RESEARCH ARTICLE

Cardioprotective Activity of *Nyctanthes arbor-tristis* Linn Extract Loaded Nanoparticles

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ABSTRACT

The study targets to develop and evaluate nanoparticles loaded with *Nyctanthes arbor-tristis* Linn stem extracts for its cardioprotective activity. The *Nyctanthes-arbor-tristis* Linn stem was extracted by continuous hot percolation method using the soxhlet apparatus and subjected to phytochemical evaluation. Nanoparticles was developed by precipitation technique with zinc nitrate and sodium hydroxide and characterized by laser light transmission test, scanning electron microscopy and dynamic light scattering. The safety profile was performed as per OECD 423 guidelines. The cardioprotective activity of novel nano-formulation has been investigated by *in-vivo* doxorubicin induced cardiotoxicity in rats. The percentage yield of *Nyctanthes-arbor-tristis* Linn stem extract was found to be 6.2% w/w and revealed the presence of various primary and secondary metabolites. The particle size of nanoparticles was in the range of 58.59 - 79.58nm with zeta potential value -37.38 and *In-vitro* drug release was 99.14%. Acute toxicity study didn't show any toxicity and mortality. Hence 200mg/kg and 400mg/kg where selected for further studies. Nano formulation showed significant reduction in the cardiac biomarkers in the doxorubicin induced cardiotoxicity. ECG showed normal cardiac rhythm and histopathological study shown a normal architecture of the cardiac tissue in nanoparticle loaded *Nyctanthes arbor tristis* Linn extract treated animals. Based on the results obtained, the nanoparticle loaded *Nyctanthes arbor tristis* Linn stem extract found to have significant cardioprotective activity.

Keywords: *Nyctanthes arbor tristis* Linn, Nanoparticles, cardiotoxicity, cardiovascular diseases.

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INTRODUCTION

Cardiovascular system plays a very important and central part of the circulatory system which involves pumping of oxygenated blood throughout the body. Any abnormalities that occur in cardiovascular system can leads to drastic changes in the normal function of other body systems and may also lead to sudden death if not treated.[1] It has been estimated that around 17 million peoples are dying because of cardiovascular diseases every year especially in middle class or low-income countries. Peoples who are smoking with tobacco are more susceptible to coronary heart diseases and cardiovascular diseases.[2] National health survey reports showed that CVDs are responsible for approximately 3.2 million deaths every year. It is predicted to be the most common and major threat to human life by 2020.[3] The drugs available for CVDs have a low therapeutic index and more side effects.[4] However, the present drugs showing intoxication is well documented. Nanoparticles are tiny particles with a dimension less than 1-100 nm, with desired physicochemical properties which improve biological function due to their improved bioavailability by enhancing aqueous solubility. The main aim of creating nanoparticle as a drug delivery system is to regulate particle size and make them to release active agents as specific site. The unique properties of Nanoparticles such as high stability and possibility of incorporation of both hydrophilic and hydrophobic substances made them better choice of administration than other dosage forms.[5] The beneficial therapeutic potential of large number of medicinal plants made researchers to look into a safe and economical cardio protective molecule. The present world is using herbal medicines when compared to synthetic drugs because of their less toxicity and side effects. Nyctanthes arbor tristis Linn is one of the useful traditional medicinal plant also known as Parijatha belonging to Oleaceae family. It is a type of ornamental plant used for decorative purposes but it has more medicinal values. Each part of the plant extract is used for treating various ailments as home remedies and has been reported to have antioxidant, anti-pyretic, anti-malarial, hepatoprotective, immunoprotective, anti-inflammatory, diuretic, and anti-filalaries property.[6] Therefore, the current investigation was conducted to assess the cardioprotective potential of Nyctanthes arbor tristis Linn extract loaded with nanoparticulates.

MATERIALS AND METHODS:

Plant Material

Nyctanthes arbor-tristis Linn stem was collected from Mayasandra, Tumkur District Karnataka. The plant was taxonomically recognized and verified by A. N. Srigeswara, curator of the Mahatma Gandhi Botanical Garden at the UAS, GKVK (Accession no. 4608).

