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PHYTOCHEMISTRY, ANTI-CANCER AND ANTI-INFLAMMATORY ACTIVITIES OF SOLVENT LEAF EXTRACTS OF *NYCTANTHES ARBOR - TRISTIS*

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Keywords:

N. arbour - tristis, Pharmacological activity, Phytochemical screening, Anti-cancer, anti-inflammatory

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ABSTRACT: Ayurveda is one of the oldest systems of medicine that uses plants and their extracts for treatment and management of various diseased states. *Nyctanthes arbour - tristis* Linn. (Family Oleaceae), which has long been used traditionally for various medicinal purposes in the Indian sub continent. Each part of the plant has some important medicinal value and is thus commercially exploitable. It is now considered as a valuable source of several unique products for the medicines against various diseases and also for the development of some industrial products. The present study is to focus on the potential phyto-chemicals and pharmacological activity of plant *N. arbor - tristis*. In view of this, the study was designed to investigate for chemical constituents, anti-cancer and anti-inflammatory activities of solvent leaf extracts. Phytochemical screening revealed the presence of alkaloid, steroids etc. The extract exhibited significant anti-cancer and anti-inflammatory properties and the observed biological activities in this study provide scientific validation of ethnomedicinal use of this plant.

INTRODUCTION: The Greek word for a plant is 'Phyto' and chemicals produced by plants are called phytochemicals/fight-o-chemicals. In plant flora, 25,000 phytochemicals were known to exist and of them 10,000 are estimated to be alkaloids and 4,000 flavonoids distributed all around [1]. The medicinal value of plants lies in chemical substances that possess physiological action. Higher plants have unique and rich collection of various biochemicals, so they are generally known as storehouses of chemo-therapeutants. As they possess lesser side effects, they can be used to develop natural drugs.

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Secondary metabolites are chemically and taxonomically diverse group of compounds with obscure functions extensively used in research, agriculture, human therapy and so on [2]. They are produced from primary metabolites such as amino acids, carbohydrates and proteins with a series of metabolic reactions. The important phytochemicals in medicinal plants utilized both by humans and by animals are alkaloids, flavonoids, glycosides, tannins, phenols, steroids, resins, saponins and so on [3]. The disease preventing nature is due to the presence of antioxidants, such as flavonoids, hydrolysable tannins, phenolic acids and so on. This reacts against free radicals responsible for cancer, coronary heart diseases, mutagenic and inflammation reactions [4].

Nyctanthes arbor-tristis (Night-Flowering Jasmine) is an erect shrub or a small tree of the family of Oleaceae growing upto 10m **Fig.1**. *Nyctanthes arbor-tristis* Linn. is a widely spread plant from

Northern Pakistan, Nepal, South and North India and Thailand. *N. arbor-tristis* is normally planted by cuttings or generates from seeds. The plant sheds its leaves during the period March to May and it puts up flowers from September to November. It usually grows in red as well in black soils, where climatic conditions of arid and semi-arid are prevalent.

FIG. 1: FRESH TWIG OF *NYCTANTHES ARBOR - TRISTIS*

N. arbor-tristis is a beautiful and fragrant plant. Its flowers bloom at night, drop off and fall early next morning for this reason it is called as "sad tree". It is mainly characterized by the presence of phenylethanoid derivatives and iridoid derivatives [5]. It is used in traditional medicine as stomachic, carminative, intestinal astringent, expectorant, in biliousness, piles, and various skin diseases and as hair tonic [6]. It has also been reported to possess hepato-protective, anti-viral, antifungal [7] and analgesic, anti-pyretic, ulcerogenic activities [8].

Bansal *et al.,* (2015) [9] reported that the bioactive compounds of *Nyctanthes arbor-tristis* are very useful to provoke menstruation, treatment of scabies and other skin infections, as hair tonic, chalogogue, laxative, diaphoretic, diuretic, treatment of arthritis, malaria, bronchititis and anti-helminthic.

In view of these reports, the present study was undertaken to investigate the phytochemistry and, anti-cancer, anti-inflammatory activities of solvent leaf extracts of *Nyctanthes arbor-tristis* for its diverse pharmacological applications in ancient and modern system of medicine.

MATERIAL AND METHODS:

Plant Material Collection and Authentication:

Fresh plant leaves of *Nyctanthes arbour - tristis* were collected from Warangal, Telangana state, India and were identified and authenticated by Botanical Survey of India, Coimbatore, Voucher Reference No. BSI/SRC/5/23/2011-12/Tech. 1443.

