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Assessment of antibacterial, anti-inflammatory and antioxidant effects of *Nyctanthes arbor tristis* - an in-vitro study

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Abstract

Background and objectives: Periodontitis is a chronic inflammatory disease of multi-factorial etiology with microbial plaque being the primary cause. Various treatment modalities have been utilized in the management of periodontal diseases. Phytochemicals in the periodontal therapy has been evolved in the recent years with diverse benefits. *Nyctanthes arbor-tristis* a plant with many medicinal properties. The aim of this study was to assess the antibacterial, anti-inflammatory and antioxidant activity of *Nyctanthes arbor-tristis* leaf extract (NAL) under laboratory condition.

Methods: In the present study, *Nyctanthes arbor-tristis* leaf extract was assessed for its antibacterial effects against *Streptococcus mutans, Streptococcus sanguis, Staphylococcus aureus and Porphyromonas gingivalis using* agar disc diffusion method and minimum inhibition concentration (MIC). The anti-inflammatory effect was assessed by lipoxygenase inhibition assay and hyaluronidase inhibition assay. The antioxidant effects were assessed by ABTS and DPPH antioxidant assays. The statistical analysis was done by One-way ANOVA test, Independent Student t-test and Mann Whitney U Test.

Results: On comparison between the groups, the test results revealed that chlorhexidine showed higher mean zone of inhibition against the tested organisms. The test results of DPPH assay and ABTS assay revealed that the quercetin had higher radical scavenging property as compared to *Nyctanthes arbor-tristis* leaf extract. The test results of lipoxygenase inhibition assay and hyaluronidase inhibition assay revealed that the control groups showed higher percentage of lipoxygenase inhibition activity and hyaluronidase inhibition activity. The difference in results obtained were not statistically significant.

Conclusion: *Nyctanthes arbor-tristis* leaf extract showed antibacterial effect on the tested organisms and insignificant anti-inflammatory and antioxidant property. Hence; further studies with large sample size and under in-vivo and vitro conditions have to be conducted.

Keywords: Periodontitis, Nyctanthes arbor-tristis, lipoxygenase, hyaluronidase, antioxidant, anti-inflammatory, DPPH, ABTS.

Introduction

Periodontitis is a common chronic infectious disease associated with pathogenic microorganisms affecting the supporting structures of teeth and leading to progressive destruction, finally leading tooth loss. It is multifactorial in etiology, dental plaque being the critical one.[1]

There are various stages in dental plaque formation that includes sequential formation of acquired pellicle, colonization of primary colonizers and then secondary colonizers. Although plaque is recognized to be the primary etiological factor, it is now recognized that the great majority of the damaging events occurring in the periodontal tissues result from activation of destructive processes that occur as a part of the host immune inflammatory response.[2]

Periodontitis being an inflammatory condition, the production of ROS oxidation products causes an imbalance in the oxidant/antioxidant activity within periodontal pockets which leads to the periodontal tissue destruction. This is characterized by increased metabolites of lipid peroxidation, DNA damage, protein damage, and oxidation of important enzymes; meanwhile, they can function as signaling molecules or mediators of inflammation.[3]

Hence, the goal of periodontal treatment is to reduce the number of periodontal pathogens, bring down the inflammation of the tissues, reduce the oxidative stress and alter the host response.

Mechanical debridement and use of chemotherapeutic agents are some of the clinical methods employed to treat periodontal disease.[4]The major disadvantage of these chemotherapeutic agents is the toxicity and development of bacterial resistance. In an attempt to overcome the limitations of mechanical debridement and adverse effects of chemotherapeutic agents, the search for newer and safer chemotherapeutic agents still continues. Natural phytochemicals isolated from plants are considered as good alternatives to synthetic chemicals.[5]

***Nyctanthes arbortristis* (*N. arbortristis*)** is one such plant that has been used to manage various systemic and oral condition. The various parts of plant like fruits, leaves, seeds, flowers, barks and stem have significant phytochemicals and they have significant antibacterial, anti-inflammatory, antioxidant, hepatoprotective, antiviral, antifungal, antipyretic, antihistamine, anti-malarial activities.[6,7]

Antimicrobial evaluation of aqueous and alcoholic extract of leaves against numerous Gram positive and Gram negative strains revealed that *Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumonia, E. coli, P. marginata and Staphylococcus epidermis* were found more susceptible to the aqueous extract whereas *Micrococcus luteus, Staphylococcus aureus, Streptococcus pyogenes* and *Bacillus subtilis* were more sensitive to methanolic extract.[8]

The alcoholic extract of leaves of N. arbortristis are reported to have acute and subacute anti-inflammatory activity.[9] Several in vitro experiments such as DPPH ((1,1-diphenyl-2- picrylhydrazyl), hydroxyl and superoxide radicals and hydrogen peroxide scavenging assays showed that acetone soluble fraction of ethyl acetate fresh leaf extract of *Nyctanthes* showed significant antioxidant activity.[10]

In various studies conducted on *Nyctanthes arbortristis*, its antimicrobial activity is established on human pathogens. But there is limited literature to support the antimicrobial activity of N. *arbortristis* against oral pathogens.

In the present study, I have explored the minimum inhibitory concentration of *Nyctanthes arbor tristis* methanolic leaf extract required to inhibit oral microorganisms (*Streptococcus mutans, Streptococcus*

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sanguis, Staphylococcus aureus and Porphyromonas gingivalis). Also, antibacterial, anti-inflammatory and antioxidant efficacy of *Nyctanthes arbor tristis* were assessed under laboratory conditions.

Materials And Methods

An in vitro study was carried out to assess the antibacterial, anti-inflammatory and antioxidant activity of ***Nyctanthes arbor tristis*** leaf extract. The leaves were procured from the local areas of Bangalore and the laboratory procedures were done at Skanda Life Sciences Private Limited, Nagarbhavi, Bangalore.

Collection of Plant Material

Nyctanthes arbor tristis leaves were procured (Figure 1) from the local areas of Bangalore. Only the fresh leaves were collected and screened. The samples were then washed thoroughly under running tap water several times. After which the leaves were placed under shade conditions for drying. Drying process for leaves took around 5 days. After the samples were completely dried, they were then crushed to powdery form using a pre sterilized mixer grinder (Figure 2). The samples were then sealed in air tight bottles and were further analysed.

