PHYTOCHEMICALS ANALYSIS, GC – MS CHARACTERIZATION AND IN-VITRO ANTI – ARTHRITIS ACTIVITY OF *NYCTANTHES ARBOR- TRISTIS LINN*. LEAF

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Abstract: Earth is a planet dominated by plants. From time in memorial, mankind has exploited nature for all kind of useful production and enjoyed the goodness in it. Medicinal plants have served as a major source of medication in alleviating human ailments due to the phytochemicals and promising pharmacological activities in them. One of the most useful plants in India, extensively used in ayurvedic medicine for the treatment of various diseases is *Nyctanthes arbor-tristis.* This present study involves phytochemical screening, gas chromatography - mass spectrometry (GC – MS) analysis and pharmacologically validation for the anti-arthritic properties using in-vitro inhibition of protein denaturation model of *Nyctanthes arbor-tristis* leaf. Extraction of powdered plant material, using the solvents; ethanol, ethyl acetate, chloroform and petroleum ether, qualitative phytochemical analysis of various extracts, GC – MS analysis of ethyl acetate extract using a Perkin Elmer GC Claurus 500 system and the determination of *In Vitro* Anti – Arthritis activity by protein denaturation method. In various concentrations of 200, 400, 600, 800, 1000µg/ml, all the extracts showed positive response compared to standard diclofenac sodium. The ethanol extract showed significant protection against denaturation of protein. The order of effect of different extract were represented as follows ethanol>ethyl acetate> chloroform. Thus we conclude that the anti-arthritic activities may be due to the effect of the phytochemicals present in the plant.

Key Terms: Anti-arthritis, phytochemicals, *Nyctanthes arbor-tristi,* protein denaturation, GC-MS Analysis

I. INTRODUCTION

Arthritis is a disorder that affects joints with symptoms such as joint pain and stiffness. There are over 100 types of arthritis (Arthritis Basics, CDC, 2018). Arthritis exists in various forms; the common ones are osteoarthritis (degenerative joint disease) and rheumatoid arthritis.

Occurrence of osteoarthritis depends on age, that is; the more advanced in age the more the likelihood of developing the disorder and this affects mainly the fingers, knees and hips.

Rheumatoid arthritis is an autoimmune disease which is characterized by chronic inflammation due to synovial hyperplasia which further progresses to massive irreversible bone destruction (Mayada *et al.,* 2014). It is an autoimmune disorder that often affects the hands and feet. Other symptoms include redness, warmth, swelling, and decreased range of motion of the affected joints (Athanasiou *et al.,* 2013).

Recent epidemiological study reveals that about 1% of people all over the world are now affected with rheumatoid arthritis and it is more prevalent among women rather than men, though this fact have not been scientifically proven. Rheumatoid arthritis is developed (almost in 80% of cases) from the mid of the fourth decade in life to the last of the fifth (DeMaria, 2002.).

The exact pathogenesis of rheumatoid arthritis still remains unclear although strong evidence suggests the involvement of cytokines are important in disease progression since cytokines play a fundamental role in various inflammatory processes, articular destruction, and rheumatoid arthritis -associated comorbidities (Frieri, 1986., Frieri *et al.,* 1994., Brennan and McInnes, 2008).

Although, there is no known cure for rheumatoid arthritis yet, the aim of treatment is thus, to reduce joint inflammation and pain, maximize joint function, and prevent joint destruction and deformity. Current treatment provides non-steroidal anti-inflammatory drugs (NSAIDs) which have several and severe adverse effects such as; edema (swelling of the feet) heartburn,

stomach upset and stomach ulcers and possibly risk of blood clots, heart attack and stroke. These undesirable side effects have force patients and researchers to look for complementary and alternative medicine (CAM).

In a quest to alleviate various human ailments, medicinal plants have played an important role in giving therapeutic aids. Nature has provided a complete store of knowledge of herbal drugs which constitute a major part of traditional medicine involving the Ayurvedic, Unani, Siddha, Charak Samhita, Sushruta samhita and Homeopathy system of medicines (Gupta *et al.*, 2005; Sandhu and Heinrich, 2005). These systems of medicine are totally based on the use of plants in the treatment of different diseases.

