Natural product is a source for bioactive compounds and has potential for developing some novel therapeutic agent. Over the last decade there has been a growing interest in drugs of plant origin and such drugs formed an important class for disease control. Herbs are staging a comeback and herbal ‘renaissance’ is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment.

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immune compromised patients in developing countries and many infectious microorganisms are resistant to synthetic drugs; hence an alternative therapy is very much needed [1]. Since ages, man has been dependent on nature for curing various body diseases. From ancient civilization various parts of different plants were used to eliminate pain, control suffering and counteract disease. Most of the drugs used in primitive medicine were obtained from plants and are the earliest and principal natural source of medicines. The plants used, as drugs are fairly innocuous and relatively free from toxic effects or were so toxic that lethal effects were well known. The nature has provided the storehouse of remedies to cure all ailments of mankind. There is no doubt that plants are a reservoir of potentially useful chemical compounds which serve as drugs, are provided newer leads and clues for modern drug design by synthesis [2].

Looking towards various pharmacological activities of the plant *Nyctanthes arbor-tristis* as revealed by the literature survey, the present study was undertaken to investigate—

(i) *In-vitro* Antibacterial Activity of β-Sitosterol isolated from the leaves
(ii) *In-vitro* Anti-inflammatory Activity of Astragalin isolated from the stem

(iii) *In-vitro* Antioxidant activity of Naringenin isolated from the stem.

**6.1 IN-VITRO ANTIBACTERIAL ACTIVITY OF β-SITOSTEROL ISOLATED FROM THE LEAVES OF Nyctanthes arbor-tristis**

Literature survey shows that the extract of different parts of *Nyctanthes arbor-tristis* have been reported to possess antibacterial activity. The ethanolic and hydro-alcoholic extracts of the leaves were investigated for its antibacterial performance against both antibiotic resistant and nonresistant strains of *Staphylococcus aureus* [3]. A benzofuranone derivative, 3, 3a, 7, 7a-tetrahydro-3a-hydroxy-6 (2H) benzo-furanone, isolated from the flowers showed significant antibacterial activity against both gram positive and gram negative bacteria. [4]. The antibacterial potential of different parts of the plant was evaluated for gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*) bacteria [5]. The zone of inhibition and minimum inhibitory concentration (MIC) of the extracts of stem bark of the plant were determined and compared with the standard drugs ciprofloxacin and fluconazole. The chloroform extract was found to have both antibacterial and antifungal activity whereas the petroleum ether and ethanol extracts possess only antibacterial activity [6]. Various extracts of bark showed significant positive control *in vitro* antifungal and antibacterial activity when compared with ketoconazole and ciprofloxacin as standard antifungal and antibacterial drug, respectively [7]. Experimental results showed that the chloroform and ethyl acetate extracts of fresh leaves, seeds and fruits have significant antibacterial activity against gram negative bacteria (*E. coli* and *K. pneumoniae*) and Gram positive bacteria (*S. aureus*), whereas dried extracts of chloroform and ethyl acetate shown significant antibacterial activity against *Pseudomonas aeruginosa* [8]. Antimicrobial
efficacy of ethanol, methanol, petroleum ether and aqueous extracts of the
leaves exhibited varying degree of inhibitory effect against different
pathogenic strains. The most susceptible bacterium and fungi were
*Pseudomonas aeruginosa* and *Rhizopus stolonifer*, respectively. Petroleum
ether extract of leaves responded more effectively to antimicrobial activity as
compared to other extracts. [9].

6.1.1 Materials and Methods:

Looking towards the antimicrobial potential of the NAT plant, the
compound β-Sitosterol, isolated from its leaves part, has been screened for its
antimicrobial effect.

**Preparation of Microorganisms:** The organisms used in this study were
*Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The
strains were maintained on nutrient agar slants at 4°C. A loopful of each
bacterial strain was inoculated into 50 ml of sterile nutrient broth in 100 ml
conical flask. The flasks were incubated on a rotatory shaker for 24 hours at
37°C to activate the strain. Nutrient agar (NA) medium was used as bacterial
culture medium in the antibacterial assays.

**Composition of Culture Media:**
The media used for growth of bacteria were:

**Nutrient broth medium:**
Beef extract - 10.0g
Peptone - 10.0g
Sodium chloride - 5.0g
Distilled water - 1000 mL
The pH was adjusted to 7.2 - 7.4
All the weighed ingredients were mixed in water and dissolved with the aid
of heat with stirring.