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Table 1: Composition of NNAT suspension

S. No	Name of ingredients	Quantity taken
1	Nanoparticles loaded NAT extract	20%
2	Sodium CMC	0.1%
3	Glycerine	1%
4	Sodium benzoate	0.01%
5	Purified water	q. s

Table 2: Phytochemical analysis of Ethanolic extract

S. No	Phytochemical constituents	Result
1.	Carbohydrates	+
2.	Glycosides	+
3.	Alkaloids	+
4.	Tannins	+
5.	Flavonoids	+
6.	Mucilage	-
7.	Phenolic compounds	+

Preparation of extract

The *Nyctanthes arbor-tristis* Linn stem was collected and shade dried. 50 grams of dried stem was ground to make a coarse powder and defatted with petroleum ether for four days to remove fatty materials then filtered through filter paper and dried at room temperature. Obtained dry powder was extracted with ethanol for 72 hours at 40 ^{0}C and concentrated extract was subjected for preliminary phytochemical screening as per standard procedure.[7]

Formulation and evaluation[8,-10]

Probe Sonication method

0.1M of Zinc nitrate was dissolved in 50ml of distilled water. 1.2grams of NAT extract was added to the above solution. After complete dissolution 0.3M of NaOH was added with the help of a syringe and stirred for 30mins using probe sonicator with an Ultrasonic horn at 40% amplitude for 2mins with a 5sec ON and 5sec OFF cycle from time t=0. Obtained NNAT extract were centrifuged at 10,000 rpm for 1hr at -4^{0}C to collect nanoparticles and dried at 40^{0}C to get dried NNAT extract by using hot air oven and characterised by Laser light transmission test (Tyndale effect), Complexometric titration method, SEM analysis, DLS, Drug loading efficiency and *In-vitro* drug release studies.[11] The composition of preparing 10ml NNAT suspension was shown in Table 1**.** The weighed quantity of NNAT extract was mixed with Sodium CMC, sweetening agent, sodium benzoate, distilled water and stirred with a magnetic stirrer for 5mins.[12]

Figure 1: *Nyctanthes arbor-tristis* Linn Tree and Stem

Figure 2: Ethanoic extract of stem

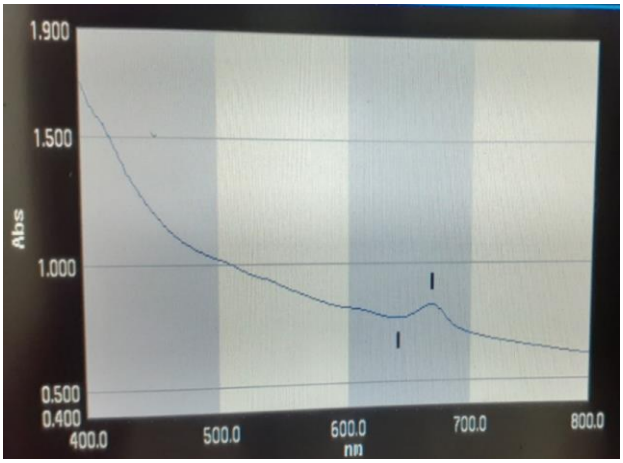


Figure 3: Standard peak of NAT extract at 668nm

Table 3: Physical state of NNAT suspension

S. No	Parameter	NNAT suspension
1	Nature	Liquid
2	Colour	Greenish Brown
3	Odor	Pleasant
4	Texture	Suspension

Table 4: Accelerated stability study of NNAT suspension

pH	Rheology	Viscosity	Sedimentation rate
7.3	1.13 ml/sec	2.81Cp	36

Accelerated stability studies

Accelerated stability studies was performed for NNAT suspension as per ICH guidelines Q1A (R2) at 40^0C/ 75RH±2. The parameters such as sedimentation volume, flow rate, viscosity, pH and rheology was determined.[13,14]

Preclinical Studies

Albino Wister rats, of either sex, weighing 150–200g, were used in the investigation. They were acquired from the Pharmacology department of M S Ramaiah University of Applied Science. Rats were kept in cages with adequate ventilation in a room that was kept between 21 and 25 degrees Celsius. During the duration of the trial, the animals were fed a rodent diet and had unlimited access to water. The Ramaiah University of Applied Sciences, Bangalore's Faculty of Pharmacy's Institutional Animal Ethics Committee (IAEC) accepted the experimental protocol (IAEC No: XXII/MSRFPH/COL/M 023).

Oral Acute Toxicity studies

Six Albino Wister Rats were used in an oral acute toxicity investigation for nanoparticle-loaded NNAT extract suspension in accordance with OECD guidelines 423. To assess acute toxicity, a 2000 mg/kg NNAT extract suspension was given orally to each rat (Limit test). Animals were housed in separate cages for a full day following medication in order to monitor for any toxic signs. Mortality was noted up to 14 days after the animals were under close observation for 48 hours.[15]

***In-vivo* cardio protective activity**

30 Healthy Albino Wister rats of either sex weighing 150-200g was selected for the study and divided in to five groups (n=6).