Despite the importance of western medicines, presently about 80% of the world population is using medicinal plants as their major source for medication in primary treatment (Kumari, 2009).

This is only possible due to the present of compounds commonly called phytochemicals. Unlike pharmaceutical chemicals these phytochemicals do not have any side effect if administered appropriately. Since the phytochemicals treats diseases without causing any harm to human beings these can also be considered as "man- friendly medicines" (Kumari, 2009).

One of the most useful plants in India, extensively used in ayurvedic medicine for the treatment of various diseases is *Nyctanthes arbor-tristis*

Nyctanthes arbor-tristis is a valuable medicinal plant which belongs to the family Oleaceae, and distributed widely in sub – Himalayan region

According to Shandhar and Kaur, traditionally, the flowers are used as stomachic, carminative, astringent to bowel, antibilious, expectorant, hair tonic and in the treatment of piles and various skin diseases. The stem bark is made into powder form and given in rheumatic joint pain, in treatment of malaria and also used as an expectorant. The leaves of *Nyctanthes arbor-tristis Linn* are used for the treatment of various diseases such as sciatica, chronic fever, rheumatism, and internal worm infections, and as a laxative, diaphoretic, diuretic, and many other treatments. The seeds are useful as anthelmintics and in treatment of alopecia, it is antibilious and an expectorant, and is also useful in bilious fevers (Shandhar and Kaur, 2011).

The leaves extract has many proved pharmacological effects like anti-bacteria (Rani *et al.*, 2012) analgesic, anti-inflammatory, anti-diabetic, anti-arthritic, antioxidant, hepatoprotective and antispasmodic activities (Ratnasooriya *et al.*, 2005).

The phytochemical analysis of the leaf, fruit and seed of *Nycthantes arbor-tristis* reveals the presence of several phytochemicals such as flavonoid, phenol, tannins, glycosides, saponins etc. the secondary metabolite such as glycosides and alkaloids are the largest group of chemicals produced by the plants (Shandhar and Kaur, 2011).

Due to all these benefits, the present study involves phytochemical screening, GC-MS analysis and pharmacologically validation for its anti-arthritic properties using in-vitro inhibition of protein denaturation model of *Nycthantes. arbor-tristis* leaf.

II. MATERIALS AND METHODS

A. COLLECTION OF PLANT MATERIAL AND AUTHENTICATION

Fresh whole plant (leaves) of *Nycthantes arbor – tristis* were collected from the place near Guduvanchery. The plant specimen was identified and authenticated by Dr. D. Arvind, Assistant professor Department of Ayush (Govt. of India), National institute of siddha. Certificate number: NISMB2872017.

B. WASHING, DRYING AND SIZE REDUCTION

The plant was washed thoroughly to remove debris with distilled water. Then shade dry for about 1 week. The dried plant material was further crushed to powder and the powder was stored in an air tight container for further analysis.

C. EXTRACTION OF POWDERED PLANT MATERIAL

The powdered plant material was subjected to extraction using the solvents; ethanol, ethyl acetate, chloroform and petroleum ether. 25g of the powdered plant sample were measured and transferred into five different beakers labelled according to the solvent. 100ml of the various solvents were measured and poured, then stirred thoroughly. The beakers were tightly covered with foil paper to prevent evaporation. These were kept for 72 hours and then filtered via filter paper. The crude extract gotten was weighed and store in vial bottle for analysis. The percentage yields of all the extracts were determined. Ethanol 16 %, ethyl acetate 17 %, chloroform 8 % and petroleum ether 5 %.

D. PRELIMINARY PHYTOCHEMICAL ANALYSIS

According to the methods described by Sahira and Cathrine, 2015; Prashant *et al;* 2011, and Mohammad *et al;* 2013, various solvent extracts were qualitatively tested for alkaloids, flavonoids, phenol, steroids, saponins, and tannins.

E. GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC – MS)

Gas Chromatography – Mass Spectrometry analysis of ethyl acetate extract was performed using a Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass spectrometer equipped with an Elite 5MS fused silica column (30× 0.25 mm ID. × 1Mm df, composed of 5% Diphenyl/95% Dimethyl poly siloxane) for GC – MS detection, an electron ionization system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1ml/min and an injection volume of 3MI was employed (split ratio of 10:1). Injector temperature 250℃; ion source temperature 280℃. The oven temperature was programmed from 110 ℃ (isothermal for 2 minutes) with an increase of 10℃/min to 200℃, then 5℃/min to 280℃ ending with a 9 min isothermal at 280℃. Mass spectra were taken at 70eV, a scan interval of 0.5 seconds and fragments from 45 to 450Da. The relative percentage amount of each component was calculated by comparing its average peak area to total areas. Software adopted to handle mass spectra and chromatograms was a Turbo Mass V.5.2.0.

F. DETERMINATION OF IN VITRO ANTI – ARTHRITIS ACTIVITY

The *in-vitro* anti-arthritis activity of ethanol, ethyl acetate and chloroform extracts of *Nycthantes arbor – tristis Linn* leaf against the standard diclofenac sodium were determined by protein denaturation (Ravi, 2009).

- Test solution (2ml): It consists of 1ml of test solution of various concentrations ranging from 200µg/ml to 1000µg/ml and 2ml of Bovine serum albumin (5% aqueous solution).
- Test control solution (2ml): It consists of 1ml of distilled water and 1m of Bovine serum albumin (5% aqueous solution).
- Product control (2ml): It consists of 1ml test solution of various concentration ranging from 200 µg/ml to 1000 µg/ml and 1ml of distilled water.
- Standard solution (2ml): It consists of 1ml of Diclofenac sodium (200 – 1000 µg/ml) and 1ml of Bovine serum albumin (5% aqueous solution).

pH of all the above solutions was adjusted to 6.3 using 1N HCl. The entire sample solution were then incubated at 37℃ for 20 minutes and kept at 70℃ in hot water bath for 10 minutes. Allow the solution to cool for some time then add 2.5ml of phosphate buffer to all the solution. The absorbance of the resulting solution was measured at 660nm using UV visible spectrophotometer (Ravi, 2009)

The Percentage inhibition of protein denaturation was calculated using the given formula.

$$100 - \frac{Optical\ Density\ of\ Test\ Solution - Optical\ Density\ of\ Product\ Control}{Optical\ Density\ of\ Test\ Control} \times 100$$

III. RESULTS

A. PHYTOCHEMICAL ANALYSIS

In the present study, the preliminary phytochemical analysis of the various extracts of *Nycthanthes arbor- tristis Linn.* were determined as presented in Table1.

Various phytochemicals such as alkaloid, saponins, tannins, flavonoids, phenol, steroids and terpenoids have been observed as the active phyto - constituents of *Nycthanthes arbor – tristis Linn.* via ethanol, ethyl acetate, chloroform and petroleum ether extracts.

Table 1: showing result of phytochemical analysis

Chemical Constituents	Ethanol Extract	Ethyl Acetate Extract	Chloroform Extract	Petroleum Ether Extract
Alkaloids				
i. Mayer's Test	_	+	+	_
ii. Hager's Test	+	+	+	+
Flavonoids				
Alkaline Reagent Test	+	+	+	+
Lead acetate Test	+	+	+	_
Phenols	+	+	-	-
Saponins	+	_	+	_
Steroids	+	+	-	-

Tannin Gelatin Test	+	+	+	+
Ferric Chloride Test	+	+	_	+
Terpenoids	++	++	+	-

Presence +, Absence –

B. GC – MS Analysis

Using ethyl acetate extract of *Nyctanthes arbor tritis*, bioactive compounds were identified and characterized by GC-MS analysis.

Identification and interpretation on GC mass spectrum (Figure 1) was conducted using Sophisticated Analytical Instrument Facility of the Indian Institute of Technology Madras, sponsored by the Department of Science and Technology Government of India.

The active compounds with their retention time (RT), molecular formula, molecular weight (MW) and biological activity in the ethyl acetate extract of the leaf of *Nyctanthes arbor tristis* are presented in (Table 2). In the present survey, fourteen compounds have been distinguished from the ethyl acetate extract of the leaves part of *Nyctanthes arbors* by Gas Chromatography-Mass Spectrometry analysis.