Extraction and Purification of the Extracts

15 g of the powdered leaves were placed in a thimble made of Whatman Filter paper No1. The thimble was kept in the inner tube of soxhlet body in such a manner, that the level of the thimble was lower than the siphoning tube. The apparatus was then connected to a round bottom flask which was filled with 200 ml of solvent to which boiling chips was added. The upper portion of the soxhlet was fitted with condenser. The whole apparatus was kept on a heating mantle for 4 hours maintaining the boiling point of the solvent. Further the extract phase was heated on a water bath and was subjected to slow evaporation of the solvent. The solid residue was then washed several times with water and acetone to remove solvent impurities. Finally, the pure extract obtained was cooled and stored in vials at room temperature for further usage (Figure 3&4)

Screening of *Nyctanthes arbor tristis leaves* extract by Disc Diffusion Method

Test organisms/cultures used were as follows, *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* MTCC 7443, *Streptococcus sanguis* ATCC 10556 and *Porphyromonas gingivalis* ATCC 33277. Positive control used was Chlorhexidine, Negative control used was Methanol. Cell suspension was prepared from *Streptococcus sanguis, Streptococcus mutans* and *Staphylococcus aureus*, *Porphyromonas gingivalis* cultures grown on Trypticase soya broth. *Porphyromonas gingivalis* cultured anaerobically in anaerobic chamber.

100µl inoculum of test cultures was then inoculated on Muller Hinton Agar plates (90 mm) for bacterial cultures. 10µl of 100 mg/mL stock extract of ***Nyctanthes arbor tristis leaves***, chlorhexidine and methanol were impregnated on 6mm sterile Whatman No. 1 discs. These discs containing the test compound, positive and negative control were placed on agar plates. The plates were then incubated at 35 ^{0}C for 24-48 hrs. After the incubation was done the agar plates were observed for zone of inhibition around the disc. This was done in triplicates for each of the organism (Figure 7)

MIC determination against the bacteria by micro broth dilution technique as per National Committee for Clinical Laboratory Standard (NCCLS) method

Cell suspension prepared from bacterial cultures grown on Trypticase soya broth was adjusted to 1-2x10^5 cells/mL. 90 µl of Nyctanthes arbor tristis leaves extract was then mixed with different test concentrations (0.0, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/ml) with 10 µl inoculum in 96 well plates in triplicates. Mix 90µl Muller Hinton Agar without leaf extract with 10 µl Inoculum was used as control. The bacterial cultures were then incubated at 35 ^{0}C. After the

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incubation process, the bacterial test plates were observed once 24-48 hours were completed. Optical density (OD) was measured in Tecan plate reader at 600 nm. MIC is determined as Minimum concentration of drug giving 50% inhibition of OD as compared with control.

Assessment of anti-inflammatory activity using Lipoxygenase inhibition assay

Preparation of working solutions

1785 µL from the enzyme stock was made up to 10 mL to get 10,000 units/mL. 50 mg of Linoleic acid was dissolved in 5 mL distilled water containing 50µL Tween-20, and made up to 50 mL with distilled water. The ingredients were added to the test tubes: are 0.8mL of test solution / reference standard (Indomethacin, 300 µg/mL dissolved in 3% methanol) of various concentrations, 0.1 mL of 2 M Borate buffer; pH 9.0 and 0.1 mL of 500-1000 units Lipoxygenase enzyme (type 1 from soybean, EC No. 1.13.11.12). The tubes were mixed and incubated at room temperature for 5 min, after which, 2.0 ml of substrate solution (50 mg of linoleic acid dissolved in 50 µL Tween 20, then made up to 50 ml with 2M Borate buffer and diluted to a concentration of 166.6 µg/ml with 2M Borate buffer pH 9.0) were added, and mixed well. The absorbance was then measured for 4 min at 234 nm. Control reaction was carried out without test sample (***Nyctanthes arbor tristis leaf*** extract).

Calculations

The percentage inhibition of Lipoxygenase is calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (leaf extract)}}{\text{Absorbance (control)}} \times 100$$

Assessment of anti-inflammatory activity using Hyaluronidase inhibition assay (Asada et al) Preparation of working solutions

- Preparation of 0.1 M acetate buffer (pH 3.8)

(A) Acetic acid (0.1 M): 5.775 mL of glacial acetic acid in 1000 mL of de-ionized water.

(B) Sodium acetate (0.1 M): 8.2g $C_2H_3O_2Na$ or 13.6g of $C_2H_3O_2Na.3H_2O$ in 1000mL.

44mL of solution A was mixed with 6 mL of solution B and made up to 100 mL with de-ionized water.

Enzyme: 4.15 mg/ml of hyaluronidase in 0.1 M acetate buffer pH 3.8, stored at – 20°C.

Substrate: 6 mg/mL of sodium hyaluronate in 0.1 M acetate buffer (pH 3.8), stored at –20°C.

Inhibitor: 3.5 mg/mL of potassium tetraborate in 0.1 M acetate buffer (pH 3.8).

- Preparation of Sodium chloride (0.15M): 26.3 mg/mL of sodium chloride in 0.1 M acetate buffer (pH 3.8).
- Preparation of Sodium hydroxide (0.4 N): 1.6 g of sodium hydroxide in 100 mL of de-ionized water.

Preparation of potassium tetraborate (0.8 M): 24.46 g of potassium tetraborate in 100 mL of de-ionized water.

Preparation of 67 mM P-dimethyl amino benzaldehyde (DMAB): 10 g of DMAB was dissolved in 100 mL of glacial acetic acid containing 12.5% 10 N HCl [85 mL of conc. HCl in 100 mL of water]. 1 mL of above stock solution was diluted with 9 mL of glacial acetic acid.

- Hyaluronidase was activated by incubating 100 µl hyaluronidase (4.15 mg/mL in 0.1 M acetate buffer, pH 3.8) with 50 µl sodium chloride (26.3 mg/mL in 0.1 M acetate buffer pH 3.8) for 20 minutes at 37 ^{0}C.
- Following activation, the enzyme mixture was pre-incubated with 200 µl of leaf extract and reference standard (cromolyn) at various concentrations for 20 minutes at 37 ^{0}C.
- Following pre-incubation, 150 µl of sodium hyaluronate (6 mg/mL in 0.1 M acetate buffer pH 3.8) was added and the reaction mixture was incubated at 37 ^{0}C for 40 minutes.

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- The reaction was stopped by addition 0.1 mL (0.4 N) sodium hydroxide and 100 µl (0.8 M) potassium tetraborate. This was followed by heating the mixture at 100 ^{0}C for 3 minutes.
- The mixture was cooled and 3 mL of 67mM DMAB (P-dimethyl amino benzaldehyde) was then added and incubated at 37 ^{0}C for 20 minutes. The absorbance was measured at 585 nm.
- Control reaction was carried out without the test sample

Calculations

The percentage inhibition of Hyaluronidase is calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (leaf extract)}}{\text{Absorbance (control)}} \times 100$$

Assessment of antioxidant effect using DPPH assay (Rajakumar et al.)