Organic Extraction: The fresh leaves of *Nyctanthes arbor-tristis* were shade-dried for 10 days at 37 ^{0}C, and then finely powdered. The powder was exhaustively extracted with solvents in 1:5 ratio using soxhlet apparatus for 6-8 hours and then centrifuged. From the centrifuged extract the supernatant was filtered over Whatman No. 1 filter paper. The filtered extract was then subjected to dryness under reduced pressure at 37 ^{0}C (not exceeding 40 ^{o}C), until usage DMSO was added and stored at -80 ^{0}C. Extracts diluted with culture media were sterilized by 0.22 µm membrane filters prior to cancer cell lines treatment. In culture medium, less than 0.2% DMSO final concentration was used. All the extracts were stored in a dessicator for further evaluation. The crude extracts were analysed for their phytochemical, thin layer chromatography and pharmacological activities.

The leaf extracts were used for phytochemical analysis as per the following standard procedures for the identification of various classes of active plant constituents [10, 11].

Medium: 10% MEM possessing L-glutamine (4 mmol/L), 10% fetal bovine serum (FBS) and antibiotics of 100 µg /mL streptomycin and 100 units/mL of penicillin must be added and incubated in a humidified atmosphere containing 5% CO_2 with 95 % air at 37 °C.

Cell Lines: MDA-MB 231 (Breast Cancer Cell Lines free of pathogens) and A 549 (Lung Cancer Cell Lines) purchased from ATCC.

Anticancer activity: A colored formazan product is formed by the yellow coloured 3-(4,5-dimethythiazol-2-yl) - 2, 5 - diphenyl tetrazolium bromide (MTT) reduction, which were taken up by the cells with the detection of the readings using spectrophotometry (λ_{max} = 562 nm). Based on the mitochondrial respiratory function, the MTT is reduced, by measuring the number of viable cells in the culture.

Cancer cells were treated with various concentrations (0, 10, 15, 20 and 30 µg) of the compound for 48h, washed with Dulbecco's

phosphate buffered saline (DPBS), the media is aspirated at the end of the treatment and incubated with 20µl of 5 mg/ml MTT solution in 500 µl of culture medium for 1 h at 37 ^{o}C. In order to solubilize the cells, 500 µl of formazan DMSO was used. By using automated TECAN multimode reader the absorbance was measured at 562 nm. The experiment is conducted in duplicates and triplicates by comparing the test results with the control in which the drug is omitted [12].

Anti-inflammatory Activity:

Experimental Animals: Using standard laboratory conditions (12: 12 h light/dark cycle at 24 ^{o}C) the experiment was conducted on Male albino wistar rats of 130 to 150 g in weight. The rats were kept in polypropylene cages. Water *ad libitum* and commercial rat diet (NIN, Hyderabad) were provided to the rats. Approval has been given by Institutional Animal Ethical Committee of IICT to conduct experiments. The animals are quarantined first, and then acclimatized to laboratory conditions for a week. During this period of testing, the animals were observed for general health and suitability.

1. Compound Dose – 250mg/kg
2. STD (Indomethacin) -10 mg/kg

Carrageenan Induced Rat Paw Edema Model: By employing a slight modification in the method described by Winter *et al.,* (1962) [13] the evaluation of test compounds in wistar rats was screened for anti-inflammatory activity. The overnight fasted animals were divided into three groups–standard, control and different test groups. A dosage of 10 mg/Kg of Indomethacin was administered orally to the standard group animals. Before this, a dosage of 250 mg/Kg/day of test compound for 10 days was administered by oral route. A suspension was made with gum acacia and weighed test compound in required quantity. Every day, at a specific time interval, the prepared suspension was orally administered to all the rats throughout the study. The control group of rats received the vehicle solution without test compound. On termination day, the test drugs are administered to the entire group of rats. An hour later they are challenged with 0.1ml of 1% Carrageenan in the sub plantar region of right hind paw. After this, the volumes of the rats' paws were measured before and after an hour each for 3h using digital plesthysmometer (Ugo Basile, Italy). The mean paw volume of control group was compared with treated groups for calculating the percent inhibition of paw volume.

$$\% \text{ Inhibition} = Vc - Vt / Vc$$

(Vc: Control Mean paw volume and Vt: Test paw volume)

