Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2012, 4(3):1686-1695

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Study on phytochemical screening and antibacterial activity of *Nyctanthes-arbor tristis*

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ABSTRACT:

In this present study, an attempt was taken to investigate the antibacterial activity of Nyctanthes arbor - tristis. The seed and fruit extracts were used for their antibacterial screening. Melanin content and stability in fruits and seeds were studied with various factors like temperature, oxidants and metal ions. Chloroform and ethyl acetate extracts of fresh leaf, seeds and fruits were shown significant antibacterial activity against Gram negative bacteria (E. coli and K. pneumoniae) and Gram positive bacteria (S. aureus), where as dried extracts of chloroform and ethyl acetate shown significant antibacterial activity against Pseudomonas aeruginosa. Phytochemical analysis shows the presence of phytosterols. Phenolics compounds, tannins, flavonoids, cardiac glycosides, saponins and alkaloids in leaf, fruit, seed content. Steroids were present only in seeds. UV spectral studies have shown high content of melanin in seeds. Melanin content was stabilized by $KmNO_4$ than $K_2Cr_2O_7$. Melanin color was increased and preserved by metal ions like of Fe^{++}, Mg^{++} and Zn^{++}.

Key words: Melanin, Anti-bacterial activity, Dimethyl sulfoxide, Streptomycin, Oxidants.

INTRODUCTION

Medicinal plants are the most exclusine sources of life saving drugs for the majority of the world's population [1]. The medicinal action of plants are unique to a particular plant species, consistent with the concept that the combination of secondary metabolites in a particular plant is taxonomically distinct [2]. Natural products have traditionally played a pivotal role in drug discovery, that make the compounds of interest in the development of active anti-hypertensive pharmaceutical agents [3]. Aqueous extracts of *Moringa oleifera* showed significant anti-inflammatory effect [4]. *Nyctanthes arbor-tristis* commonly known as Night jasmine or coral jasmine, characterized by the presence of phenyl ethanoid derivatives and iridoid glucosides. Earlier studies on this plant have resulted in isolation of a number of glycosides from leaves, seeds and flowers. Leaves contains tannic acid, methyl salicylate, amorphous glucosides, mannitol, resin, ascorbic acid, carotene and traces of volatile oil [5] along with ß-amyrin, ß-sitosterol, nyctanthic acid and iridoid glucosides, arborside D and E (Minor iridoid arbortristosides). The iridoid arbortristoside A has been reported to have anti - proliferative activity [6].

The decoction of leaves is widely used in ayurvedic medicine for treating arthritis [7]. It has also been reported to possess hepatoprotective, antileishmanial, antiviral and antifungal activities [8] and analgesic, antipyretic and ulcerogenic activities [9]. The powdered seeds are recommended to treat scurvy [10]. Roots are used for emaciation and stem bark is taken to cure dysentery, ulcer of palate and internal injuries [11], anti-inflammatory [12], anti-malarial [13], leishmanicidal [14], amoebicidal [15], tranquilizing activity [16], antihelminthic [17] activities. The acetone soluble fraction of *Nyctanthes arbor - tristis* showed impressive antioxidant activity in several *in vitro* experiments such as hydroxyl and superoxide radicals and hydrogen peroxide scavenging assays. In addition, this property is reported due to the presence of high phenolics and flavanoids [18]. Melanin has been intensively studied for a long time [19]. Melanin has been shown to resist decay and biodegradation and to confer protection against

UV irradiation and lytic enzyme attack [20]. The natural melanin from plants or animals possesses a broad spectrum of biological activity [21].

The present study was undertaken to investigate the antibacterial activity of *Nyctanthes arbor - tristis* plant parts in view of its diverse pharmacological application in ancient and modern system of medicine. Phytochemical constitutents of plant extracts using specific qualitative methods have been carried out, Spectrophotometric analysis of phenolics and glycosides, thin layer chromatographic separation of glycosides, isolation and characterization of melanin from the plant parts such as fruit and seeds have been investigated.