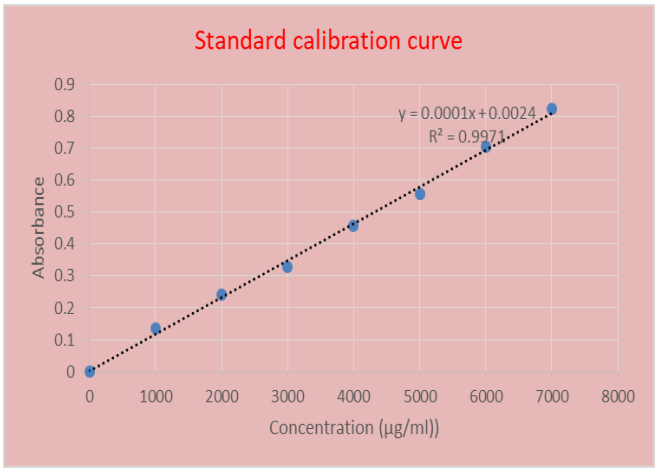
Group 1: Normal control receiving the vehicle

Group 2: Disease Group (Doxorubicin 12 mg/kg, intraperitoneally)

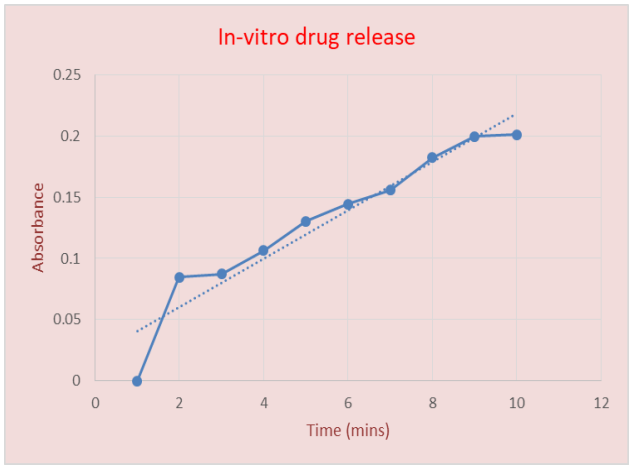
Group 3: Standard drug-treated (250mg/kg, orally) + Doxorubicin (12mg/kg, intraperitoneally)

Group 4: NNAT suspension (200mg/kg, orally) + Doxorubicin (12mg/kg, intraperitoneally)

Group 5: NNAT suspension (40mg/kg, orally) + Doxorubicin (12mg/kg, intraperitoneally)



Graph 1: Standard Callibration Curve



Graph 2: Graph representing the *In-vitro* release pattern

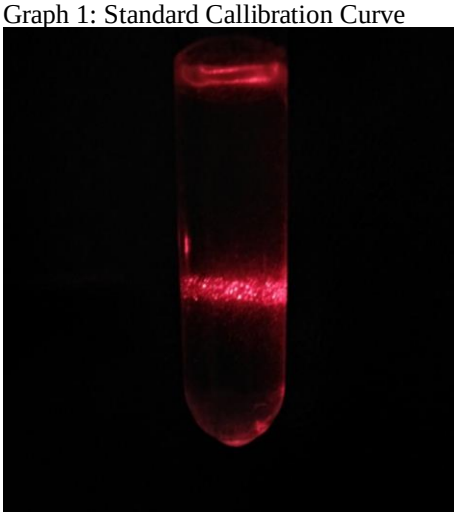


Figure 4: Laser Light Transmission

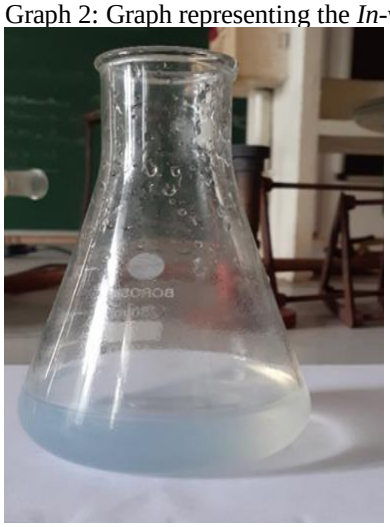


Figure 5: Complexometric titration of NNAT extract

Table 5: Effect of NNAT extract on Cardiac biomarkers

S. No	Biomarkers	Normal Control	Positive Control	Standard group	Low Dose group	High Dose group
1	CKMB	233.03 ±1.05	503.65 ±0.97	364.15 ±1.908***	425.31 ±1.96***###	342.54 ±0.78***###
2	LDH	932.39 ±8.84	1250.03 ±4.7	986.29 ±1.06***	1094.88 ±1.62***###	1050.03 ±4.7***###
3	SGPT	63.785 ±2.122	117.55 ±1.06	85.34 ±0.64***	97.19 ±0.64***#	91.09±0.68***#
4	ALP	478.36 ±3.12	778.52 ±1.65	694.78 ±3.31***	743.09 ±1.26**###	650.04 ±11.37s***###
5	Cardiac Troponin T	-	+	-	-	-

Values are expressed in Mean± SEM (n=6). *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001 compared with control group and ###p<0.001, ## p<0.01, #p<0.05 in comparison with standard. One-way ANOVA followed by Tukey-comparison test.