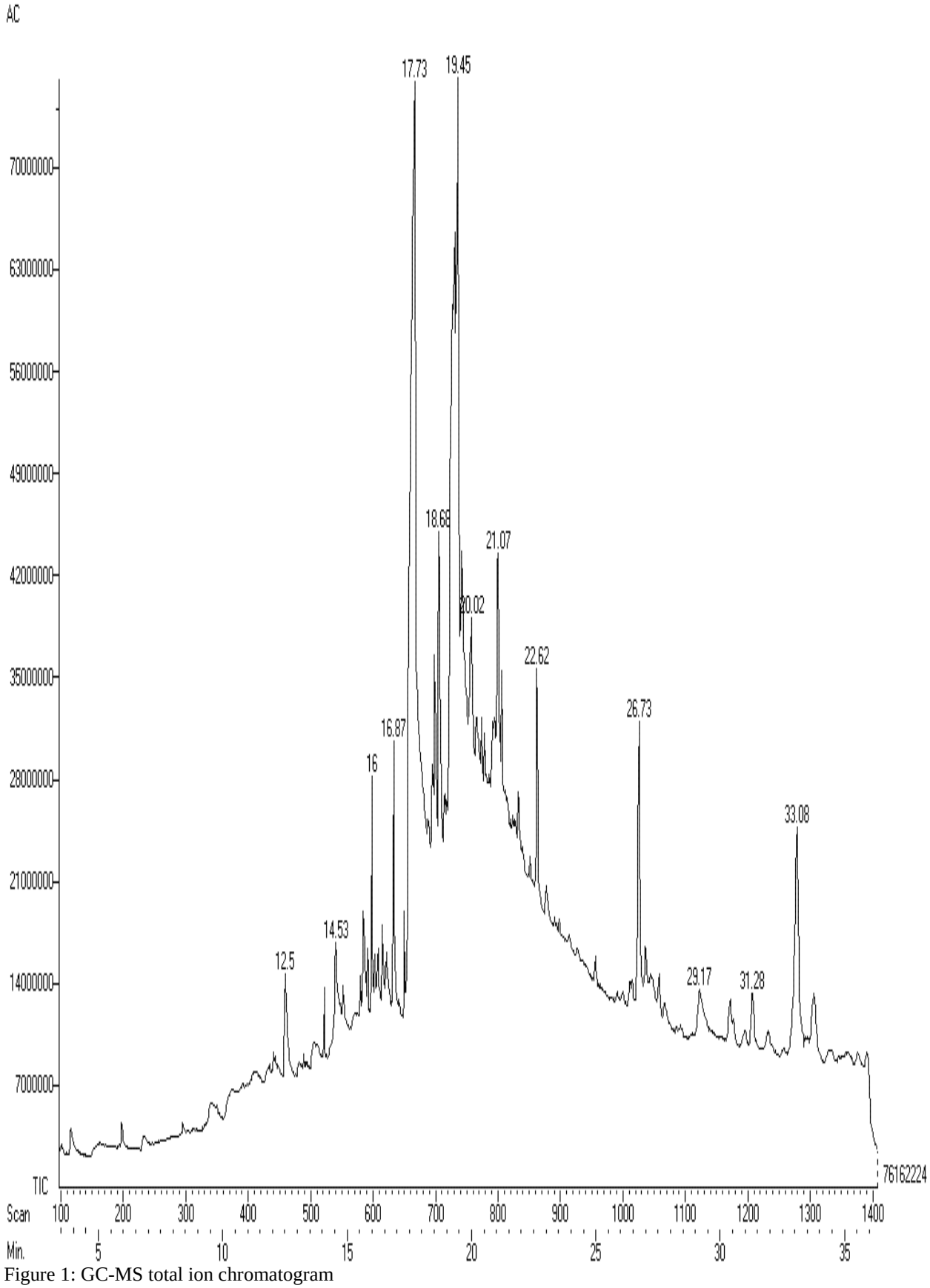

Figure 1: GC-MS total ion chromatogram

Table 2: Bioactive Constitutes from GC – MS Analysis of Ethyl acetate extract of *Nyctanthes arbor tristis linn* leaf