EXPERIMENTAL SECTION

Plant materials

The plant parts viz., flowers, leaves, fruits and seeds of *N. arbor-tristis* were collected from Coimbatore, Ramanathapuram, Namakkal, Erode and Trichy districts of Tamil Nadu.

Solvent extraction

Fresh flowers, leaves, fruits and seeds were collected, washed and weighed (4 g each). The materials were then macerated in 10 ml of ethyl acetate and chloroform separately. The mixtures were kept for 6 hours at room temperature. The mixtures were then filtered through sterile Whatmann filter paper No: 1. Filtrate, thus obtained were centrifuged at 5000 rpm for 5 minutes. The supernatants were collected in the beaker and the solvents were evaporated to dryness, and the extract was stored at 4˚C in refrigerator. At the time of antibacterial assays the extracts were dissolved in 1-3 ml of Dimethyl sulfoxide (DMSO). Similarly dried materials (leaves, fruits and seeds) were extracted using ethyl acetate and chloroform. Dried, powdered plant materials (3 g each) were suspended in 10 ml of ethyl acetate and chloroform separately and incubated for 24 hours at room temperature. The mixtures were then centrifuged at 5000 *g* for 5 min.

Microorganisms

The test organisms used in this study were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC 2719) and *Staphylococcus aureus* (ATCC 25923). Strains were maintained on Nutrient agar slants. Cultures were inoculated into each 50 ml of sterile nutrient broth. The flasks were incubated on shaker for 24 hours for lag phase strain.

Antibacterial activity assay

Agar well diffusion method

The antibacterial activity of *Nyctanthes arbor - tristis* was evaluated by their ethyl acetate and chloroform extracts through agar well diffusion method. 24 hours broth cultures of test organisms were used for assay. Cultures were spreaded in the Meuller-Hinton agar surfae. After drying, wells were made on the seeded plates using sterile metal borer (8mm). The plates were allowed to dry for 5 min. DMSO solubilised extracts (100, 200 and 300 µl) were dispensed into each well. DMSO and streptomycin (10 µl) was used as negative and positive controls. The plates were incubated overnight at 37˚C.

Extraction and separation of Glycosides

The dried leaves, fruits and seeds (1 g each) were kept overnight in 20 ml warm hexane. Extraction was done twice with warm hexane (2×20 ml), and then centrifuged at 5000 rpm for 5 min. Hexane extract was spin at 5000 *g* for 5 min and pellets were extracted twice with warm methanol. Aqueous phase was collected and methanol was evaporated to dryness. The dried extract was dissolved in 1 ml warm methanol and stirred well in cyclomixer. 5 and 10 µl extracts were loaded on pre-coated silica gel 60 F254 TLC plate, developed in solvent system (ethyl acetate (60): methanol (14): water (10),v/v/v). Plates were developed to a height of 10 cm and dried. The spots were visualized by spraying vanillin sulphuric acid reagent, incubating the plates at 110˚ C for 5 min.

Extraction and estimation of phenolics

Dried leaves, fruits and seed powder (1g each) were suspended in 10 ml of 0.3 M methanolic hydrochloric acid and kept overnight. Mixtures were centrifuged at 5000 rpm for 5 min. Aqueous phase was collected and evaporated. Residues were dissolved in 5ml of distilled water. 0.1ml of extracts were transferred to test tubes and made up to 7.0ml with distilled water and stirred well. The Folin - phenol reagent was added to the extracts and shaken vigorously. After 3 minutes, 1 ml of 35% sodium carbonate solution was added and incubated for one hour. Absorbance was measured at 630nm.

Qualitative analysis of phytochemicals
Specific qualitative tests were performed to investigate the chemical constituents of *Nyctanthes arbor - tristis* leaf, fruit and seeds.

Alkaloids
One ml of ethyl acetate extract was dried in a test tube and mixed with 1-2ml of dilute hydrochloric acid with constant stirring. The mixture was filtered and three drops of Dragendorff's reagent was added.

Saponins
1ml extract was dried in a test tube and diluted with distilled water up to 5ml. The suspension was shaken for 15 minutes.