**Nutrient agar Medium:**
Beef extract - 10.0 g
Peptone - 10.0 g  
Sodium chloride - 5.0 g  
Agar-agar - 1-2%(w/v)  
Distilled water - 1000 mL  
The pH was adjusted to 7.2 - 7.4

All the above weighed ingredients were mixed in water and heated on water bath with stirring till agar completely dissolved. The media were sterilized by autoclaving at 15 lb/sq inch pressure at 121°C for 15 minutes.

**Preparation of test solution:**

The test solution of the compound (5 mg/mL) was prepared in chloroform and the filter paper disc soaked in chloroform was used as control one. Streptomycin was used as standard.

**6.1.2 Screening of Antibacterial Activity:**

The antibacterial activity of β sitosterol isolated from the ethanolic extract of leaves of *Nyctanthes arbor-tristis* was evaluated by Agar Well Diffusion Method [10]. Twenty four hours broth cultures of the bacteria were used for the assay. A sterile cotton swab was dipped into the bacterial suspension and evenly streaked over the entire surface of sterile nutrient agar plate to obtain uniform inoculum. Wells were punched on the seeded plates using sterile borer (8 mm). The plates were allowed to dry for 5 min. The compound (200 and 300 µL) was dispensed into each well using sterile micropipette. The plates were incubated overnight at 37°C. Antibacterial activity was determined by measuring the diameter of zone of inhibition (mm).

**6.1.3 Result:**

Antibacterial potential of the compound was assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial activities are presented in [Table 6.1]. The antibacterial activity of the compound increased linearly with increase in volume of the extract. 200 µL and 300 µL
volume of the compound showed significant antibacterial activity against the microorganism tested. The results revealed that the gram negative (Escherichia coli and Pseudomonas aeruginosa) bacteria were more sensitive as compared to those of gram positive bacteria (Staphylococcus aureus). The growth inhibition zone measured ranged from 15 to 25 mm for all the sensitive bacteria.

6.1.4 Discussion:

The interest in medicinal and aromatic plants has been shown all over the world because of safe and effective constituents of plant products. Table 6.1 shows the results of antimicrobial activity against the tested microorganisms. Isolated compound, β-Sitosterol showed higher zone of inhibition against Pseudomonas aeruginosa as compared with the other tested bacteria. In the present study, our observations and results clearly shows the antibacterial activity of the isolated compound and justifies the traditional use of the plant for the treatment of different bacterial infections [11]. Thus, further test is recommended on large number of bacterial strains to decide their potential as candidates in development of antibacterial drugs.

6.2 IN-VITRO ANTI-INFLAMMATORY ACTIVITY OF ASTRAGALIN ISOLATED FROM METHANOLIC EXTRACT OF STEM

Inflammation is a normal protective response to inactive or destroy the invading organisms, to remove the irritants and set the stage of tissue repair [12]. It involves a complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair [13]. Inflammation is characterised by heat, redness, pain, swelling and disturbed physiological functions. It increases vascular permeability, protein denaturation and membrane alterations [14]. The critical role of inappropriate inflammation leads to onset of many diseases like cardiovascular disease, autoimmune disorders, neurodegenerative conditions, infection and cancer [15]. The
inflammatory chemical mediators come from plasma proteins, platelets, neutrophils. These mediators bind to specific receptors on target cells and increases vascular permeability, stimulate smooth muscle contraction, induce pain or mediate oxidative damage. Histamine, serotonin, arachidonic acid, cytokines are some chemical mediators [16]. The commonly used drug for management of inflammatory conditions are non-steroidal anti-inflammatory drugs (NSAIDS), which have several adverse effects like gastric irritation leading to formation of gastric ulcers [17].

Erythrocytes have been used as a model system for the study of interaction of drugs with membranes [18, 19]. Drugs like anesthetics, tranquilizers and nonsteroidal anti inflammatories stabilize erythrocytes against hypotonic haemolysis at low concentrations [20]. When RBC is subjected to hypotonic stress the release of haemoglobin (Hb) from RBC is prevented by anti-inflammatory agents. Membrane stabilization of HRBC membrane by drugs (anti-inflammatory agents) against hypotonicity induced haemolysis serves as a useful in vitro method for assessing the anti-inflammatory activity of various compounds [21].

Medicinal plants have a wide variety of chemicals from which novel anti-inflammatory agents can be discovered. Research on the biological activities of plants during the past two centuries has yielded compounds for the development of modern drugs [22]. The novel anti-inflammatory compound called C-glucosyl chromone was isolated from gel extracts of Aloe vera (Aloe barbadensis miller) [23].

Brahmi (Bacopa monnieri) is reported to show sedative, antiepileptic, vasoconstrictor and anti-inflammatory activity. It has been reported that the plant contains tetra cyclic triterpenoid saponins, bacosides A and B, hersaponin, alkaloids viz herpestine and brahmine and flavonoids [24].