S/No	Name of compound	RT	Molecular formula	Molecular Weight (g/mol)	Biological Activity
1.	Flavone	14.53	$C_{15}H_{10}O_2$	222.243	Anti – inflammatory, antioxidant, antiseptic, anti-tumor.
2.	Oxacyclopentadecan-2-one, 15, 15- dimethyly	17.73	$C_{16}H_{30}O$	254.414	Anti – microbial, anti - cancer
3.	Isopropyl stearate	22.62	$C_{21}H_{42}O_2$	326.565	Anti – microbial, anti – viral, anti - inflammatory
4.	6- [Diethylamino] benzofuran – 3[2H] – one	12.5	$C_{12}H_{15}NO_2$	205.257	Anti – bacterial, anti - inflammatory
5.	Strychnidin-10-one, 2, 3- dimethoxy 19- oxide	33.08	$C_{23}H_{26}N_2O_5$	394.471	Analgesic, adjuvants, immunologic
6.	Tetradecanoic acid, 2- phenyl-1, 2- dioxin-5-yl ester	31.28	$C_{15}H_{30}O_2$	242.3975	Anti – fungal, anti – malaria, antioxidant
7.	Corynan – 17- ol, 18, 19- didehydro- 10- methoxy acetate	29.17	$C_{22}H_{28}N_2O_3$	368.477	Antitumor, Antiulcer, Cytotoxic, Gastro protective, Hepatoprotective, Insectifuge
8.	Phenol, 2, 6 bis (1, 1 – dimethylethyl) – 4 – [(4- hydroxyl – 3, 5 – dimethylphenyl) methyl]	26.73	$C_{54}H_{78}O_3$	775.215	Anti – mitotic, anti – proliferation, anti-oxidant
9.	Coumarine 3 – (2, 4 – dinitrophenyl)	21.07	$C_{19}H_{16}O_4$	308.3279	Anti – cancer, anti – inflammatory, anticoagulant, anti-neurodegenerative, analgesic, antidiabetic,
10.	E, E Z- 1, 3, 12- Nonadecatriene – 5, 14 - diol	20.02	$C_{19}H_{34}O_2$	294.479	Antimicrobial activity
11.	10 – methyl- 8- tetradecen- 1-ol acetate	18.68	$C_{17}H_{32}O_2$	268.435	Insect pheromone
12.	Pentadecanoic acid, 13 – methyl; methyl ester	16.87	$C_{17}H_{34}O_2$	270.457	Antioxidant
13	5- Cyclohexadecen- 1- one	16.00	$C_{16}H_{28}O$	236.399	Cosmetics
14.	9- oximino- 2, 7- diethoxyfluorene	19.45	$C_{17}H_{17}NO_3$	283.322	Anti- pathogenic activity

C. *IN VITRO* ANTI – ARTHRITIS ACTIVITY

The plant extract fractions used for this activity are; ethanol, ethyl acetate and chloroform extract of leaf of *Nyctanthes arbor-tristis Linn.*

These extracts showed positive response compared to standard diclofenac sodium for its anti-arthritic properties using *in-vitro* inhibition of protein denaturation model. The activity was showed in a concentration dependent manner, (i.e.) 200, 400, 600, 800, 1000µg/ml. Hence the maximum activity was reported at the highest concentration taken for evaluation. (Table3).

Table 3: Percentage inhibition of protein denaturation

Concentration (µg/ml)	PERCENTAGE (%) INHIBITION OF PROTEIN DENATURATION			
	Diclofenac Sodium	Ethanol Extract	Ethyl Acetate Extract	Chloroform Extract
200	68.57± 0.03	48.75±0.04	40.23±0.02	19.86±0.08
400	70.68 ± 0.02	53.62±0.04	50.45±0.01	28.43±0.3
600	76.32±0.05	60.76±0.04	57.87±0.01	35.75±0.09
800	82.98±0.02	67.54±0.03	66.32±0.03	46.34±0.04
1000	88.87±0.07	75.35±0.07	71.35±0.03	52.7±0.04

Values are expressed as Mean ± S.E.M.

Ethanol extract showed the highest percentage inhibition of 75.35%, ethyl acetate followed by 71.35% and chloroform showed by 52.7% all at a concentration of 1000µg/ml.