Phytosterols
Dried extract was dissolved in 2ml of acetic anhydride. 2 drops of concentrated sulphuric acid was added slowly along the sides of tube.

Phenolics
One ml of extract was taken in a test tube and dried. The extract was dissolved in 5 ml of distilled water. Then 1ml of neutral ferric chloride solution was added.

Tannins
0.5 g of dried, powdered samples were boiled in 20ml of water in a test tube and then filtered. 1ml of 0.1% ferric chloride solution was added and observed for brownish green or a blue black coloration.

Phlobatannins
Deposition of red precipitate when an aqueous extract of each sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

Flavonoids
To 5ml of dilute ammonia solution, a portion of the aqueous filtrate of each extract followed by the addition of concentrated sulphuric acid.

Steroids
2ml of acetic anhydride was added to dried extract which was dissolved in 2ml of water, of each sample with 2ml of sulphuric acid.

Terpenoids
1ml of extract was taken in separate test tubes and then dried. 2ml of chloroform was added to each extract and concentrated sulphuric acid was added to it.

Cardiac glycosides
5ml of each extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. 1ml of concentrated sulphuric acid was added to the mixtures.

Isolation and characterization of melanin
Extraction and purification of melanin from dried fruits and seeds were performed by standard method [22]. Fruit and seed powder (10 g each) were treated with 75ml of 2M sodium hydroxide, pH 10.5 for 24 hours. Mixture was centrifuged at 4000 g for 15 minutes. Aqueous was acidified with 2M hydrochloric acid to pH 2.5 and incubated at room temperature for 2 hours, and centrifuged at 4000 g for 15 minutes. Pellet was purified by acid hydrolysis using 6M hydrochloric acid at 100˚C for 2 hours. Precipitate was then treated with chloroform, ethyl acetate and ethanol. Pellet was then dried at room temperature and dissolved in 2M sodium hydroxide, spin at 4000 *g* for 15 minutes. Supernatant was precipitated with 1M HCl. Pellet was then washed with distilled water and stored at room temperature. To determine the UV - visible light absorption spectrum of melanin, 50 mg of melanin extracted from fruits and seeds were dissolved in 2M sodium hydroxide (pH 7.5) and the solution was scanned with a spectrophotometer (Hitachi U - 2800 spectrophotometer) at wavelength ranging from 190 to 800 nm.

Effect of temperature on melanin
Heat stability of melanin was measured following treatment in a thermostatically controlled water bath at 25˚, 50˚and 75˚C. The samples were held at each temperature for specific times and cooled to room temperature. Absorption of the solutions was recorded at λ max.

Effect of oxidant and metal ion on the stability of melanin

The effect of oxidant and metal ion was evaluated as described [23] with minor modification with respect to concentration. Melanin solution (50 mg/15 ml), an aliquot of this melanin solution and different concentration of potassium permanganate and potassium dichromate (8.3 and 16.9 µg/ml) and metal ions ($MgCl_2$, $ZnSO_4$: 5 mg/ml; $FeCl_3$, 0.05 mg/ml) were mixed in final volume of 3.0 ml. the absorbance of homogenate was determined at λ max.

RESULTS

Antibacterial activities of ethyl acetate extract

Antibacterial potential of ethyl acetate extract was assessed in terms of zone of inhibition in presence of extract. The growth inhibition zones were measured by agar well diffusion assay. In general, the inhibitory effects of extracts used were more or less similar against the microorganisms tested. The growth inhibition zone obtained was ranged from 15 to 25 mm (Table 1). Only flower extract had the significant inhibitory effect against gram-negative (*E. coli, P. aeruginosa* & *K. pneumoniae*) and gram-positive (*S. aureus*) bacteria. Leaf extract had antibacterial activity against all the gram negative bacteria tested. Extracts of fruits and seeds have shown the inhibitory effect against only two of the gram negative bacteria (*P. aeruginosa* & *K. pneumoniae*) which are used for this assay. These studies revealed that the gram negative (*P. aeruginosa* & *K. pneumoniae*) bacteria were more sensitive to all the extracts than the gram positive bacteria (*S. aureus*) (Table 1). The inhibitory effect of the combined extracts (leaf, fruit and seed extracts) had less effect against the microorganisms tested as compared to that of individual extracts. *S. aureus* was found to be resistant to all extracts except flower extract. The extracts of dried leaves and seed powders prepared in ethyl acetate have shown broad antibacterial activity. Fresh fruit and seed extract had no activity against *E. coli* (Table 2). Dried leaf extract had lost antibacterial properties against *P. aeruginosa*. *S. aureus* was sensitive to dried seed extract. All these data demonstrated that extract of fresh plant parts had more antibacterial activity as compared to that of dried plant parts (Table 2). Further, only fresh flower, leaf and fruits had more antibacterial activity compared with standard streptomycin.