Preparation of working solutions:

- **DPPH:** 1.3 mg/ml of DPPH in HPLC (high performance liquid chromatography) grade methanol.
- **Quercetin:** 1 mg of quercetin dissolved in 1 ml methanol.
- 90 µl of DPPH solution was treated with 180 µl of various concentration of leaf extract.
- The different concentrations tested for reference standard were 0.5, 1.0, 1.5, 2.0, 2.5 mcg/ml.
- The reaction mixture was mixed and incubated at 25°C for 15 minutes and the absorbance was measured at 510 nm using semi-auto analyzer.
- A control reaction was carried out without the test sample.
- The antioxidant property is then analyzed in Tecan Plate reader (figure 5and 6)

Assessment of antioxidant activity using ABTS assay (Vani et al)

- PBS (Phosphate Buffered Saline -125mM NaCl in 10mM Sodium phosphate buffer, pH 7.4):0.14196g of Disodium hydrogen orthophosphate, 0.1560g of sodium dihydrogen orthophosphate and 0.7305g of sodium chloride were dissolved in 25ml of distilled water and pH was adjusted to 7.4 using dilute sodium hydroxide solution and the volume was made upto 100ml with de-ionized water.
- ABTS (2, 2'-azino bis-ethyl-benzothiozoline-6-sulphonic acid) (7mM):38.4mg of ABTS was dissolved in PBS and the volume was made upto 10ml.
- APS (Ammonium per sulfate) (2.45mM):5.59mg of APS was dissolved in PBS and the volume was made upto 10ml.
- ABTS (2,2'-azino bis-ethyl-benzothiozoline-6-sulphonic acid) Radical solution :
- Mother stock: 10ml of ABTS (7mM) and 10ml of APS (2.45mM) solutions were mixed and allowed to maintain at room temperature in dark for 16 hours.
- Working solution: 1.7ml of the mother stock was made up to 50ml with PBS (Figure 13), so as to give an absorbance of 1.000
- Quercetin (1mg / ml) (Reference standard) – It is commercially available which contains flavonoids.
- Radical cations were produced by reacting ABTS and APS on incubating the mixture at room temperature in dark for 16 hours.
- The solution thus obtained was further diluted with PBS to give an absorbance of 1.000.
- Different concentrations of the leaf extract and the reference standard i.e. quercetin (highest volume taken was 50µl) were added to 950 µl of ABTS

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working solution to give a final volume of 1ml, made up by adding PBS.

- The absorbance was recorded immediately at 734nm

Statistical Analysis

- **Statistical technique used:** One way ANOVA test followed by post hoc analysis test for disk diffusion method, independent student t test for minimum inhibitory concentration (MIC), Mann Whitney U test for lipoxygenase inhibition assay(LIA), hyaluronidase inhibition assay(HIA), ABTS [2, 2'-azino-bis (ethylbenzothiazoline-6-sulphonic acid]) assay and DPPH (2, 2 diphenyl – 1 picrylhydrazyl) antioxidant Assays.
- **Decision criterion:** The decision criterion is to reject the null hypothesis if the p-value is less than 0.05. Otherwise we accept the null hypothesis.

Results

Screening of Antibacterial activity using disc diffusion method

Methanol extract of *Nyctanthes-arbor tristis leaves* showed significant mean zone of inhibition against *Streptococcus mutans, Streptococcus sanguis*, *Staphylococcus aureus* and *Porphyromonas gingivalis* but Chlorhexidine showed superior action as compared to the leaf extract. The aqueous leaves extract showed maximum antibacterial activity against *Porphyromonas gingivalis* with (7.87 ± 0.32. mm) maximum degree of zone of inhibition and least antibacterial activity was observed against *Staphylococcus aureus* with (5.67± 0.21.) minimum zone of inhibition (Table 1 and graph 1)

Determination of MIC using micro broth dilution technique

The test results of micro-broth dilution technique for MIC determination revealed that chlorhexidine demonstrated MIC for *Streptococcus sanguis* at a concentration of 1.00 µg/mL and for *Streptococcus mutans* and *Staphylococcus aureus* at a concentration of 0.5 µg/mL and for *Porphyromonas gingivalis* at a concentration of 2 µg/mL whereas *Nyctanthes-arbor tristis* demonstrated MIC for *Streptococcus mutans*, *Staphylococcus aureus* at a concentration of 8 µg/mL and for *Streptococcus sanguis* and *Porphyromonas gingivalis* at a concentration of 16 µg/mL.

An independent student t-test was used to compare the mean percentage of inhibition of *Streptococcus sanguis, Streptococcus mutans, Staphylococcus aureus* and *Porphyromonas gingivalis* bacteria between two study groups (Chlorhexidine and *Nyctanthes-arbor tristis*). The test results revealed that chlorhexidine group showed a higher mean percentage of inhibition against the tested microorganisms.

The test results revealed that chlorhexidine group showed a higher mean percentage of inhibition *against Streptococcus sanguis, Staphylococcus aureus, Streptococcus mutans, Porphyromonas gingivalis* with a mean percentage of 60.93±32.47, 65.88±32.65, 65.02±31.26, 48.59±28.21 as compared to the *Nyctanthes-arbor tristis* group (With a mean percentage of 23.91±22.03, 30.54±28.21, 31.18±27.35, 23.78±22.67). (Table 2 and graph 2)

Assessment of anti-inflammatory activity

The results obtained from the lipoxygenase and hyaluronidase inhibition assays are presented in **Table 3 and 4** respectively.

Lipoxygenase inhibition assay

The results obtained from the Lipoxygenase inhibition assay revealed that indomethacin showed 58.47% of lipoxygenase inhibition activity at a concentration of 10 µg/mL and *Nyctanthes-arbor tristis* showed 47.51% of lipoxygenase inhibition activity at a concentration of 20µg/mL

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Mann Whitney U Test was used to compare the mean percentage of Lipoxygenase inhibition activity between Indomethacin and *Nyctanthes-arbor tristis* groups and indomethacin group showed higher mean percentage lipoxygenase inhibition activity with a mean percentage of 38.42± 25.06 as to the *Nyctanthes-arbor tristis* compared group with a mean percentage of 29.85 ± 23.10 with a mean difference of 8.57.This mean difference was statistically not significant with a P-value= 0.58**[Table 3, Graph III]**

Hyaluronidase inhibition assay

The results obtained from the hyaluronidase inhibition assay revealed that cromolyn showed 51.22% of hyaluronidase inhibition activity at a concentration of 40μg/mL whereas, *Nyctanthes-arbor tristis* showed 41.62% of hyaluronidase inhibition activity at a concentration of 80μg/mL.