Extract of different plant parts of Nyctanthes arbor-tristis has been reported to possess anti-inflammatory activity in a variety of experimental
models [25, 26, 27, 28]. Astragalin (Kaempferol-3-O-glucoside) is an important flavonoid found in many plants. In our study Astragalin, isolated from the extract of stem part of NAT [29], has been assayed for its anti-inflammatory activity.

Denaturation of proteins is also a well documented cause of inflammation in conditions like rheumatoid arthritis. So, ability of extracted compound to bring down thermal denaturation of protein is also evaluated due to its possible contribution for anti-inflammatory activity [30].

6.2.1 Materials and Methods:

Stock Solution Preparation: Astragalin (25 mg) extracted from the stem of the plant, was dissolved in 10 mL of ethanol. It was centrifuged at 2500 rpm for 10 minutes. The supernatant thus obtained was named as Astragalin Stock Solution, abbreviated as ASS and used in experiments.

Drug used as Standard: Acetylsalicylic acid available in the commercial name of Ecosprin-75 marketed by USV Limited, Mumbai, Maharashtra was used as a standard.

HRBC Suspension Preparation: Fresh blood was collected from a healthy human volunteer and mixed with equal volumes of sterilized Alsever solution (containing 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride), stored at 4°C and used within 5 hours. Saline at two different concentrations were prepared (isosaline 0.85% and hyposaline 0.25%). Blood samples were centrifuged at 300 rpm and the packed cells obtained were washed with isosaline (pH 7.2) three times and 10% (v/v) suspension was made with isosaline.

6.2.2 Screening of Anti-inflammatory Activity:

Hypotonic solution-induced haemolysis or membrane stabilizing activity

This test was done according to the method described by Shinde et al. with slight modifications [31]. The test sample consisted of stock erythrocyte (RBC) suspension 0.30 mL mixed with 2 ml of hyposaline and 1ml of
phosphate buffer (150 mm, pH 7.4) containing stock solution (ASS) ranging from concentration 100-500 µg/mL. In control sample 0.30 mL RBC suspension was mixed with hypotonic buffered solution alone. The standard drug acetylsalicylic was treated similar to test at 100 and 200 µg/mL concentrations. The mixtures were incubated at 10 minutes at room temperature, centrifuged for 10 minutes at 3000 rpm. The percentage inhibition of haemolysis or membrane stabilization was calculated by following equation–

% Inhibition of haemolysis = 100 × \[ \frac{A_1 - A_2}{A_1} \]

Where: \( A_1 \) = Absorbance of hypotonic buffered solution alone (control)

\( A_2 \) = Absorbance of test / standard sample in hypotonic solution

**Effect on Protein Denaturation:**

Protein denaturation was performed as described [32] with slight modifications. Test solution consisting of 1mL of different concentrations of stock solution (ASS) ranging from 100-500 µg/ml and standard acetylsalicylic acid 100 and 200 µg/mL was mixed with 1mL of egg albumin (from fresh hen’s egg) solution (1mM) and incubated at 37 ±2°C for 15 minutes. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 minutes. After cooling, the turbidity was measured spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated from control where no drug was added.

**6.2.3 Result:**

ASS protected the erythrocyte membrane against lysis induced by hypotonic solution in a concentration dependent manner significantly (100-500 µg/mL). Acetylsalicylic acid in the concentration of (100-200 µg/mL) was used as standard and also offered significant protection of RBC’s membrane against damaging effect induced by hypotonic solution [Table-6.2].
The inhibitory effect of different concentrations of stock solution (ASS) on protein denaturation is shown in (Table 6.2). ASS (100-500 µg/mL) and acetylsalicylic acid (100-200 µg/mL) showed significant inhibition of denaturation of egg albumin in concentration dependent manner.

Both membrane stabilization activity and effect on protein denaturation contribute to the \textit{in-vitro} anti-inflammatory activity of the stock solution (ASS) used in our study.

\textbf{6.2.4 Discussion :}

It is therefore expected that the isolated compound Astragalin with membrane stabilizing property should offer significant protection of cell membrane against injurious substances. Also the compound prevented protein denaturation effect on egg albumin solution. Therefore astragalin leads to effective RBC membrane stabilization and inhibition of protein denaturation both contributing to it’s \textit{in-vitro} anti-inflammatory activity.