Table 1. Antibacterial potential of ethylacetate extract of *Nyctanthes arbor - tristis* fresh flowers, leaves, fruits and seeds

Plant extract (300 µl)	Diameter of zone of inhibition (mm)			
	E. coli	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
Flowers	25	19	22	14
Leaves	20	21	20	R
Fruits	R	20	17	R
Seeds	R	15	16	R
Combined extract	19	19	21	R
Streptomycin* (10 µg/disc)	16	R	18	22

*R – Resistant., * Standard*

Table 2. Antibacterial potential of ethyl acetate extract of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds

Plant extract (300 µl)	Diameter of zone of inhibition (mm)			
	E. coli	*P. aeruginosa*	*K. pneumonia*	*S. aureus*
Leaves	19	R	17	R
Fruits	21	18	19	R
Seeds	17	24	15	13
Combined extract	19	19	21	R
Streptomycin* (10 µg/disc)	16	R	18	22

*R – Resistant., * Standard*

Table 3. Antibacterial potential of chloroform extract of *Nyctanthes arbor - tristis* fresh flowers, leaves, fruits and seeds

Plant extract (300 µl)	Diameter of zone of inhibition (mm)			
	E. coli	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
Flowers	22	R	26	R
Leaves	17	18	14	R
Fruits	14	20	14	R
Seeds	R	26	16	15
Combined extract	20	21	17	13
Streptomycin (10µg/disc) *	16	R	18	22

*R – Resistant., * Standard*

Antibacterial activities of chloroform extract

Antibacterial activities of fresh flower, leaves, fruits and seeds chloroform extract was almost similar to that of ethyl acetate extract. However, *E. coli* which was resistant to ethyl acetate fruit extract was found to be sensitive to

chloroform fruit extract (Table 3). Similarly, combined chloroform extract have shown inhibitory effect against all the microorganisms tested. *P. aeruginosa* and *K. pneumoniae* were found to be more sensitive against flower and seed extract respectively. *S. aureus* was found to be sensitive to seed and combined extracts. Chloroform extract of dried leaves, fruits and seeds have shown a strongest antibacterial activity against *E. coli, P. aeruginosa* and *K. pneumoniae. E. coli and P. aeruginosa* were most sensitive to the fruit and seed extracts respectively. *S. aureus* was less sensitive to leaf and combined extract.

Table 4. Antibacterial potential of chloroform extract of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds

Plant extract (300 µl)	Diameter of zone of inhibition (mm)			
	E. coli	*P. aeruginosa*	*K. pneumonia*	*S. aureus*
Leaves	15	18	13	11
Fruits	27	20	16	R
Seeds	20	24	17	R
Combined extract	20	22	19	11
Streptomycin* (10 µg/disc)	16	R	18	22

*R – Resistant., * Standard*

Table 5. Dosage dependent effect of ethyl acetate extract of *Nyctanthes arbor - tristis* fresh flowers, leaves, fruits and seeds against some selected bacteria