Mann Whitney U Test was used to compare the mean percentage of hyaluronidase inhibition activity between cromolyn and *Nyctanthes-arbor tristis* groups and cromolyn group showed a higher mean percentage of hyaluronidase inhibition activity with a mean percentage of 29.40± 20.82 as compared to the *Nyctanthes-arbor tristis* group with a mean percentage of 15.84± 12.48 with a mean difference of 13.56. This mean difference was not statistically significant with a P-value= 0.20. **[Table 4, Graph IV]**

Assessment of anti-oxidant activity

1. DPPH assay

The percentage of radical scavenging capacity obtained from DPPH assay revealed that Quercetin showed 80.03% of radical scavenging capacity at a concentration of 10μg/mL *Nyctanthes-arbor tristis* whereas showed 66.22% of radical scavenging capacity at a concentration of 10μg/mL.

Mann Whitney U Test was used to compare radical scavenging capacity between quercetin and *Nyctanthes-arbor tristis* groups.

The test results revealed quercetin group showed a higher mean percentage of radical scavenging activity with a value of 46.53 ± 31.16 as compared to the *Nyctanthes-arbor tristis* group with a mean percentage of 35.53 ± 23.41 with a mean difference of 10.99. This mean difference was statistically not significant with a P-value= 0.39**. (Table 5, graph V)**

2. ABTS assay

The percentage of radical scavenging capacity obtained from ABTS assay showed that Quercetin showed 95.85% of radical scavenging capacity at a concentration of 10μg/mL whereas *Nyctanthes-arbor tristis* showed 83.83 % of radical scavenging capacity at a concentration of 10μg/ml**.**

Mann Whitney U Test was used to compare radical scavenging capacity between quercetin and *Nyctanthes-arbor tristis* groups.

The test results revealed quercetin group showed a higher mean percentage of radical scavenging with a value of 54.65 ± 33.54 as compared to the *Nyctanthes-arbor tristis* group with a mean percentage of 52.24± 26.27 with a mean difference of 2.42. This mean difference was statistically not significant with a P-value= 0.87. **(Table 6 and Graph VI)**

Discussion

Periodontitis is considered as one of the most common diseases of the oral cavity and is the main cause of tooth loss in adults. With respect to periodontitis, dental plaque biofilm shows succession of microbial colonization with changes in microbial flora observed from health to disease. A number of microbial complexes were identified that were associated with periodontal disease.[11]

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Although plaque is recognized to be the primary etiological factor, it is now recognized that the great majority of the damaging events occurring in the periodontal tissues result from activation of destructive processes that occur as a part of the host immune inflammatory response.[12]

Thus, periodontitis is a multifactorial, complex disease, and an up-regulated or maladapted immune-inflammatory response to bacterial plaque which predisposes patients to periodontal breakdown. This will stimulate the production of pro-inflammatory cytokines and inflammatory mediators. These cytokines and inflammatory mediators stimulate the release of tissue derived enzymes, the matrix metalloproteinases, which cause destruction of the extracellular matrix and bone.[13]

The upregulation of pro-inflammatory cytokines also leads to increased production of reactive oxygen species (ROS) like superoxide anion, hypochlorous acid, hydrogen peroxide etc. Reactive Oxygen Species (ROS) encompasses other reactive species which are not true radicals but are nevertheless capable of radical formation in intra and extra cellular environment.[3]

Mechanical debridement and use of chemotherapeutic agents are some of the clinical methods employed to treat periodontal disease. The major disadvantage of these chemotherapeutic agents is the toxicity and development of bacterial resistance. In an attempt to overcome the limitations of mechanical debridement and adverse effects of chemotherapeutic agents, the search for newer and safer chemotherapeutic agents still continues. [12]

Natural phytochemicals isolated from plants are considered as good alternatives to synthetic chemicals. The various plants with medicinal properties known from the ancient times are Azadirachata indica (Neem), Curcuma longa (Turmeric), Psidium guajava (Guava), Salvadora persica (Meswak), Aloe barbadensis (Aloe vera), Acacia catechu Wild (AC), Cymbopogon Citratus (Lemon grass etc.[14]

Nyctanthes arbor-tristis is one of the most useful traditional medicinal plants in India. It is usually grown in sub-Himalayan regions and Southwards to Godavari. Each part of the plant has some medicinal value and is thus commercially can be used. Therefore each part of the plant is considered as source of several unique products for the medicines against various ailments and also for the development of some industrial products.[15]

Nyctanthes arbor-tristis belongs to family Oleaceae. The tree is sometimes called the "tree of sorrow".[15]

Scientific Classification

. Kingdom: Plantae
. Order: Lamiales
Family: Oleaceae
. Genus: Nyctanthes
. Species: N. arbor-tristis

A search in the literature showed that there are various studies done on *Nyctanthes arbor-tristis* and its medicinal value. The various parts of plant like fruits, leaves, seeds, flowers, barks and stem have significant phytochemicals and they have significant antibacterial, anti-inflammatory, antioxidant, hepatoprotective, antiviral, antifungal, antipyretic, antihistamine, anti-malarial activities.[16]

Phytochemical Constituents[17]

Leaves: Leaves contain D-mannitol, flavone glycosides, ß-sitosterol, astragalin, oleolonic acid, nyctanthic acid, tannic acid, ascorbic acid, methyl salicylate, lupeol, volatile oil, glucose, fructose, carotene and benzoic acid.

Therefore Nyctanthes arbor tristis is used in the prevention and treatment of various diseases. The bitter leaves are used as cholagogue, laxative, diaphoretic and diuretic. The leaf juice is used to expel roundworms and threadworms in children, to treat loss of appetite, piles, liver disorders,

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biliary disorders, chronic fever, malarial fever, obstinate sciatica and rheumatism. The leaf extract is widely used in Ayurveda to treat arthritis and malaria. The leaves are also used in mycotic skin infection and in dry cough. The young leaves are used as female tonic and in reducing gynecological issues.17

A study was conducted by Vyas AL[18] et al in 2013 to evaluate the antimicrobial activity of Nyctanthes arbor-tristis leaf extract against some gram positive bacteria. Phytochemical screening of the extract showed the presence of alkaloids, carbohydrates, flavonoids, tannins and saponins. Results obtained in this study indicated that Nyctanthes arbor-tristis possess compounds with antimicrobial properties that can be used for plant based antimicrobial agents.

A study was conducted by Goyal S[19] et al in 2013 to evaluate the anti-inflammatory effect of various fractions (50 mg/kg) of *Nyctanthes arbor-tristis* leaves in carrageenan induced rat paw edema model. Both butanolic and chloroform fraction highly and moderately significantly ($P<0.01$) inhibited carrageenan induced paw edema model. They also concluded that the ant-inflammatory potential of *Nyctanthes arbor-tristis* leaves may be due to presence of active constituents like flavonoids and iridoid glycosides present in butanolic fraction and terpanoids in chloroform fraction.