Table 6: QT & RR intervals in Different treated Groups

S.No	Groups	QT (msec)	RR (msec)
1	Normal Control	307.75±6.86	214.25±10.379
2	Doxorubicin	209.375±10.674	609±13.943
3	Standard	250.32±5.85***	300.154±10.25***
4	NNAT 200mg/kg	225.125±8.781***	303.375±13.840**
5	NNAT 400mg/kg	271.875±5.985*#	274.75±11.412*#

All values are expressed as Mean±SEM using one way ANOVA followed by Tukey comparison test. Here ***p<0.001, **p<0.01, *p<0.05 in comparison with positive control and ###p<0.001, ## p<0.01, #p<0.05 in comparison with standard.

The study was carried out for a period of 3 weeks and few general observations were made like mortality rate and body weight. On the 21st day, all the rats was anesthetized as per the standard procedure by using Ketamine Hydrochloride 80mg/kg and lignocaine 20mg/kg. Blood samples was collected by a retro-orbital puncture for estimation of cardiac biomarkers CK-MB, LDH, SGPT, ALP and Cardiac Troponin T. ECG was taken in anesthetized rats to analyse Q-T and R-R interval. All the rats was sacrificed by euthanasia technique to isolate heart and to perform histopathological studies to observe cardiac damage.[16-19]

Statistical analysis

All values were reported as the mean ± standard error of the mean (SEM). The statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons.

RESULTS AND DISCUSSION

Preparation of *Nyctanthes arbor tristis* Linn stem extract

The ethanolic extract *of Nyctanthes arbor-tristis* Linn stem was prepared by continuous hot percolation method with 70% ethanol and percentage yield was found to be 6.2% w/w (Figure 2). The extract was found to contain flavonoids, cardiac glycosides, alkaloids, Carbohydrates, tannins and phenolic compounds as shown in Table 2.

Development and characterization NNAT

Nanoparticles loaded with *Nyctanthes arbor tristis* Linn extract were developed as per the standard procedure and its optical properties were determined.

Optical properties

Absorbance v/s Concentration was plotted on the y-axis and x-axis respectively by using an excel sheet and R^2 value was found to be 0.992 with y=0.0001x + 0.0024 shown in Figure No 3 and Graph 1.

Characterization of Nanoparticle loaded *Nyctanthes arbor tristis* Linn extract

Laser light transmission test (Tyndall effect)

A clear Tyndall effect was seen through the solutions. Hence, confirming presence of fine particulate matter, as the nanoparticles produced were causing the Tyndall effect by scattering the laser light in a straight line and shown in figure 4.

Complexometric titration method

Appearance of white precipitate indicates the presence of zinc in a formulation when titrated against EDTA solution using Eriochrome Black T as indicator is depicted in figure 5.

Scanning electron microscopy (SEM analysis)

The particle size was found to be 58.59nm to 79.58nm with no agglomeration, shown in Figure 6.

Dynamic light scattering analysis (DLS) studies

The average particle size of developed NNAT formulation was found to be 194.1nm with PDI- 0.258 and Zeta potential was found to be 37.38. DLS results are high compared to SEM data shown in figure 7.

Drug entrapment

Entrapment efficiency of NNAT extract for 1:9 concentration of Zinc nitrate and NaOH was found to be 99.14%.

***In-vitro* drug release studies**

The extract released from the zinc nanoparticles was found to be 89.37% at 7 h. Results are tabulated in Graph 2.

Accelerated stability studies of NNAT suspension

Physical test

Physical stability of NNAT suspension is stable in both temperature and can be stored at room temperature (25^0C and 40^0C) for a longer time. Results are showed in the Table 3.

Sedimentation rate, rheology, pH and Viscosity

NNAT suspension accelerated stability studies was performed and all the parameters was in normal range and acceptable form where pH was 7.3. Rheology was found to be 1.1.ml/sec, viscosity was 2.81Cp and sedimentation rate was found to be 36. This proves that the developed Nano formulation was stable

Oral Acute toxicity studies

NNAT suspension doesn't show any abnormalities or mortality in oral acute toxicity studies. Thus 200mg/kg as low dose and 400mg/kg as high dose was selected for further studies.