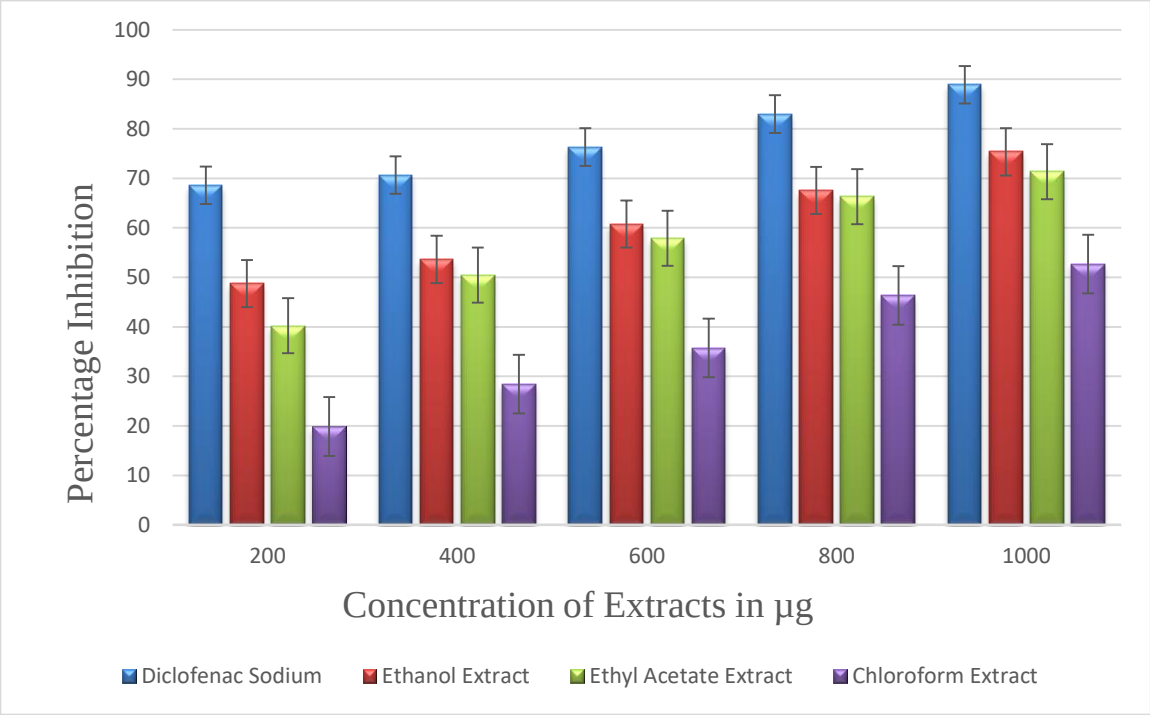

Figure 2: Graph comparing the percentage inhibition of various extracts

IV. DISCUSSION

Flavonoids have been demonstrated to have antibacterial, anti-inflammatory, anti-allergic, anti-viral activity (Alan and Miller, 1996). Alkaloids with pharmacological effects are used as medication and recreational drugs (Roger and Wink, 1998). Saponins have been discovered to show hypercholesterolemia and tumor inhibiting activity in experimental animals (John, 1996). Tannins and phenols, which together constitute the polyphenolic group, are known to have antioxidant, anticancer, anti-inflammatory and antimicrobial activities. As suggested by Mehta *et al.,* 2012, terpenoids in hydroalcoholic extract, as well as in ethyl acetate fraction has protective activity against arthritis.