A study was conducted by Kar B[20] et al in 2015 to evaluate the *in vitro* antioxidant activities of methanol extract of *Nyctanthes arbor-tristis* (MENA) leaves. MENA on ROS and RNS were evaluated in different *in vitro* methods like 1, 1-diphenyl-2-picrylhydrazil radical, lipid peroxidation assay. Total phenolic content and total flavonoid content were also measured by standard assay method. The extract showed significant antioxidant activities in a dose dependent manner. From the above study it is concluded that the methanol extract of *N. arbor-tristis* leaves is a potential source of natural antioxidant.

There are various studies done to know the activity of *Nyctanthes arbor-tristis,* as the studies done by Show S et al[21], Kumar A R[8] et al, Gharti Kul P et al[22] for antibacterial activity. Similarly studies were done by Pattanayak C et al[23], Saxena RS et al[9], Kumari TS et al[24] to understand the anti-inflammatory activity and also similarly studies done by Rathod N et al[25], Rathee JS et al[26], Dhinakaran DI et al[27] to understand antioxidant activity. Yet there are paucity of studies on anti-inflammatory, antioxidant and antibacterial activities of *Nyctanthes arbor-tristis* in dentistry. Thus, this study was aimed to reaffirm antibacterial, anti-inflammatory and antioxidant activity of *Nyctanthes arbor-tristis* in vitro.

The antibacterial activity was assessed by disc diffusion method followed by determination of minimum inhibitory concentration (MIC). In this in vitro study, organisms used in the study were *Streptococcus mutans*, *Streptococcus sanguis*, *Staphylococcus aureus and Porphyromonas gingivalis.*

These organisms were selected as:

- *Staphylococcus aureus* is one of the important oral pathogen.
- *Streptococci* are the primary colonizers of the oral cavity and these organisms synthesize extracellular gummy dextrans from sucrose, and it has been postulated that such polymers are responsible for the ability of these *Streptococci* to form gelatinous plaques on the surfaces of teeth.[28] These organisms are found to be the main etiological agents in the initiation of dental caries and periodontal diseases.[29]
- Also, unspecified cell wall components from *Streptococcus mutans* and *Streptococcus sanguis*, sonicated extracts of *Staphylococcus aureus* and

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Staphylococcus epidermidis stimulate bone resorption.[30]

- Among major periodontal pathogens, *P. gingivalis* considered as one of the main etiological agents in the pathogenesis and progression of the periodontal disease (Hajishengallis et al., 2012). This periodontopathic bacterium was found in 85.75% of subgingival plaque samples from patients with chronic periodontitis (Datta et al., 2008).[31,32]

Evaluation of anti-inflammatory activity of *Nyctanthes arbor-tristis leaf* extract by lipoxygenase inhibition assay and hyaluronidase inhibition assay and antioxidant activity by DPPH antioxidant assay and ABTS radical scavenging assay was carried out.

Nyctanthes arbor tristis leaves were procured from the local areas of Bangalore and laboratory procedures were done in Skanda Life Sciences, Bangalore. Only the fresh leaves were collected and screened. The samples were then washed thoroughly under running tap water several times. After which the leaves were placed under shade conditions for drying.

Drying process for leaves took around 5 days. After the samples were completely dried, they were then crushed to powdery form using a pre sterilized mixer grinder. The samples were then sealed in air tight bottles and were further analysed.

Methanol is used as solvent in our study as plant extracts from organic solvents have been found to give more consistent antioxidant and anti-inflammatory activity. This is in accordance with the study done by **Kar B** et al.[20] In their study, use of methanolic extract of ***Nyctanthes arbor tristis*** showed higher antioxidant properties in both DPPH and ABTS assays. They also reported that methanolic extract can show higher anti-inflammatory properties.

Hence, in this study the extract was prepared by adding methanol to it as methanol is one of the most commonly used organic solvent due to its properties of low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate.

Once the extract was prepared, they were used for the screening procedure for antimicrobial activity by agar disc diffusion method against bacterial strains. American Type Culture Collection (ATCC) strains of *Streptococcus mutans* (ATCC), *Streptococcus sanguis* (ATCC) and *Porphyromonas gingivalis (ATCC*), and microbial type culture collection (MTTC) strains of *Staphylococcus aureus* were used at the research laboratory for microbiological assay.

The Agar disc diffusion method of antimicrobial test was developed in 1940 by Heatley.[33]This procedure was accepted by NCCLS (National Committee for Clinical Laboratory Science). [34,35]The diameter of the inhibition zone was measured at three different planes on the under surface of the agar plate using a transparent scale. This is in accordance with the study conducted by Chandrashekar et al.[36]

Chlorhexidine was used as a positive control as it is the gold standard and one of the most effective antiplaque agents in dentistry against which other antiplaque and anti-gingivitis agents are measured while methanol acted as a negative control.[37] This selection of positive and negative controls were in accordance with the study done by Dalirsani et al [38]in the year 2011, where Chlorhexidine discs were used as positive controls while methanol and blank discs were used as negative controls to evaluate the antimicrobial activity of Streptococcus mutans.

Chandrashekar B R in the year 2014 conducted a study where 0.2% chlorhexidine and DMSO (dimethyl sulfoxide) were used as positive and negative controls, to evaluate the antimicrobial activity of Streptococcus

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mutans, Streptococcus sanguis and Streptococcus salivarius.[36]

The present study was done in accordance to the methodology used in studies conducted by Jenner et al[39] in 2013 where the efficacy of commercially available herbal toothpastes against different periodontopathogens was evaluated. After screening for the zone of inhibition, the Minimum Inhibitory Concentration (MIC) was assessed.

MIC is the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation. MIC is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agent. MIC determination against bacteria was done by micro broth dilution technique as per National Committee for Clinical laboratory standard (NCCLS) method.

For assessing the anti-inflammatory activity, lipoxygenase inhibition assay and hyaluronidase inhibition assay was done. Lipoxygenase inhibition assay is very useful in screening large number of drugs for anti-inflammatory activity within short time. In this assay, lipoxygenase activity of ***Nyctanthes arbor tristis*** was compared to Indomethacin as it is a strong anti-inflammatory drug. It is a NSAID (non-steroidal anti-inflammatory drug) and is a highly potent inhibitor of prostaglandin synthesis (non-selective COX inhibitor) and acts by suppressing neutrophil motility. This is in accordance with the study conducted by Shinde et al in 1999 using Indomethacin as the standard or positive control.[40]

Another test used for assessing anti-inflammatory activity was hyaluronidase inhibition assay. This assay was performed in accordance with the study conducted by Asada et al [41] in 1999. Hyaluronidase is one of the mucopolysaccharide-splitting enzymes and is related to inflammation and thus is found to increase in periodontitis. In this assay, hyaluronidase activity is determined by measuring the amount of N-acetyl glucosamine split from hyaluronic acid by the cleavage of glycosidic linkage spectrophotometrically. Cromolyn or disodium cromoglycate (DSCG) is a mast cell stabilizer, and synthetic chromone derivative is used as a positive control in this assay as it has strong inhibitory activities for hyaluronidase.[42]