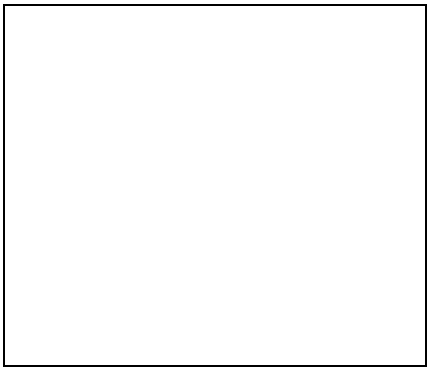
RESULTS: The phytochemical analysis of the dried leaves of *N. arbor-tristis* revealed that the alkaloids were present in the solvent leaf extracts of hexane, chloroform, petroleum ether and ethyl acetate (**Table 1)**. Flavoinods were only traced in the extract of petroleum ether, while steroids were identified only in methanol. Saponins were observed in the solvent extracts of chloroform and ethyl acetate. Glycosides, tannins, phenols, resins were completely absent in all the solvent extracts of *N. arbor-tristis.*

The TLC analysis clearly indicated that the solvent extracts of dried leaves of hexane was with brown, blue, purple and yellow colours having R_f values ranging from 0.20 to 0.96, while chloroform extracts were with green colour and R_f values were 0.50 and 0.53 (**Table 2)**. The dried leaf extract with petroleum ether of *N. arbor-tristis* was with green and violet colours and R_f range was 0.18 to 0.74. The ethyl acetate extracts were with green colour and R_f range was 0.11 to 0.53. The methnol extracts of dried leaves of *N. arbor-tristis* showed green and ash colours with R_f values between 0.21 to 0.75. The chemical elucidation of the methonolic leaf extract of *Nyctanthes arbor-tristis* was carried out and presented. There are several peaks observed in chromatograms for the presence of various compounds between Rt 2.474 to 15.294 min **Fig. 2**.

A peak observed at Rt 2.781 min corresponded to be (1S,4aS,5R,6S,7S,7aS)-ethyl1,5,6-trihydroxy-7-(propionyloxy)- 1, 4a, 5, 6, 7, 7a- hexahydrocyclo - penta[c] pyran-4-carboxylate, characterized as glycoside in NAT leaves and a second peak observed at Rt 2.530 min corresponded to be Methylenenona-2,5-dien-4-yl) - 4, 4, 10, 13, 14-pentamethyl-2,3,4,7,8,9,10, 11,12,13,14,15,16,17 – tetradecahydro - 1H cyclo penta [a] phenanthren-3-ol, characterized as steroid in leaves of methanolic extract.

The HPLC and mass spectrometry of leaf extract I revealed glycoside isolated structure had the molecular weight of 317 which corresponds to a molecular formula, $C_{14}H_{20}O_8$ as represented in **Fig. 3**. Mol. wt = 317 ($C_{14}H_{20}O_8$), (M+= 317, 100%)

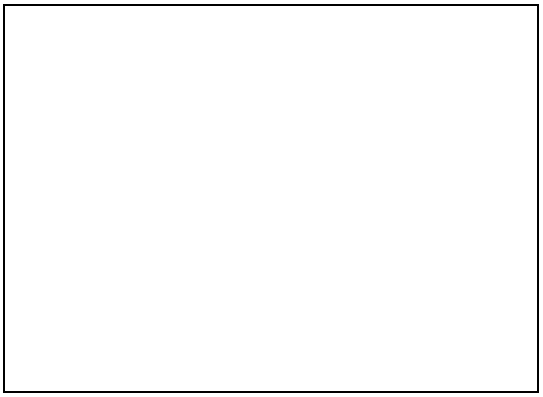
Predicted structures: (1S, 4aS, 5R, 6S, 7S, 7aS)-ethyl1,5,6-trihydroxy-7-(propionyloxy)-1,4a, 5,6, 7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate. Mol. Wt: (M+H) = 317 ($C_{14}H_{20}O_8$)



GLYCOSIDE

The HPLC and mass spectrometry of leaf extract II revealed steroid isolated structure had the molecular weight of 477 which corresponds to a molecular formula, $C_{34}H_{54}O_1$ as represented in **Fig. 4.** Mol. wt = 477 ($C_{34}H_{54}O_1$), (M-= 477, 100%)

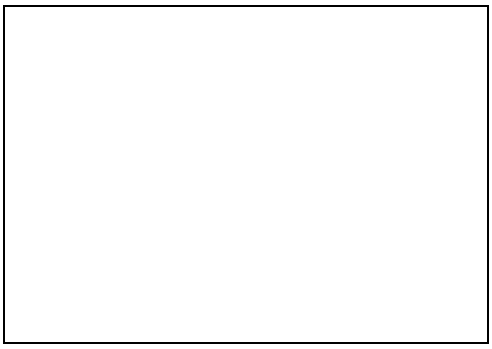
Predicted structures: Methylenenona-2,5-dien-4-yl)-4,4,10,13,14-pentamethy 2,3,4,7,8, 9, 10, 11, 12, 13,14,15,16,17-tetradecahydro- 1H - cyclopenta [a] phenanthren-3-ol. Mol. wt: (M-H) = 477 ($C_{34}H_{54}O_1$)



STEROID

The HPLC and mass spectrometry revealed phenol isolated structure had the molecular weight of 331 which corresponds to a molecular formula, $C_{21}H_{14}O_4$. Mol. wt = 331 ($C_{21}H_{14}O_4$), (M+= 331, 100%).