\textbf{6.3 \textit{IN-VITRO} ANTIOXIDANT ACTIVITY OF NARINGENIN ISOLATED FROM THE STEM OF \textit{Nyctanthes arbor-tristis}}

The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidise nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidants are radical scavengers which protect the human body against free radicals or inhibit the formation of free radicals or prevent the damage caused by free radicals. However, it has been suggested that synthetic compounds have shown toxic effects like liver damage and mutagenesis. Different plants secondary metabolites like flavonoids and some other phenolic compounds have been already reported as possessing antioxidant activity. Therefore, it’s the time to find the natural safe antioxidant molecules that are gaining a great importance in the field of free
radical biology [33]. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxide and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Different therapeutic approaches using antioxidant from both natural and synthetic origin has been proposed for use in the treatment of various human diseases [34]. One of such natural antioxidant compound is Naringenin which has been isolated from the stem of Nyctanthes arbor-tristis in our findings [35]. It is a grapefruit flavanone and has been reported to reduce oxidative damage to DNA [36]. A powerful antioxidant and potential therapeutic agent in the treatment of oxidative inflammatory diseases is Vitamin C, chemically known as L-Ascorbic acid or L-Ascorbate. It is an essential nutrient for living organisms having antioxidant property and thus protect the body against oxidative stress [37]. The aim of the present study was to compare the antioxidant potential as well as free radical scavenging activity of the plant derived compound, Naringenin with the synthetic compound vitamin C (Ascorbic acid) towards the reactive oxygen species.

6.3.1 Materials and Methods:

Chemicals:

Vitamin C and DPPH were obtained from Merck Mumbai. All the other reagents were used of high analytical grade. 100 μM solution of DPPH in methanol was prepared. Test solution of Naringenin was prepared by dissolving 10 mg of it in 10 mL of methanol.

6.3.2 Screening of Anti-Oxidant Activity:

DPPH Radical Scavenging Activity:

The DPPH (2, 2'-diphenyl-1-picrylhydrazyl) assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour).
When antioxidants react with DPPH, which is a stable free radical, becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance’s decreases from the DPPH radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured [38] [Fig-6.3.1]. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of new drug.

\[(\text{DPPH}) + \text{H-A} \rightarrow \text{DPPH-H} + \text{A}\]

(Purple) \hspace{2cm} (Yellow)

![Figure 6.1: Reduction of DPPH](image)

DPPH radical scavenging activity was measured using the method of Cotelle et al. [39]. 3.0 mL of reaction mixture containing 0.2 mL of DPPH (100 µM in methanol) and 2.8 mL of test solution of compounds (Naringenin and Vitamin-C standard) at various concentrations (5-50 µg/mL) and incubated at 37°C for 30 min and absorbance of the resulting solution was measured at 517 nm using spectrophotometer (UV-MINI-1240, SHIMADZU). IC\textsubscript{50} values of Naringenin and Vitamin C for DPPH were found to be 27.63 and 30.34 µg/mL respectively. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not incubated with compounds) using the following formula:

\[\text{Percentage Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100\]
% Inhibition of DPPH radical = C-T/C X 100

where, C is Absorbance of control and T is Absorbance of Test Sample

From the above obtained Radical Scavenging Capacity (RSC) the IC₅₀ were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization.

6.3.3 Result:

Antioxidant activity of test solution (naringenin in methanol) was evaluated by using free radical scavenging activity on DPPH, a stable free radical with Vitamin C as standard compound. It was observed that free radicals are scavenged by naringenin extracts in a concentration dependent manner and the results are comparable with synthetic antioxidant vitamin C [Fig-6.3.2]. IC₅₀ values for Naringenin and Vitamin C were found to be 27.63 and 30.34 µg/mL respectively. From the result of DPPH, it was found that Naringenin displayed strong antioxidant activity compared to vitamin C.

![Graph showing inhibitory activity of Naringenin and Vitamin C](image)

**Figure. 6.2 : 1,1-Diphenyl-2-picrylhydrazil (DPPH) scavenging activity of Naringenin and Vitamin C.**
6.3.4 Discussion:

Oxidative stress refers to a situation where the production of oxidants exceeds the capacity to neutralize them, leading to damage to cell membranes, lipids, nucleic acids, proteins and constituents of the extracellular matrix. Different therapeutic approaches can be used to decrease the oxidative stress and include scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or by targeting the signaling routes and expression of molecules involved in the inflammatory cascade [40]. Although, such approach can easily be available due to synthetic drugs which protect against oxidative damage but still safety is an important aspect now a days. Therefore, an alternative solution to solve this problem is the abundant area of research that mainly focused on the readily available non toxic natural antioxidants from food supplement and natural products. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. Our results on scavenging activity on DPPH, clearly shows that Naringenin could have great importance as therapeutic agents in preventing or showing the progress of aging and age associated oxidative stress related degenerative diseases.
REFERENCES


[18] Sessa G, and Weisman G. Effect of components of the polyene antibiotic, Fillipin on phospholipids spherules (liposomes) and