***In-vivo* cardioprotective activity**

DOX treated animal showed increasing in cardiac biomarker levels (CK-MB, LDH, SGPT, ALP, and cardiac Troponin T), abnormal ECG and necrosis in cardiomyocytes. All the cardiac biomarkers levels such as CK-MB, LDH, SGPT, ALP and cardiac Troponin T have declined significantly in both NNAT suspension 200 & 400 mg/kg treated groups when compared to the DOX treated group. It also showed negative signs in Cardiac Troponin T test. 400mg/kg of NNAT suspension showed highly significant cardioprotective action than that of 200mg/kg. Standard Ascorbic acid (250mg/kg) also showed significant reduction in cardiotoxicity. Therefore, based on the results the NNAT suspension significantly

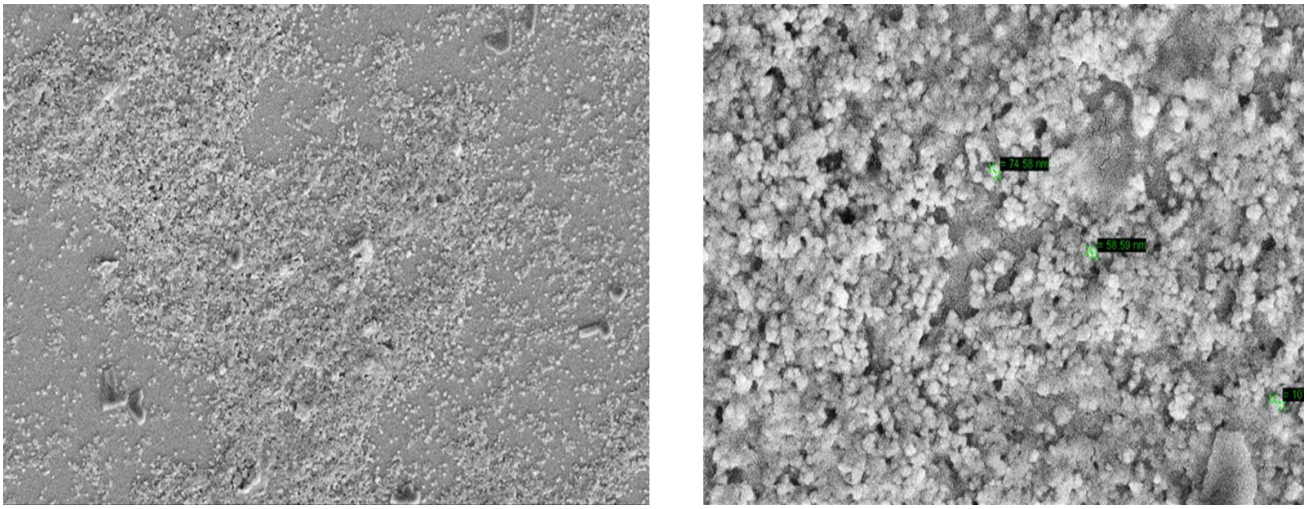


Figure 6: Scanning Electron Microscopy of NNAT formulation

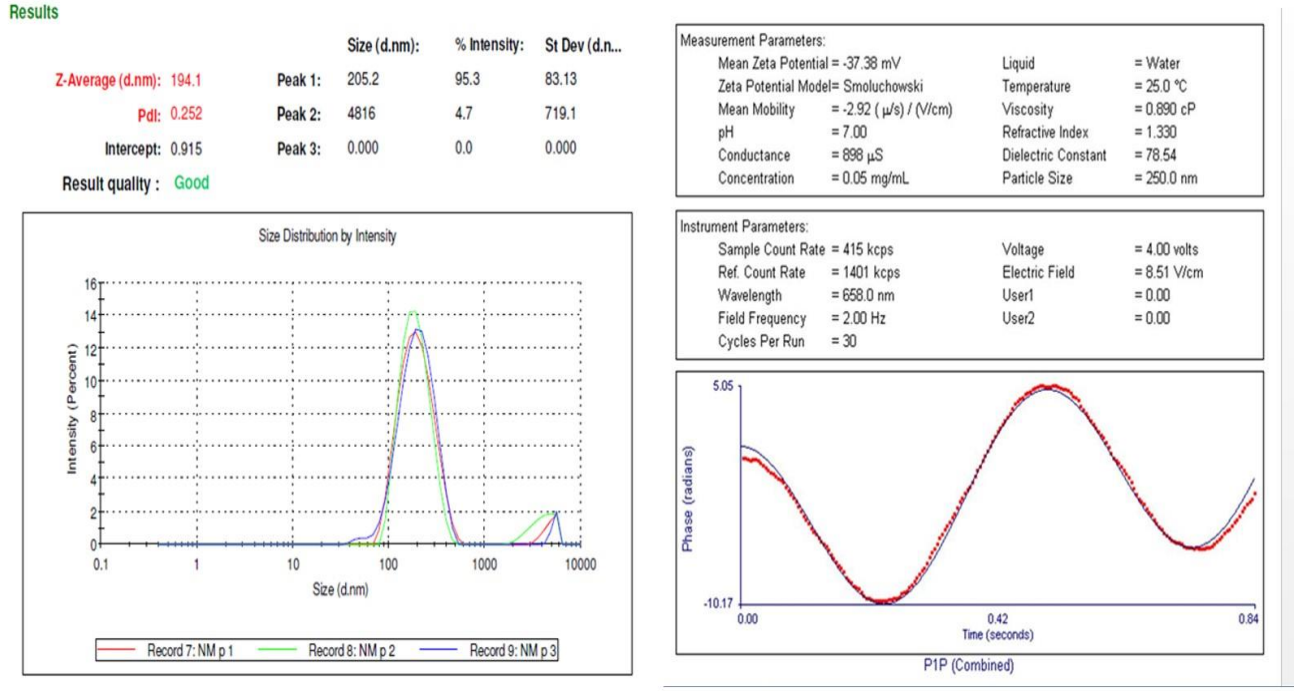


Figure 7: DLS of NNAT formulation

shows dose dependent cardioprotective activity against doxorubicin induced cardiotoxicity. Results are shown in table 5 and figure 8. The normal control animals showed normal ECG with regular rhythm, normal p waves, QRS width and constant PR intervals. Doxorubicin treated animals showed opposite action which has wider and almost diminished QRS complex giving signs of ventricular fibrillation. The QT and RR interval of the normal group animals was found to be 307.75±6.86 and 214.25±10.379 respectively. Doxorubicin treated group animals showed nearly diminished and prolonged intervals; QT was 209.375±10.674 and RR was 609±13.943. Ascorbic acid treated animals showed significant reduction in QT and RR intervals whereas NNAT suspension treated animals showed significant improvement in function and reduced prolongation in QT & RR interval in comparison with DOX-treated animals. All the results are shown in table 6 and figure 9.

Histopathological study

The sections of the cardiac tissue was examined after staining with hematoxylin and eosin dye. Normal control group animals showed normal architecture of cardiac muscles without any infiltration by inflammatory cells (Fig No 10.1). The positive control animals which are exposed to doxorubicin showed moderate myocycotic necrosis with accumulation and infiltration of inflammatory cells, disorganization of cardiac myocytes, separated bundles (Fig No 10.2). The standard treated animals did not showed any sign of necrosis (Fig No 10.3) and NNAT Suspension treated animal heart have shown no indication of necrosis, showing normal architecture similar to the normal rat's heart (Fig No 10.4 and 10.5) with proper branching and organized muscle fibers, without any signs of intercalated disks, discontinued