Predicted structures: 8-(5,7-dihydroxy-3-vinyl naphthalen-1-yl) – 2 H - chromen-2-one Mol. wt: (M+H) = 331 ($C_{21}H_{14}O_4$).



PHENOL

The HPLC and mass spectrometry revealed glycoside isolated structures had the molecular weights of 552.52 (2 compounds), 566.55, 568.52 and 536.53 (2 compounds) which corresponds to the molecular formulas, $C_{26}H_{32}O_{13}$, $C_{27}H_{34}O_{13}$, $C_{26}H_{32}O_{14}$, $C_{26}H_{32}O_{12}$, as represented in **Fig. 5**.

The TLC analysis clearly indicated that the solvent extracts of dried leaves of hexane was with brown, blue, purple and yellow colours having R_f values ranging from 0.20 to 0.96, while chloroform extracts were with green colour and R_f values were 0.50 and 0.53. The dried leaf extract with petroleum ether of *N. arbor-tristis* was with green and violet colours and R_f range was 0.18 to 0.74. The ethyl acetate extracts were with green colour and R_f range was 0.11 to 0.53. The methnol extracts of dried leaves of *N. arbor-tristis* showed green and ash colours with R_f values between 0.21 to 0.75.

The methonolic leaf extract of *Nyctanthes arbor-tristis* was further investigated for its anti-cancer, anti-inflammatory activities.

The effect of *N. arbor-tristis* leaves extracted in methanol were evaluated using MTT assay on human cancer cell lines. The growth inhibitory potential of *N. arbor-tristis* extracts was screened against two cancer cell lines which are of epithelial origins.

The cell lines such as lung (A549) and breast (MDA MB-231) represents the major organ sites. Hence, they were selected for testing the effect of *N. arbor-tristis* extracts on their proliferation.

A solvent control (methanol) in different concentrations (10µl, 20µl and 30µl) were tested against MDA-MB 231 human breast cancer cell lines and checked for cell viability percentage. The percentage of cell viability decreased with increased concentration of methanol solvent (**Table 3)**. The 10ug of methanol solvent concentration showed 83.1% and 20 ug showed 79.0% of cell viability while 30 ug caused for 71.8% cell viability. Similarly the decrease in cell viability was also examined with cell line A549 (**Table 4)** and noticed that the 10 ug was responsible for 91.8%, 20 ug was for 74.1% while 30 ug was showed 68% of decrese in cell viability. DMSO was used as another control showed no activity against cell lines. The cancer cell lines sensitivity was determined by using a positive control, etoposide. The potency of *N. arbor-tristis* extracts was compared with the conventional anti-cancer drugs by using this anti-proliferative assay. The control used to screen the cancer cell lines susceptibitility is etoposide and observed that all the cell lines were susceptible to standard drug treatment. A comparison made between anti-proliferative activity of *N. arbor-tristis* extracts and standard anti-cancer drug, showed that the growth arresting activity of *N. arbor-tristis* was higher than that of etoposide, and thus displayed its efficacy as a cancer preventing agent.

The anti-inflammatory activity of methonolic extract of *Nyctanthes arbor-tristis* of the carrageenan induced wistar rats in terms of their body weight index with control and NAT methanol leaf extract were compared on the 1st and the 11th day and found no significant change. On the 1st day the control is 154.80±2.63 and on the termination day159.95±2.42 whereas the extract is 156.68±2.94 on the 1st day and 158.82±0.03 on the termination day (**Table 5)**.