Plant parts	Extract (µl)	Diameter of zone of inhibition (mm)			
		E. coli	*P. aeruginosa*	*K.pneumoniae*	*S. aureus*
Flowers	100	10	15	17	R
	200	23	17	19	R
	300	25	19	22	14
Leaves	100	R	R	R	R
	200	12	R	14	R
	300	20	21	20	R
Fruits	100	R	R	R	R
	200	R	14	R	R
	300	R	20	17	R
Seeds	100	R	R	R	R
	200	R	14	R	R
	300	R	15	16	R
Streptomycin*	10µg	16	R	18	22

*R – Resistant., * Standard*

Table 6. Dosage dependent effect of ethyl acetate extract of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds against some selected bacteria

Plant parts	Extract (µl)	Diameter of zone of inhibition (mm)			
		E. coli	*P.aeruginosa*	*K.pneumoniae*	*S.aureus*
Leaves	100	10	R	R	R
	200	15	R	R	R
	300	19	R	17	R
Fruits	100	R	12	R	R
	200	16	17	13	R
	300	21	18	19	R
Seeds	100	R	R	R	R
	200	15	22	14	R
	300	17	24	15	13
Streptomycin*	10µg	16	R	18	22

*R – Resistant., * Standard*

Phytochemical composition

Phytochemical compositions of *Nyctanthes arbor - tristis* leaves, fruits and seeds were studied by specific qualitative tests (Table 9). Nine different compounds were identified in seeds. Leaf and fruits have been found to accumulate eight various secondary metabolites. The major secondary metabolites accumulated were alkaloids, phytosterols, phenolic compounds, flavonoids, steroids, cardiac glycosides and saponins. However, alkaloids and steroids were absent in leaf and fruit respectively.

Glycosides content have been further investigated by thin layer chromatography. The number of glycosidic bands appeared ranges from 3 to 5. Maximum five glycosidic bands were appeared in seed extract. Leaf and fruit extracts have shown the presence of 4 and 3 glycosidic bands respectively. Leaf and seed glycosidic banding patterns were found to be identical. The banding pattern of fruit did not match to those of leaf and seed glycosidic patterns. Glycosidic bands were common to all the materials. Glycosides band was exclusively present in fruit extract.

Table 7. Dosage dependent effect of chloroform extract of *Nyctanthes arbor - tristis* fresh flowers, leaves, fruits and seeds against some selected bacteria

Plant parts	Extract (µl)	Diameter of zone of inhibition (mm)			
		E. coli	*P.aeruginosa*	*K.pneumoniae*	*S.aureus*
Flowers	100	R	R	13	R
	200	20	R	21	R
	300	22	R	26	R
Leaves	100	R	R	R	R
	200	R	13	R	R
	300	17	18	14	R
Fruits	100	R	R	R	R
	200	12	13	12	R
	300	14	20	14	R
Seeds	100	R	13	R	R
	200	R	22	12	11
	300	R	26	16	15
Streptomycin*	10µg	16	R	18	22

*R – Resistant., * Standard*

Table 8. Dosage dependent effect of chloroform extract of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds against some selected bacteria

Plant parts	Extract (µl)	Diameter of zone of inhibition (mm)			
		E. coli	*P.aeruginosa*	*K.pneumoniae*	*S.aureus*
Leaves	100	R	10	R	R
	200	12	13	11	10
	300	15	18	13	11
Fruits	100	R	14	R	R
	200	23	16	14	R
	300	27	20	16	R
Seeds	100	R	20	R	R
	200	16	22	R	R
	300	20	24	17	R
Streptomycin*	10µg	16	R	18	22

*R – Resistant., * Standard*

Table 9. Phytochemicals composition of *Nyctanthes arbor - tristis* leaf, fruit and seed

Phytochemicals	Plant extract		
	Leaf	Fruit	Seed
Alkaloids	-	+	+
Phytosterols	+	+	+
Phenolics	+	+	+
Tannins	+	+	+
Phlobatannins	-	-	-
Flavonoids	+	+	+
Steroids	+	-	+
Cardiac glycosides	+	+	+
Saponins	+	+	+
Terpenoids	-	-	-

Table 10. Spectrophotometric analysis of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds glycosides mixtures