DPPH [1,1-diphenyl-2-picryl hydrazyl] is a stable free radical with a purple colour. DPPH assay is based on the principle that antioxidants reduces DPPH to 1,1-diphenyl-2-picryl hydrazine, colorless compound which is measured at an absorbance of 510 nm.[43]

This assay is performed as per Rajkumar et al using Quercetin as positive control. Quercetin was used as a reference standard in the study as is most importantly known for its ability to act as antioxidant. It seems to be the most powerful flavonoid for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or induced by exogenous damage. The DPPH scavenging assay is a simple chemical experiment for the primary evaluation of any compound for its simplicity and low cost for free radical scavenging activity.[44]

Another assay used for assessing antioxidant activity is ABTS radical scavenging assay. The assay is performed as per Auddy et al[45] (2003). 95ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption which can be followed spectroscopically. The relatively stable ABTS radical has a green colour and is quantified spectrophotometrically at 734nm. When an antioxidant is added into the mixture, it will stabilize the ABTS radical and the absorbance is then measured spectrophotometrically.[46]

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The results of disc diffusion revealed that the Chlorhexidine group showed a higher mean zone of inhibition against *Streptococcus mutans, Streptococcus sanguis, Staphylococcus aureus and Porphyromonas gingivalis*. The major reason devoted to the antibacterial action of chlorhexidine is its way in inactivating non sporulent bacteria. *Nyctanthes arbor tristis* also has shown significant mean zone of inhibition against *Streptococcus mutans, Streptococcus sanguis, staphylococcus aureus and Porphyromonas gingivalis*. This is in accordance with the study done by Vyas AL et al.[18]

According to B Gulshan[47] phenolic compounds and tannins present in the methanolic extract of leaves are responsible for its antimicrobial activity. But on comparison, chlorhexidine showed greater zone of inhibition than *Nyctanthes arbor tristis* and the difference found was statistically significant.

After screening for the zone of inhibition, the minimum inhibitory concentration of *Nyctanthes arbor tristis* extract was assessed and compared with that of positive control chlorhexidine. The test results revealed that chlorhexidine group showed a higher mean percentage of *Streptococcus mutans* inhibition with a mean percentage of 65.02±31.26, *Streptococcus sanguis* inhibition with a mean percentage of 60.93±32.47, *Staphylococcus aureus* inhibition with a mean percentage of 65.88±32.65 and *Porphyromonas gingivalis* with a mean percentage of 48.59±28.21 as compared to the *Nyctanthes arbor tristis* with a mean percentage of 31.18±27.35, 23.91±22.03, 30.54±28.21, 23.78±22.67 for *Streptococcus mutans, Streptococcus sanguis, Staphylococcus aureus and Porphyromonas gingivalis* respectively.

The results of the lipoxygenase inhibition assay revealed that the Indomethacin group revealed a significant mean percentage of lipoxygenase inhibition activity with a mean of 38.42± 25.06. This is in accordance with the study done by Kumari TS et al[24] in the year 2017, where indomethacin showed higher percentage inhibitory activity in lipoxygenase assay.

The major reason devoted to the anti-inflammatory activity of indomethacin in Lipoxygenase pathway is to shunt the mechanism of arachidonic acid to 5-LOX pathway, thus reducing prostaglandins and subsequently anti-inflammatory effect. *Nyctanthes arbor tristis* did not show any significant result.

The results of the Hyaluronidase inhibition assay revealed that the cromolyn group revealed a significant mean percentage of hyalourinidase inhibition activity with a mean of 29.40± 20.82. This is in accordance with the study done by Saxena RS et al.[23]

The major reason devoted to its anti-inflammatory activity is its potent inhibitory activity on mast cells, also it is said to inhibit macrophages, eosinophils, monocytes, and platelets that believed to play a role in the inflammatory response.[48] *Nyctanthes arbor tristis* did not show any significant result.

The test results of DPPH radical scavenging assay showed that the quercetin had a significant percentage of radical scavenging with a mean of 46.53 ± 31.16. This is in accordance with the study done by Ladumor VC et al in the year 2017. [49]

The possible mechanism of action of quercetin, being, its ability to act as a free radical inhibitor, particularly with respect to the peroxy radical, which is the propagator of the autoxidation processes in fats and oils. [50] *Nyctanthes arbor tristis* did not show any significant result.

In ABTS radical scavenging assay, the test results revealed that Quercetin group had a higher percentage of radical scavenging with a mean of 54.65 ± 33.54. This is in accordance with the study done by Vyas S et al in the year 2013 where the author described that the substitution

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of the hydroxyl groups of quercetin by the conjugating substituents resulted in a decrease in the antioxidant activity with regard to the parent compound.[51] *Nyctanthes arbor tristis* did not show any significant result.

Figures and tables

Figure 1: *Nyctanthes-arbor tristis* leaves,2:- *Nyctanthes arbor tristis* leaves pulverized into fine powder