TABLE 1: PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACTS OF *NYCTANTHES ARBOR - TRISTIS*

Phytochemicals	Hexane	Chloroform	Petroleum Ether	Ethyl Acetate	Methanol
Alkaloids	+	+	+	+	-
Flavonoids	-	-	+	-	-
Glycosides	-	-	-	-	-
Tannins	-	-	-	-	-
Phenols	-	-	-	-	-
Steroids	-	-	-	-	+
Test for Resins	-	-	-	-	-
Test for Saponins	-	+	-	+	-

TABLE 2: THIN LAYER CHROMATOGRAPHY OF SOLVENT EXTRACTS OF LEAF OF *N. ARBOR-TRISTIS*

Name of the Extract	Colour	Rf Value
Hexane	Brown	0.20
	Brown	0.25
	Blue	0.27
	Purple	0.35
	Purple	0.92
	Yellow	0.96
Chloroform	Green	0.50
	Green	0.53
Petroleum Ether	Green	0.18
	Green	0.22
	Violet	0.74
Ethyl Acetate	Green	0.11
	Green	0.48
	Green	0.53
Methanol	Green	0.21
	Green	0.33
	Green	0.45
	Ash	0.75

TABLE 3: EFFECT OF DIFFERENT CONCENTRATIONS OF METHANOL EXTRACTS OF *N. ARBOR-TRISTIS* AGAINST MDA MB-231 HUMAN BREAST CANCER CELL LINES

Crude Plant Extract		R1	R2	Average	% of Cell	STDEV Viability
	Control	**1.0843**	**1.0317**	**1.058**	**100**	**0.010**
Dried Leaf Methanol	10µg	0.89726	0.86281	0.880035	83.17911	2.302441
	20µg	0.83467	0.83727	0.83597	79.01418	0.173769
	30µg	0.75854	0.76174	0.76014	71.84688	0.21387
Dried Stem Methanol	10µg	0.92846	0.89151	0.909985	86.00992	2.469527
	20µg	0.93741	0.88177	0.90959	85.97259	3.71866
	30µg	0.85832	0.87703	0.867675	82.01087	1.25047
Dried Fruit Methanol	5µg	0.61068	0.59328	0.60198	56.89792	1.162917
	10µg	0.51037	0.51991	0.51514	48.68998	0.637599
	15µg	0.4958	0.47847	0.487135	46.04301	1.158238

[a] All data were average (± SD) of three replicates.

TABLE 4: ANTICANCER ACTIVITY OF *N. ARBOR-TRISTIS* AGAINST A-549 HUMAN LUNG CANCER CELL LINES

Plant Extract		R1	R2	Average	% of Cell	STDEV Viability
Control		0.4165	0.4016	0.40905	100	0
	10µg	0.3802	0.3712	0.3757	91.84696	1.55579
Methanol	20µg	0.3097	0.2969	0.3033	74.14741	2.21268
	30µg	0.2843	0.2727	0.2785	68.08459	2.005241

TABLE 5: ANTI-INFLAMMATORY ACTIVITY OF METHANOLIC EXTRACT OF *NYCTANTHES ARBOR-TRISTIS* (BODY WEIGHT INDEX)

Compound Code	1st Day	11th Day
Control	154.80±2.63	159.95 ±2.42
Extract	156.68±2.94	158.82±0.03

Values are mentioned as Mean ± S.E.M (n=6).