GC –MS analyses are one of the initial steps aimed to determine the nature of active compounds in medicinal plants. Previous studies by B. Ramachandran et.al (2014) showed that five compounds have been distinguished from the ethanol extract of the whole plant of *Nyctanthes arbor tritis* by gas chromatography-mass spectrometry analysis. The five compounds were Ar-tumerone, Curlone, Dibutylphthalate, Hexadelanoic acid –Ethyl ester, 1, 2-Benzene dicarboxylic acid-Diisooctyl ester and are experimented to have anti-inflammatory activity alongside antimicrobial, anticancer, insecticidal, antifouling and antioxidant activities. The present study revealed a number of active compounds through GC-MS analyses of ethyl acetate extract of *Nyctanthes arbor tritis* leaf some having anti – arthritis/anti – inflammatory activity which is of interest in this studies (Table 2)

Tissue proteins denaturation is one of the well-known causes of arthritic diseases. In certain arthritic diseases auto antigens may be produced due to denaturation of proteins (Umapathy *et al.,* 2010, Volluri, 2011). Alteration of hydrogen, hydrophobic, disulphide and electrostatic bonds in proteins is the possible mechanism underlying denaturation (Volluri, 2011). The agents preventing protein denaturation would be beneficial for the development of anti-arthritic drugs.

Also, in the previous in-vivo study, it was reported that the irregular expression of tumor necrosis factor-α (TNF-α) in experimental animals has been shown to cause destructive arthritis. The development of arthritis was markedly suppressed in interleukin-β (IL-1β) deficient collagen-induced arthritis (CIA). Interleukin-6 (IL-6) gene disrupted mice were resistant to antigen and collagen-induced arthritis. These studies indicated that pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) play a role in arthritis and are potential targets for therapy (Rathore, 2007).

Adjuvant induced arthritic model was used to test the efficacy of seed, leaves and fruit ethanolic extract of *Nyctanthes arbour tristis*. The results showed that the mice receiving two doses of FCA (Freud's complete adjuvant): one on 0th day and other on 12th day, treated with extracts of leaves and fruits reduced TNF α, IL-1, IL-6 from 14th day. Also, a shift in balance was noted between pro-inflammatory and anti-inflammatory cytokines in adjuvant-induced mice thus favouring inflammation. Therefore, the extract of leaves and fruit was found to possess anti-arthritic properties (Rathore, 2007).

Hence, in this study, the *in vitro* anti-arthritic activity of ethyl acetate, ethanol and chloroform leaf extracts of *Nyctanthes arbour tristis* evaluated in terms of inhibition of protein denaturation method showed that the plant extracts exhibits significant level of anti-arthritic activity.

The major constituents of *Nyctanthes arbor-tristis Linn.* were found to be polyphenolic compounds which are well known to possess several biological properties. In the present study, the in vitro anti-arthritic activity of the plant can be attributed to its constituents like phenols, tannins, flavonoids and terpenoids and to bioactive compounds such as Flavone, Isopropyl stearate, Coumarine 3 – (2, 4 – dinitrophenyl), 6- [Diethylamino] benzofuran – 3[2H] – one and Strychnidin-10-one, 2, 3- dimethoxy 19-oxide.

V. CONCLUSION

Research has indicated that people suffering from chronic pain, as in rheumatoid arthritis, and those dissatisfied with current treatment are very likely to seek alternative treatments, and an estimated 60–90% of persons with arthritis use complementary and alternative medicine. Due to the growing interest in herbal therapies among patients with rheumatoid arthritis, there is a need for investigation into their safety and efficacy.

Hence, herbal medicines have gained ground and popularity for the treatment of rheumatoid arthritis worldwide recently.

One of the herbal medicinal plants that interact with the mediators of inflammation used in the treatmentof rheumatoid arthritis is *Nyctanthes arbor-tristis Linn*

The presence of phytochemical constituents in this plant has been reported to have proven medicinal implications which have potential therapeutic roles in amelioration of rheumatoid arthrithis symptoms and even possibly rheumatoid arthritis itself. For further studies, the determination of the molecular mechanisms behind the action of these phytochemicals can be looked into, this will not only lead to discovery of new drugs for symptomatic relief of rheumatoid arthritis conditions like inflammation and pain, but also may make it possible to stop further progress or even reverse the damage caused by rheumatoid arthritis.

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Figures

Figure 1: GC-MS total ion chromatogram
Figure 2: Graph comparing the percentage inhibition of various extracts

Tables

Table 1: Showing Result of Phytochemical Analysis
Table 2: Bioactive Constitutes from GC – MS Analysis of Ethyl acetate extract of *Nyctanthes arbor tristis linn* leaf
Table 3: Percentage inhibition of protein denaturation