Wavelength	Peak area		
	Leaf	Fruits	Seeds
205	-	75.82	-
227	313.98	-	-
234	-	-	368.18
282	98.85	19.40	-
313	-	-	148.44

Table 11. Phenolics content of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds

Plant parts	Phenolic content (mg/g dry wt)
Leaves	0.167
Fruits	0.084
Seeds	0.336

Phenolics of *Nyctanthes arbor - tristis* dried leaves, fruits and seeds were extracted and determined quantitatively. Phenolic content markedly fluctuates among different plant parts. Phenolic content (mg/g dry weight) was found to be maximum in seeds and minimum in fruits. Leaf phenolics content was observed maximum value (0.167 mg/g dry weight). In conclusion, seed phenolic content (mg/g dry weight) was almost 2 and 4 fold higher than that of leaf and fruit's phenolic content respectively.

Spectroscopic analysis of the melanin

After purification with organic solvents (chloroform, ethyl acetate and ethanol), acid hydrolysis and repeated precipitation the yield of melanin extracted from fruits and seeds were 0.5 g/100g dry weights. Ultraviolet spectrogram of both melanin from fruit and seed are presented in Fig.1 Melanin solution in 2M NaOH, pH 7.5 exhibiting strong optical absorbance in a wide spectral range (190 - 800 nm). The absorption spectrum of the melanin shows a typical peak at 285 nm.

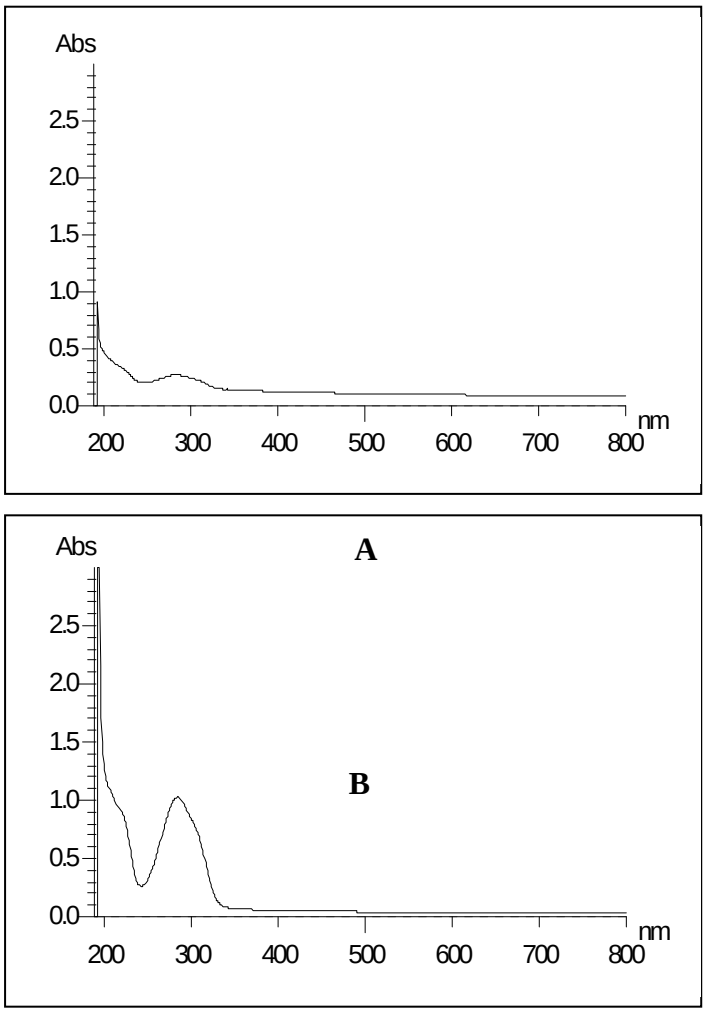

Fig. 1 Ultraviolet - visible spectrogram of fruit [A] and seed [B] melanin.