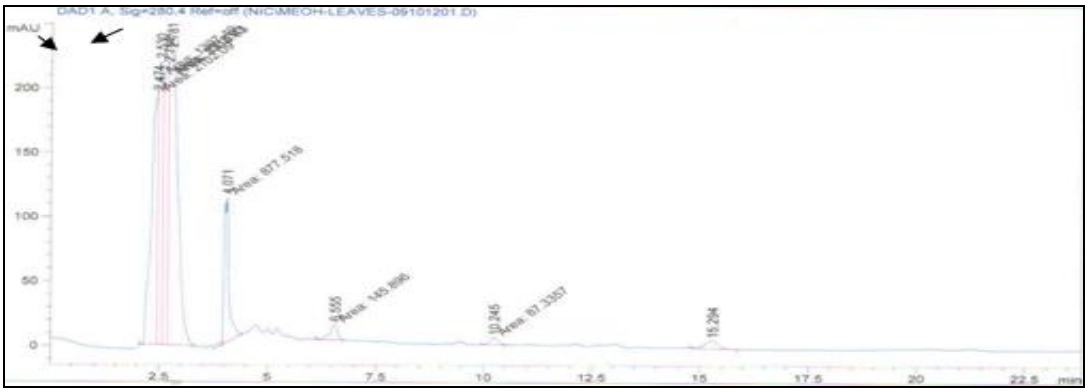


FIG. 2: HPLC CHROMATOGRAM OF METHANOL FRACTION OF *N. ARBOR - TRISTIS* LEAF EXTRACT

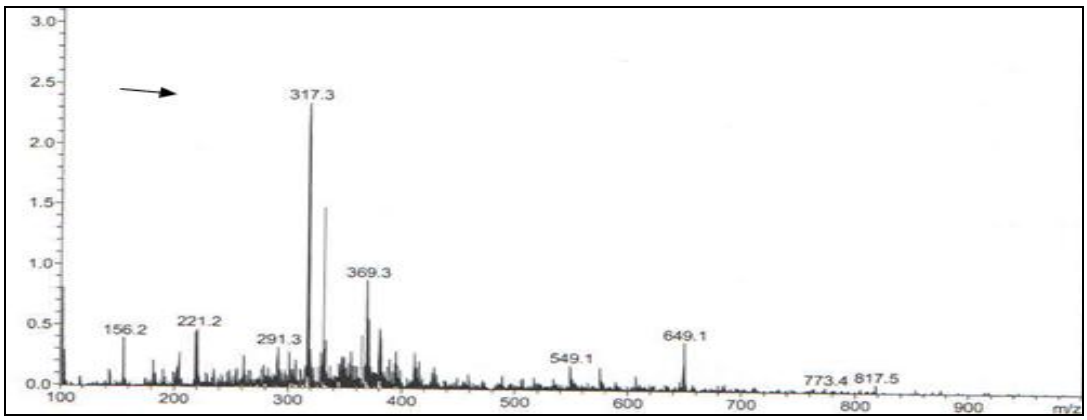


FIG. 3: MASS SPECTRUM OF METHANOLIC LEAF EXTRACT I OF *NYCTANTHES ARBOR - TRISTIS*

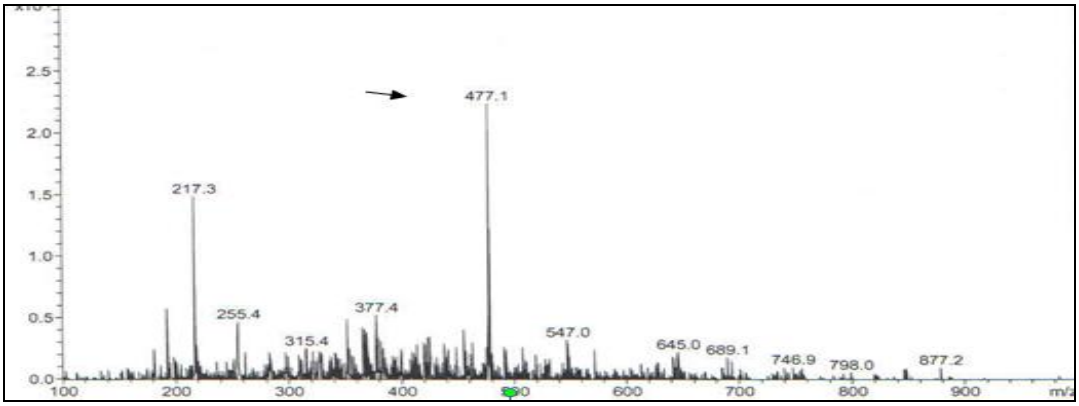
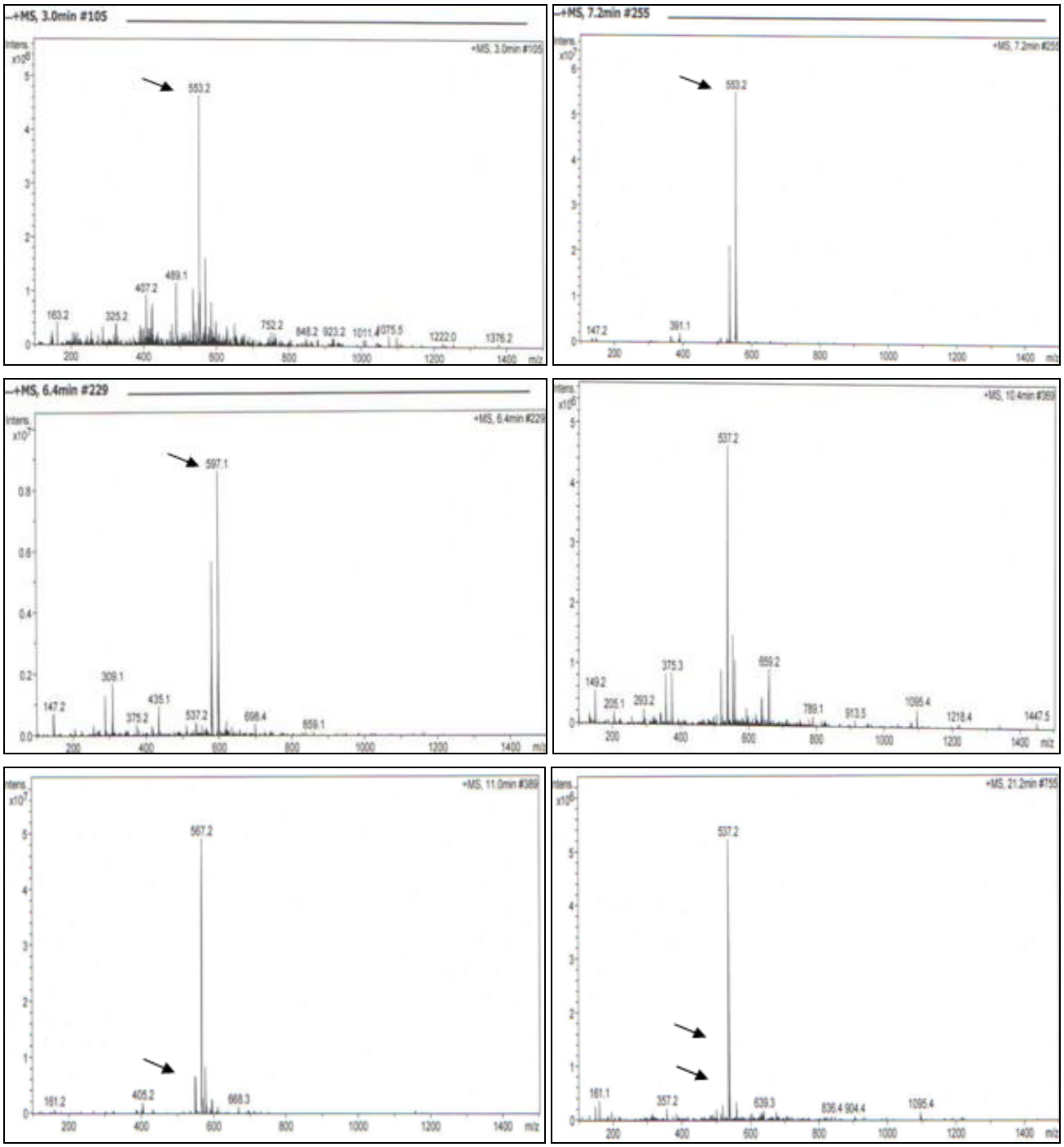