Figure 3: Obtained extract heated on a water bath

Figure 4: Obtained pure extract cooled and stored in vials

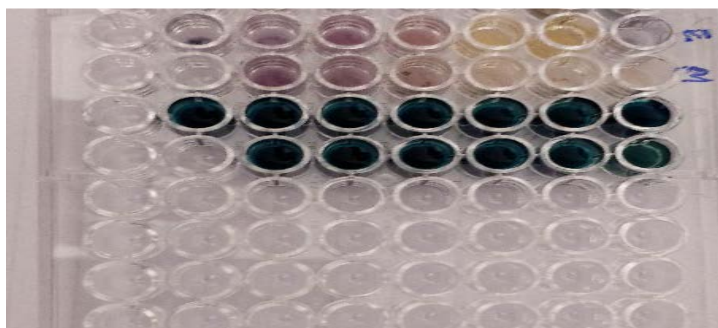

Figure 5: DPPH inhibition essay carried out on a 96 well plate

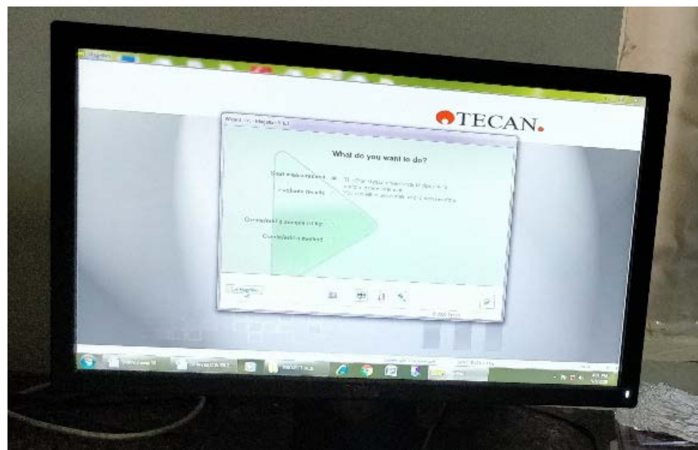

Figure 6 : Tecan plate reader

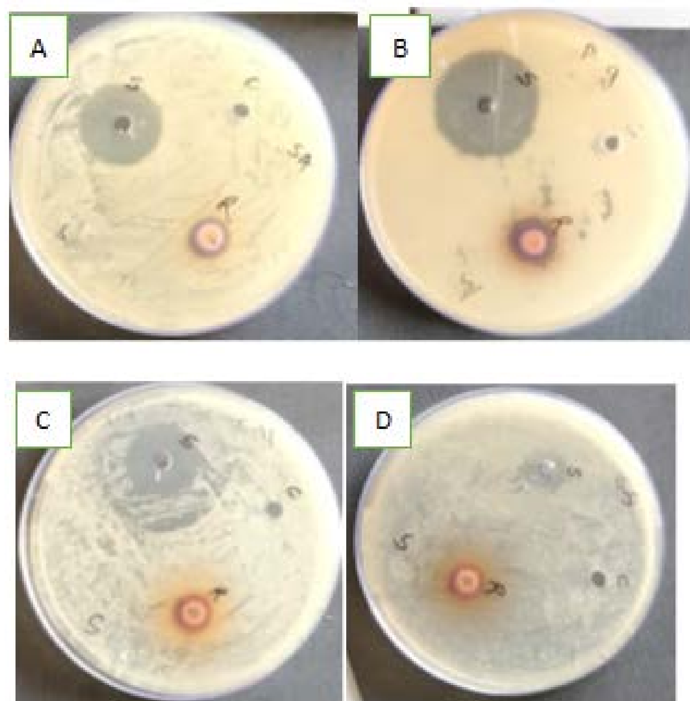

FIG 7:- Inhibitory activity of *Nyctanthes*, against (A) *Staphylococcus aureus*, (B) *Porphyromonas gingivalis*, (C) *Streptococcus mutans,* (D)*Streptococcus sanguis ;* S-chlorhexidine, R-Nyctanthes, C- methanol.

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Tables:

Table 1:-Comparison of mean zone of inhibition (in mm) between Nyctanthes Extract, -ve & +ve Controls using One-way ANOVA test followed by Tukey's Post hoc Analysis test

Bacteria	Group	N	Mean	SD	Min	Max	P-Value	Sig. Diff	P-Value
S. Aureus	NY	3	5.67	0.21	5.5	5.9	<0.001*	NY Vs NC	<0.001*
	NC	3	0.00	0.00	0.0	0.0		NY VS PC	<0.001*
	PC	3	17.03	0.25	16.8	17.3		NC Vs PC	<0.001*
P. Gingivalis	NY	3	7.87	0.32	7.5	8.1	<0.001*	NY Vs NC	<0.001*
	NC	3	0.00	0.00	0.0	0.0		NY VS PC	<0.001*
	PC	3	17.00	0.10	16.9	17.1		NC Vs PC	<0.001*
S. Mutans	NY	3	6.03	0.15	5.9	6.2	<0.001*	NY Vs NC	<0.001*
	NC	3	0.00	0.00	0.0	0.0		NY VS PC	<0.001*
	PC	3	9.80	0.26	9.5	10.0		NC Vs PC	<0.001*
S. Sanguis	NY	3	6.03	0.25	5.8	6.3	<0.001*	NY Vs NC	<0.001*
	NC	3	0.00	0.00	0.0	0.0		NY VS PC	<0.001*
	PC	3	9.83	0.47	9.3	10.2		NC Vs PC	<0.001*

Note: NY- Nyctanthes Group, NC - Negative Control, PC - Positive Control [CHX]

* - StatisticallySignificant

Table 2:-Comparison of mean Minimum Inhibition Concentration [MIC] values between two groups using Independent Student t test

Organisms	Groups	N	Mean	SD	S.E.M	Mean Diff	t	P-Value
S. Sanguis	CHX	8	60.93	32.47	11.48	37.02	2.668	0.02*
	Nyctanthes	8	23.91	22.03	7.79			
S. Aureus	CHX	8	65.88	32.65	11.54	35.34	2.317	0.04*
	Nyctanthes	8	30.54	28.21	9.97			
S. Mutans	CHX	8	65.02	31.26	11.05	33.84	2.305	0.04*
	Nyctanthes	8	31.18	27.35	9.67			
P. Gingivalis	CHX	8	48.59	28.21	9.98	24.81	1.939	0.07
	Nyctanthes	8	23.78	22.67	8.01			

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* - Statistically Significant

Table 3:-Comparison of Mean % of Lipoxygenase Inhibition Activity between Indomethacin & Nyctanthes group using Mann Whitney U Test						
Group	N	Mean	SD	Mean Diff	Z	P-Value
Indomethacin	5	38.42	25.06	8.57	-0.548	0.58
Nyctanthes	6	29.85	23.10			

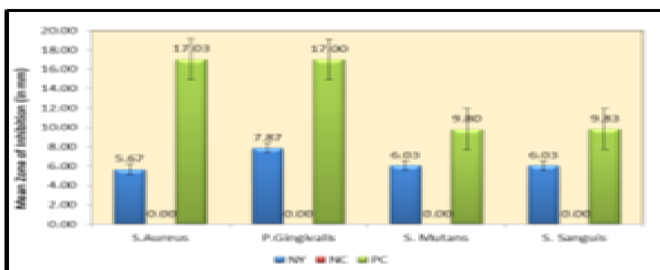
Table 4:-Comparison of Mean % of Hyaluronidase Inhibition Activity between Cromolyn Std. & Nyctanthes group using Mann Whitney U Test						
Group	N	Mean	SD	Mean Diff	Z	P-Value
Cromolyn Std.	7	29.40	20.82	13.56	-1.286	0.20
Nyctanthes	6	15.84	12.48			

Table 5:-Comparison of % of Anti-Oxidant activity [DPPH Assay] between Quercetin & Nyctanthes group using Mann Whitney U Test						
Group	N	Mean	SD	Mean Diff	Z	P-Value
Quercetin	7	46.53	31.16	10.99	-0.857	0.39
Nyctanthes	6	35.53	23.41			