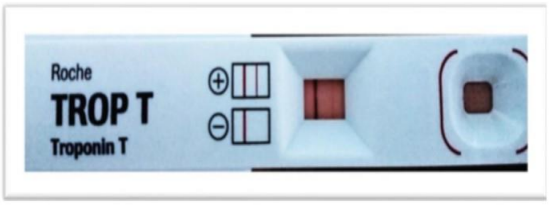


Fig No 8.1: DOX Treated Group

Fig No 8.2: Standard Treated Group

Fig No 8.3: NNAT 200 mg/kg Treated Group

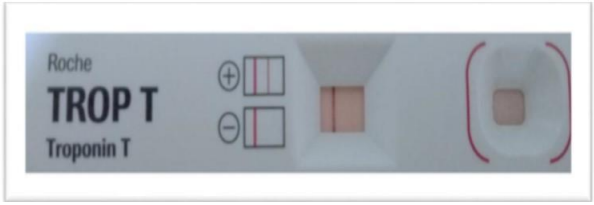


Fig No 8.4: NNAT 400mg/kg Treated Group

Figure 8: Cardiac Troponin T –test in *in vivo* cardio protective activity

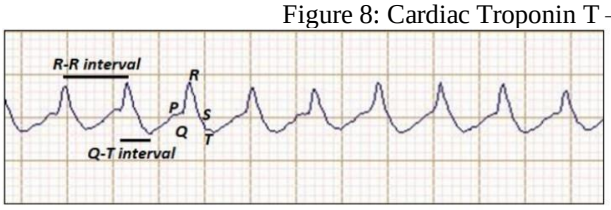


Fig No 9.1: Normal group

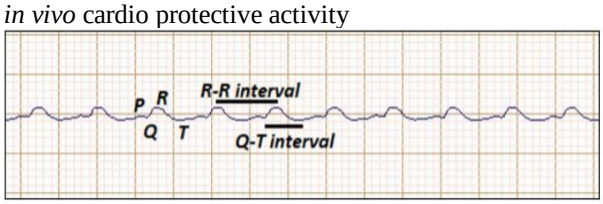


Fig No 9.2: DOX Treated Group

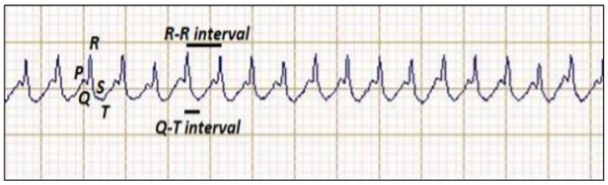


Fig No 9.3: Standard Treated Group

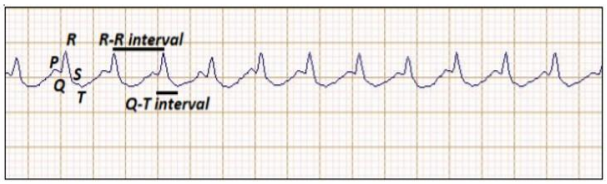


Fig No 9.4: NNAT 200mg/kg Treated Group

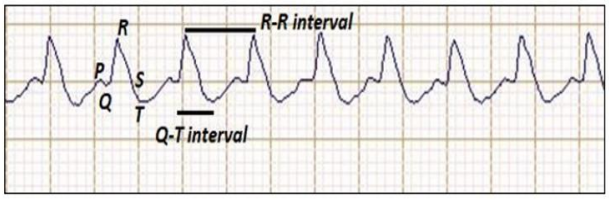


Figure 9: ECG in Cardio protective activity

cardiac myocytes were observed.

DISCUSSION

CVDs include Hypertension, Angina, Congenital Heart Disease, Coronary heart diseases, Heart attack, stroke, etc..[20] Nanoparticles drug delivery systems have more advantages compared to other systems because they have unique properties such as high stability, high carrier capacity, the likelihood of variable routes of administration and feasibility of incorporation of both hydrophilic and hydrophobic substances.[5] *Nyctanthes arbor tristis* Linn is one of the traditional medicinal plant and each part of the plant is used for various ailments as home remedies.[6] The *Nyctanthes arbor-tristis* Linn stem was extracted with 70% ethanol and preliminary phytochemical analysis confirmed the presence of flavonoids, cardiac glycosides, alkaloids, carbohydrates, tannins and phenolic compounds. Cardio protective activity of NNAT suspensions maybe due to the presence of cardiac glycosides.[21] Nanoparticle loaded with *Nyctanthes arbor tristis* Linn extract was developed and characterized by various parameters such as Laser light transmission test showed the presence of nanoparticles by scattering the laser light in a straight line. SEM is an analytical tool which captures high resolution images of objects which are in the size range of 100nm by means of producing a focused beam of electrons on scanning samples by providing topographical, morphological and compositional information of a nanoparticle and NNAT formulation were done to determine the three-dimensional structure of a nanoparticle to estimate particle size range, surface area and composition. NNAT formulation and particle size range was found to be 58.59nm-79.58nm with no agglomeration. DLS studies a well-established latest technology for measuring particle size and particle size distribution in a solution typically in the submicron region i.e., lower than 1nm. This is mainly used to the characterization of particles to analyze whether the particles are dispersed or dissolved in a liquid sample. If the particles having Brownian motion in a solution or suspension causes the laser light to be scattered at different intensities. The average particle size was 626.2nm, PDI - 0.258 and zeta potential was -37.38, this could be due to surface binding of extract on zinc nanoparticles. Drug entrapment efficiency of NNAT extract for 1:9 concentration of Zinc nitrate and NaOH was found to be 99.14%. NNAT extract released from the zinc nanoparticles showed at 7 hour with 89.37%. These values indicated the controlled release rate pattern of NNAT extract.[22] NNAT suspension such as physical state was performed at 25 ^{0}C and 40 ^{0}C and concluded that the physical stability of NNAT suspension is stable in both temperature and can be stored at room temperature for a longer time, Sedimentation rate was 36, rheology was 1.13 ml/sec, pH was 7.3 and Viscosity was 2.81Cp. Most of the pharmaceutical formulations requires additives like preservatives, coloring agents, flavoring agents. Hence in our NNAT extract suspension contains glycerin as a sweetening agent, sodium CMC as suspending agent and sodium benzoate for preservative. Where Sodium CMC acts as a suspending agent and improves the viscosity of the suspension. At present biggest challenge facing by the pharmaceutical nano suspension is mainly stability issue. Good stability refers to how long a stabilizer maintains the original and uniform sizes of nanoparticles in a suspension. In this study preliminary short term accelerated stability study for NNAT extract suspension was performed throughout 12days at specified temperature as per ICH guidelines of about 40^{0}C/ 75RH±2 in the environmental stability chamber and confirmed that suspension was stable by measuring the following physical parameters as well as pH, rheology, viscosity and

