monoclonal antibodies, immunoglobulin, small molecule used for

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immunotherapy for gene therapy but not any permanent treatment are available to cure complete disease. Research and development of anti-inflammatory medicinal agents have proven beneficial. From effectiveness to low cost, ease of availability and less or no side effect, all aspects are proving beneficial. Exploration of novel method of extraction and investigation of phyto constituents from these medicinal plants are undergoing for better evaluation of active principles despite the presence of wide variety of therapeutics for RA, the major challenges lies in their successful delivery to the affected area. Along with this, most of this therapeutics often causes side effects and drug resistance. The most important reason behind this is the nonspecific delivery of drug molecule. So nowadays the research is turned towards the development of targeted delivery strategies to the inflamed joints. The changed properties of the inflammatory site, such as a change in pH, temperature, EPR effect and over expression of various cells have the greatest potential for targeting. Therefore, different delivery system is establishing their arena by targeting the drug to the site, reducing the amount of drug & adverse effect. In-vitro & In-vivo evaluation of anti-inflammatory potential & application of medicinal plants with being explored for proper prophylactic & therapeutic evaluation. Promising result has been obtained by the application of medicinal plants in inflammatory disease & arthritis. Hence many patents have been granted in this field. However, evaluation at the molecular level of both phyto-constituents of medicinal plants & their physiological & pharmacological activities & mechanism of action & their role as anti-inflammatory agents in In-vivo studies need to be effectively explored.

In present study the attempt was made to investigate the folklore use of NAT commonly known as arijatak for its anti- inflammatory & anti-arthritic effect in scientific light.

The dried leaves of NAT were extracted with ethyl acetate. The yield of extracted NAT was found to be 15.92 % w/v. the preliminary phytochemical study of NAT showed presence of alkaloid, iridoid lycosides, phenol, saponnins, carbohydrates & flavonoids.

In-vivo anti-inflammatory property of NAT was screened by carrageenan induced paw edema model. A decrease of paw volume serves as a positive indicator in the estimation of anti-inflammatory esponse. In this study liposomes of NAT showed a significant ($p<0.05$) reduction of edema in the delayed phase of inflammation than early phase.

In-vitro anti-arthritic activity of NAT was evaluated by inhibition of protein denaturation assay. The result of this show significant ($p<0.05$) inhibition of protein denaturation in both std. & extract treated groups as compared to control group. The finding of the study exhibited a conc. dependent inhibition of protein denaturation by NAT throughout the conc. range of 100- 500µg/ml std. drug diclofenac sodium at conc. 100 & 200µg/ml showed less effect compared to 100 & 200 µg/ml ethyl acetate extract of liposomes of NAT.

NAT is one of the wealthy medicinal plant consist of various phyto-constituents like iridoid glycosides, alkaloids, polyphenol, flavonoids, tannins, saponnins. Phytochemicals are imparting pharmacological effects is one of the well-established fact.

The extract mode of reduction of inflammation, inhibition of protein denaturation by ethyl acetate extract of NAT is not clear. In reported study of anti- inflammatory activity of NAT was attributed to iridoid glycosides of the plant. [Ahasan Sharif, et.al] iridoid glycosides are present in NAT might be responsible for the different activity. [Maliha Uroos, et.al]

From the result of the present study it can be stated that liposomes of NAT may control the production of pro-inflammatory mediator's enzymes by decreasing paw volume & inhibiting protein denaturation. Hence it can suggest that the inflammatory & anti- arthritic activity of NAT might be due to iridoid glycosides.

V. CONCLUSION:-

Result obtained after successful research work contribute towards the validating the use of liposomal NAT formulation in the treatment of RA & Inflammation. From the results of phyto-chemical analysis it was found that the major chemical constituents present were iridoid glycosides, phenol, flavonoids, alkaloids & other constituents. The quantitative analysis of iridoid glycosides & arbortristoside- A, B, C are responsible for respected activity.

In present study liposomes of NAT extract treated test group reduce the paw volume & inhibit protein denaturation than plain extract of NAT. The present study concluded that liposomes of NAT possess anti-inflammatory & anti- arthritic activity.

From result analysis we found that liposome of NAT extract show more inhibition

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than plain extract in rat.
All experimental work shows significant effect but further study is needed in this research work.

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