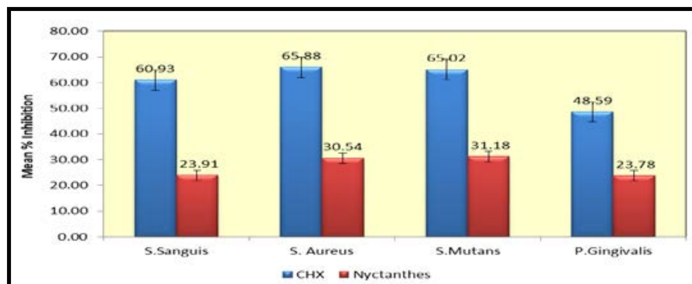
Table 6:-Comparison of Mean % of Radical Scavenging activity [ABTS Assay] between Quercetin & Nyctanthes group using Mann Whitney U Test						
Group	N	Mean	SD	Mean Diff	Z	P-Value
Quercetin	6	54.65	33.54	2.42	-0.160	0.87
Nyctanthes	6	52.24	26.27			

Graphs

Graph 1: Comparison of mean zone of inhibition between *Nyctanthes arbor tristis* Extract, Positive and Negative controls



Graph II: Comparison of mean concentration of inhibition between chlorhexidine and *Nyctanthes arbor tristis* extract for *S. sanguis, S. aureus and S. mutans and P.gingivalis*



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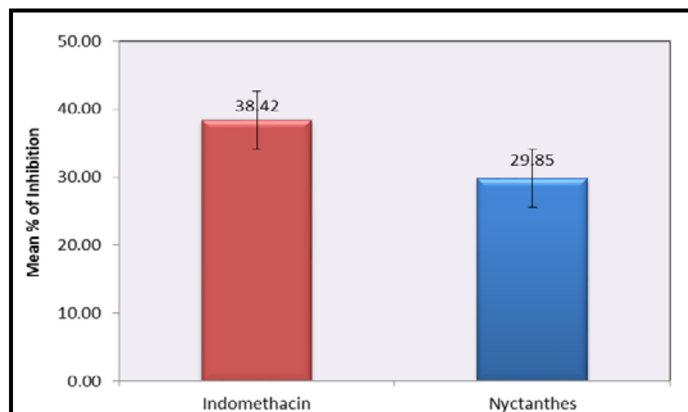
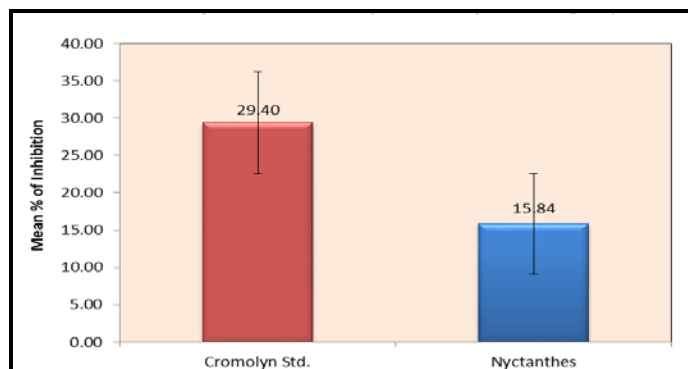
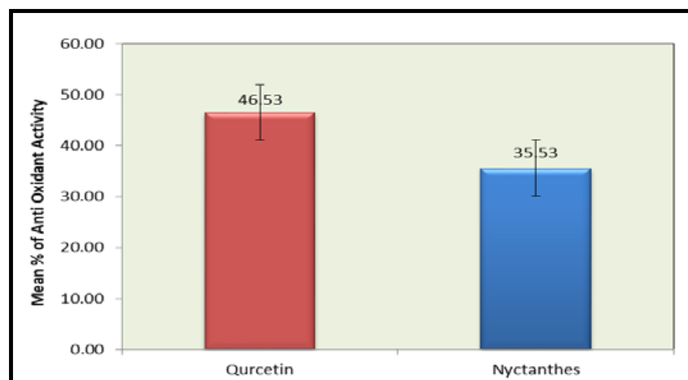
Graph III: Comparison of mean percentage of Lipoxygenase inhibition activity between indomethacin and *Nyctanthes arbor tristis* extracts



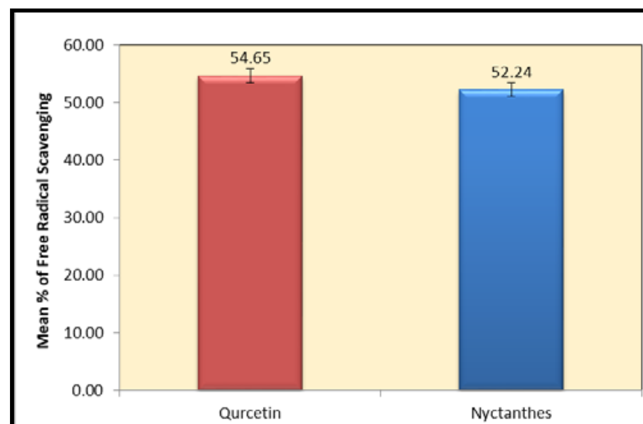
Graph IV: Comparison of mean percentage of Hyaluronidase inhibition activity between cromolyn standard and *Nyctanthes arbor tristis* extracts



Graph V: Comparison of the mean % of radical scavenging between Quercetin and *Nyctanthes arbor tristis* groups using DPPH assay



Graph VI: Comparison of the mean % of radical scavenging between Quercetin and *Nyctanthes arbor tristis* groups using ABTS assay



Limitations of the present study

It is an in-vitro study conducted to study the antimicrobial, anti-inflammatory and antioxidant property of *Nyctanthes arbor tristis* extract. The outcome of the study cannot be correlated with the in vivo scenario.

The other limitation of the study was that, other periodontal pathogens like *Aggregatibacter actinomycetemcomitans* were not evaluated.

Conclusion

The following conclusions can be drawn from the study:

1. Methanol extract of *Nyctanthes-arbor tristis* showed significant mean zone of inhibition against *Streptococcus mutans, Streptococcus sanguis*, *Staphylococcus aureus* and *Porphyromonas gingivalis* but Chlorhexidine showed superior action as compared to the leaf extract.

2. Lipoxygenase inhibition assay results revealed that the Indomethacin group showed higher percentage of lipoxygenase inhibition activity as compared to the *Nyctanthes-arbor tristis* and the differences obtained were statistically insignificant. Hyaluronidase inhibition assay results revealed that the Cromolyn group showed higher percentage of inhibition activity compared to *Nyctanthes-arbor tristis* leaf extract with a statistically insignificant difference.

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Therefore, standards exhibit more anti-inflammatory activity than methanol extract of *Nyctanthes-arbor tristis*.

3. DPPH assay results revealed that *Nyctanthes-arbor tristis* showed lower radical scavenging property as compared to quercetin group with statistically insignificant difference. ABTS assay results revealed that the Quercetin group had higher radical scavenging property as compared to *Nyctanthes-arbor tristis* with statistically insignificant difference.

Therefore, standards exhibit more anti-oxidant activity than methanol extract of *Nyctanthes-arbor tristis*.

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