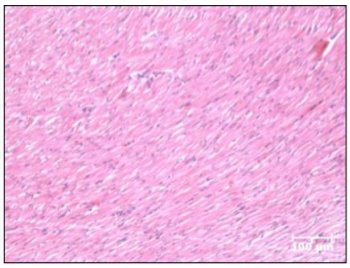


Fig No. 10.1: Normal heart

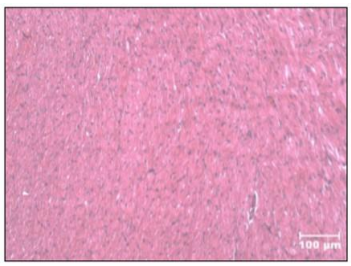


Fig No.10.2 Isoproterenol Treated Heart

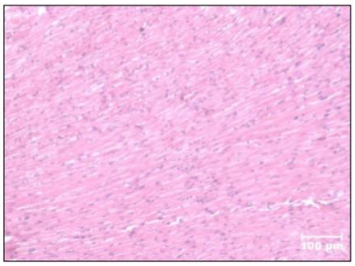


Fig No.10.3: Standard Treated Heart

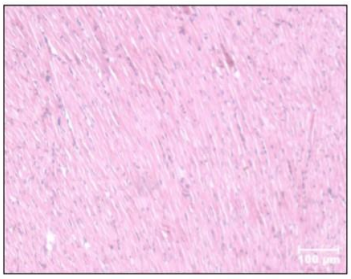


Fig No. 10.4: Tretinoin 2.5mg/kg Treated Heart

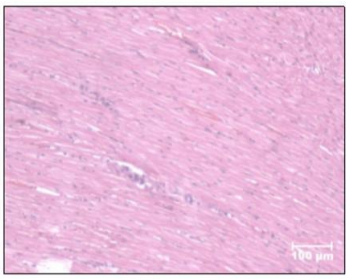


Fig No.10. 5: Tretinoin 5mg/kg Treated Heart

Figure 10: Histopathological studies on heart (10X)

sedimentation rate after 12 days. Hence, we concluded that NNAT suspension is stable up to 12days and can be stored for a long period. Oral acute Toxicity studies were performed to evaluate the safety profile of NNAT extract suspension. NNAT extract suspension doesn't show any kind of abnormalities or death, Hence, we have considered that NNAT extract suspension is safe and a dose of 200mg/kg and 400mg/kg were selected as low dose and high dose for further studies.[15] Cardio protective activity was evaluated using Doxorubicin (DOX) induced cardiotoxicity. Both the doses of NNAT suspension treated animals showed significant reduction in cardiotoxicity occurs due to administration of doxorubicin. The cardiac biomarker levels such as CK-MB, Cardiac troponin T, LDH, ALP, and SGPT are used to evaluate cardioprotective activity. Cardiac biomarkers in the blood are increased whenever the heart undergoes any severe stress due to cardiac injury such as heart attack and analyzing the levels of the cardiac biomarkers often helps to find out the size of the injury to the heart.[23] Doxorubicin, an anthracycline which is responsible for causing cardiotoxicity and elevating the levels of the cardiac biomarkers.[24] Therefore, based on the results, the NNAT extract showed dose dependent cardio protective activity against Doxorubicin-induced cardiotoxicity. Cardiac troponin T is a regulatory protein in the heart that plays a crucial role in regulating the interaction between actin and myosin in response to calcium. Testing cardiac troponin T levels aids in diagnosing myocardial infarction. All treated groups, except for the positive control group, exhibited negative results in the cardiac troponin T test. ECG is an ideal tool to analyze the function of the heart of the animal without any incision or dissection of the animal.[25] The variation in QRS complex, QT and RR intervals reflects the abnormality in the contractile activity. For normal animal heart, the contractility of the heart is adequate but the depleted contractility is showed in doxorubicin-treated animal heart explaining QRS complex, QT, RR widening and bradycardia.[19] We did not observe the normal QRS complex and QT & RR intervals; the changes in these parameters clearly indicate that there is an abnormality in conductivity and contractility of the heart. The rhythm of the normal, standard and test drug-treated animals ECG are having normal, same, regular with p waves, having normal and same QRS width, also has constant PR intervals. The QRS of normal animal, low dose and high dose test drug were regular and seems normal, as it is seen a little narrowing in standard treated but no readings were with dropped beats. In Doxorubicin treated animals ECG the above mentioned parameters were opposite, has wider and almost diminished QRS complex giving signs of ventricular fibrillation. The treatment of NNAT suspension significantly prevented the pathological changes in the ECG after doxorubicin-induced cardiotoxicity. Histopathological examination of the myocardium of normal group animals showed the normal architecture of heart muscles and NNAT suspension treated animal heart samples also showed normal architecture similar to the normal rat's heart. All the above results advocates the cardio protective efficacy of NNAT against DOX induced cardiotoxicity.

CONCLUSION

In our study, Nanoparticles loaded *Nyctanthes arbor tristis* Linn extract significantly inhibited the cardiac toxicity and decreased the levels of cardiac biomarkers with normal heart architecture with graded dose revealing its cardioprotective activity. Based on the above data, NNAT extract at 400mg/kg was found to have significant cardio protective effect than 200mg/kg. Hence, it could be concluded that the formulation at 400 mg/kg b.w is effective in preventing cardiac toxicity.

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