FIG. 4: MASS SPECTRUM OF METHANOLIC LEAF EXTRACT II OF *NYCTANTHES ARBOR - TRISTIS*



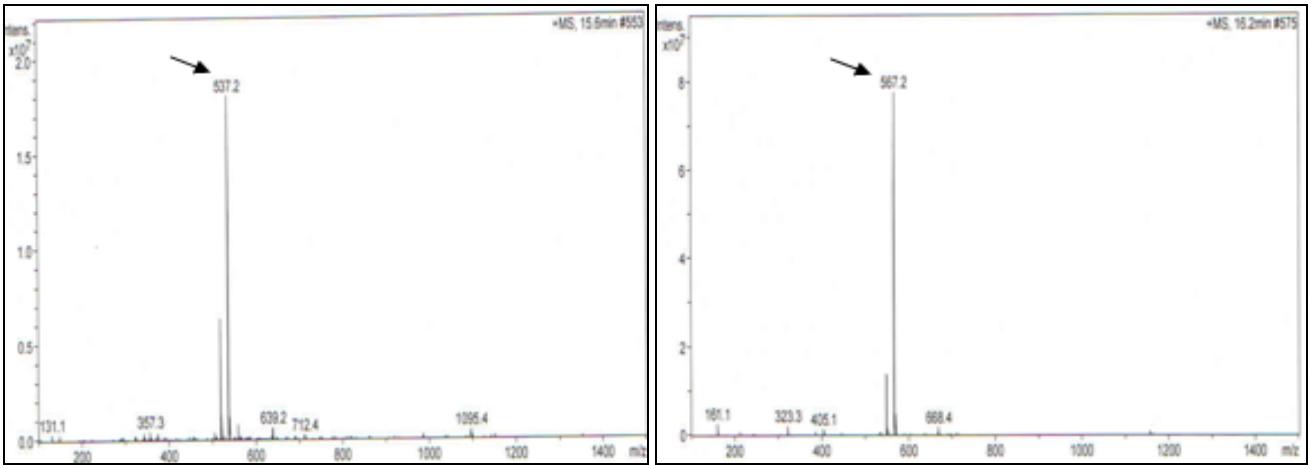


FIG. 5: MASS SPECTRUMS OF METHONOLIC LEAF EXTRACT OF *NYCTANTHES ARBOR-TRISTIS*

DISCUSSION: Number of reports are available on the phytochemistry and other pharmacological assessment of leaf extract of *Nyctanthes arbor-tristis* [14-22]. Saxena *et al.,* (1984) [14] screened the water soluble portion of the alcoholic extract of the leaves of *Nyctanthes arbor-tristis* for its anti-inflammatory activity. The extract inhibited the acute inflammatory oedema produced by different phlogistic agent *viz.* carrageenan, formalin, histamine, 5-hydroxytryptamine and hyaluronidase in the hind paw of rates. Acute and chronic phases of formaldehyde induced arthritis were significantly inhibited.