Effect of temperature on stability of melanin

The effect of temperature on melanin stability was studied. The melanin solutions were incubated for specific time period at temperature 25º, 50º and 75ºC. Fruit melanin have shown small increase in absorption at 285 nm, up to 2 hours incubation at all the temperature. At 3 hour, the absorption was decreased. Similarly seed melanin has shown small variation in absorption at different temperatures. Comparatively seed melanin had the higher absorption values than the fruit melanin at 225 nm.

Effect of oxidant on stability of melanin

The seed melanin exhibited very high absorbance at 285 nm following the treatment with oxidant ($K_2Cr_2O_7$ and $KMnO_4$ solution). Four more absorption peaks were also observed in the range of 220 - 600 nm. In case of fruit melanin, the absorption spectrum was obtained in the range of 200 - 800 nm. However no absorption peak was

observed at 285 nm. A peak at 275 nm was observed in the presence of $KMnO_4$, however, it was absent in the presence of $K_2Cr_2O_7$.

Table 12. Effect of temperature on stability of fruit melanin

Temperature		Absorbance (nm)			
(˚C)	Time (h)	0 hour	1 hour	2 hours	3 hours
25		0.236	0.259	0.327	0.288
50		0.256	0.294	0.290	0.266
75		0.365	0.337	0.272	0.360

Table 13. Effect of temperature on stability of seed melanin

Temperature		Absorbance (nm)			
(˚C)	Time (h)	0 hour	1 hour	2 hours	3 hours
25		1.519	1.281	1.331	10.00
50		1.206	1.087	1.260	1.039
75		1.276	1.220	0.881	1.527

Table. 14. Effect of oxidant to stability of fruit melanin

Oxidant	Concentration (µg/ml)	Melanin (absorbance)					
		λ = 223	λ = 275	λ = 307	λ = 341	λ = 398	λ=711
$K_2Cr_2O_7$	8.3	0.477	-	0.250	-	-	-
	16.9	0.494	-	0.557	-	-	-
$KMnO_4$	8.3	-	1.09	-	0.427	0.607	-
	16.9	-	1.02	-	0.528	0.681	0.261

Table. 15. Effect of oxidant to stability of seed melanin

Oxidant	Concentration (µg/ml)	Melanin (absorbance)				
		λ = 207	λ =220	λ =286	λ =411	λ=532
$K_2Cr_2O_7$	8.3	10.00	-	10.00	-	0.125
	16.9	10.00	-	10.00	-	-
$KMnO_4$	8.3	10.00	-	10.00	0.335	-
	16.9	-	1.044	1.830	0.271	-

Effect of metal ion on stability of melanin

The fruit melanin has shown the decrease in the absorbance at 285 nm while the seed melanin exhibited small increase in absorption following the treatment with $MgCl_2$ and $ZnSO_4$ (Table 14 and 15). In addition to 285 nm, both the fruit and seed melanin exhibited 3 - 4 other absorption peaks in the range of 200 - 400 nm. No absorption was observed at 285 nm following the treatment of both of the melanin with $FeCl_3$.

Table .16. Effect of metal ion to stability of fruit melanin

Metal ion	Concentration (mg/ml)	Melanin (absorbance)				
		λ = 199	λ =224	λ =275	λ =283	λ=376
$FeCl_3$	0.005	-	-	-	-	0.056
$MgCl_2$	5	10.00	-	-	0.136	-
$ZnSO_4$	5	-	10.00	0.294	-	0.252

Table .17. Effect of metal ion to stability of seed melanin

Metal ion	Concentration (mg/ml)	Melanin (absorbance)			
		λ = 226	λ =285	λ =310	λ =370
$FeCl_3$	0.005	-	-	0.200	-
$MgCl_2$	5	0.853	1.172	-	-
$ZnSO_4$	5	10.00	1.214	-	0.230