Puri *et al*., (1994) [23] reported the hepato-protective, anti-leishmania, anti-viral, anti-fungal activities of ethanolic extracts of flowers and leaf of *Nyctanthes arbor-tristis.* A strong stimulation of antigen specific and non-specific immunity and increase in humoral and delayed type of hyper sensitivity was demonstrated in mice fed with 50% ethanolic extracts. Singh *et al*., (1995) [24] examined the leaves of *Nyctanthes arbor-tristis* led to isolation and identification of a new minor iridoid glucoside, arborside - D as its acetyl derivative. They concluded that the structure of arborside D as 10-benzoylnyctanthoside. Similar to the present observations, Khatune *et al.,* (2001) [25] recorded cytotoxic activity with chloroform and ethyl acetate flower and leaf extacts of *Nyctanthes arbor-tristis.* The fractionation of an ethanol extract from the leaf and flowers of *Nyctanthes arbor-tristis* to the isolation of an antiplasmodial cyclohexylethanoid; a new iridoid glucoside, 6-0-trans cinnamoyl, 7-0-acetyl 6-b-hydroxyloganin and three known iridoid glucosides were elucidated and reported [15].

Singh *et al.,* (2010) [26] studied the immune-pharmocological properties of ethanolic extracts of *Nyctanthes arbor-tristis* and after administration of ethonolic leaf extracts in doses of 0.25 and 0.5 g/kg body weight, a significant increase in phagocytic index, leucocyte count and spleenic antibody cells were noticed. They further, identified through HPLC the presence of methoxylated flavonoid quercetin - 3, 3’ dimethoxy - 7 – O -rhamnogluco pyranose. The phytochemical investigations of solvent extracts of leaf of *Nyctanthes arbor-tristis* revealed the presence of alkaloids, carbohydrates, glycosides, phytosterols, fixed oil, tannins, flavonoids, proteins and amino acids, gums and mucilages in varied quantities (Suresh and Arunachalam, 2012) [27]. Rani *et al*., (2012) [28] recorded *Nyctanthes arbor-tristis* as one of the most useful traditional medicinal plant in India and reported its distribution widely in sub-himalayan regionsand southwards to Godavari. Each part of the plant has some medicinal value and is thus commercially exploitable. Further, they reported on the comprehensive information on chemical constituents, biological activities of important compounds, pharmacological actions, medical applications and micro propagations.

Agarwal and Pal (2013) [29] noticed that the crude extracts and isolated compounds from *Nyctanthes arbor-tristis* were shown to be pharmacologically active against inflammation, malaria, viral infection, leishmaniasis and immuo-stimulant. They isolated arbortristoside A, B and C and other molecules such as, calceolarioside A, 4-hydroxy-hexahydrobenzofuran-7one and beta-sitosterol and demonstrated their active role as anti-leishmanial, anti-cancer and anti-inflammatory activities.

Recently, Santhosh and Patil (2016) [22] presented a review on *Nyctanthes arbour - tristis* and recorded phytochemicals like flavanol glucoside, oleanic acid, essential oils, tannic acid, carotene, friedeline, lupeol, glucose, benzoic acid for their significant hepato-protective, anti-leshmaniasis, anti-viral, anti-fungal, anti-pyritic, anti-histaminic, anti-malarial, anti-bacterial, anti-inflammatory, anti-oxidant activities.

Thus, the present study has shown the anti-cancer and anti-inflammatory activities of leaf of *Nyctanthes arbor-tristis* and suggested its therapeutic usefulness. The plant is rich resource of biologically active compounds which would attract the attention of drug discovery groups to discover novel bioactive molecules for safer and effective treatment of various diseases.

CONCLUSION: *Nyctanthes arbour-tristis* widely used in traditional system of medicine for varied ailments are supported by various studies involving its pharmacological evalutions. The above article documented and revealed phytochemicals study and pharmacological activities of *Nyctanthes arbor -tristis* and proved as a unique source of metabolites such as alkaloids, phytosterols, phenolics, tannins, flavonoids, glycosides and saponins which is obtained from crude extract of plant and showed effective anti-cancer and anti-inflammatory.

CONFLICT OF INTEREST: The authors declare that there is no conflict of interest regarding the publication of this paper.

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