DISCUSSION

The interest in medicinal and aromatic plants has been shown all over the world for safe and effective constituents of plant products and in particularly the presence of active principles of medicinal plants. In the present study antibacterial properties of *Nyctanthes arbor - tristis* fresh and dried flowers, leaves, fruits and seeds have been investigated. Earlier studies [24] have shown antibacterial properties of *N. arbor-tristis* fresh and dried flower ethyl

acetate and chloroform extract. Both extracts had significant antibacterial activity against gram-negative and gram-positive bacteria. The results of present studies have indicated that fresh plant material extracted with ethyl acetate and chloroform have shown slightly higher antibacterial activity compared to the extract of dried plant materials. Though, it is observed that these extracts had strongest antibacterial activity against gram-negative bacteria. However, fresh flower and dried ethyl acetate extract has shown reasonable activity against gram-negative bacteria. Antibacterial effect of the extract was dosage - dependent and varies with types of extracts. Only fresh flower ethyl acetate extract at 100 µl have shown considerable antibacterial activity against gram negative bacteria. Chloroform extract of fresh plant material (flower, leaves, fruits and seeds) at 100 µl had virtually no activity against microorganisms tested. However, *K. pneumoniae* is sensitive to fresh flower extract. Similarly, ethyl acetate and chloroform extracts of dried leaf, fruit and seed had no activity against gram-negative (*E. coli* and *K. pneumoniae*) and gram - positive (*S. aureus*) bacteria. *P. aeruginosa,* however, is sensitive to all the dried plant extracts. The differences in the antibacterial effects of plant materials are expected due to differences in plant parts. Moreover the fresh plant part's extracts are more effective as compared to the dried ones. The drying may have caused conformational changes to occur in some of the chemical constituents present in these plant parts [25]. Also, the results indicate more or less similar antibacterial activity between extracts obtained with ethyl acetate and chloroform, and these extracts are equally effective against gram - negative bacteria as compared to those of gram positive bacteria. The reason for the different sensitivity between gram-positive and gram - negative bacteria may be attributed to morphological differences between these microorganisms. Cell wall of gram-negative bacteria is consisted of phospholipids and lipopolysaccharide components, hence impermeable to lipophilic solutes [26]. In spite of this permeability barrier, ethyl acetate and chloroform extract exert strong inhibition on gram-negative bacteria. Gram-positive bacteria however should be more susceptible because of only an outer layer of peptidoglycan have shown resistance against both ethyl acetate and chloroform extract. However, flower, leaf and seed extracts exerts some inhibition on gram - positive bacteria and have broader spectrum of inhibitory activity than the other (fruit) extract [21].

Phytochemical analysis of leaf, fruit and seeds of *Nyctanthes arbor - tristis* revealed the presence of phytosterols, phenolics, tannins, flavonoids, cardiac glycosides and saponins. Fruit and seed extracts revealed the presence of alkaloids while that of it is absent in leaf extract. Similarly, leaf and seed extract have shown the presence of steroids while it is absent in fruit extract [27]. Presence of glycosides and phenolics in leaf, fruits and seed measured spectrophotometrically. Moreover, thin layer chromatographic analysis of glycosidic mixtures has shown the presence of 3 to 5 various glycosides. Secondary metabolites present in *Nyctanthes arbor - tristis* are known to be biologically active. Tannins have been found to form irreversible complexes with proline rich proteins, resulting in the inhibition of the cell protein synthesis. This activity was exhibited against test organisms with all plant extracts. Tannins have important roles such as stable and potent antioxidant [28]. Flavonoids are phenolic structures containing one carbonyl group. Flavonoids complexes with extra - cellular and soluble protein and with bacterial cell wall. Thus, they exhibit antibacterial activity [29]. Spectral properties of *Nyctanthes arbor - tristis* fruit and seed melanin are similar to those of *Osmanthus fragrans*, *E. coli* and fungal melanin, and exhibits absorption spectra between 200 - 800 nm [30]. Seed and fruit melanin of *Nyctanthes arbor - tristis* are similar to melanin from black tea leaves and synthetic melanin [18]. The *Nyctanthes arbor - tristis* fruit and seed melanin pigments shown the spectral properties than that of natural melanin following treatment with $KMnO_4$ and $K_2Cr_2O_7$. Metal ion effects studies are consistent to those of earlier reported in *Osmanthus fragrans* [19], indicated that Fe^{++}, Mg^{++} and Zn^{++} ions were found to preserve melanin color.

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