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Short Communication

Preliminary Characterization of Melanin Isolated from Fruits and Seeds of *Nyctanthes arbor-tristis*

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Abstract

Melanin from *Nyctanthes arbor-tristis* fruits and seeds was isolated and purified by alkaline extraction, acid hydrolysis and organic solvents. Each, fruit and seed yielded melanin 50 mg/100g tissue weight. The melanins of fruit and seeds were analyzed by UV-visible and infrared (IR) spectroscopy for characterization. The IR spectrum of fruit and seed melanins did not match with each other and the seed melanin could be distinguished by their sharp peat at 285 nm from the fruit melanin in IR spectrum. Two melanins have shown absorptions above and below 1600 cm^{-1} in their respective IR spectrum. The melanins thus characterized were tested for their stability after incubating at different temperatures and in presence of oxidants ($KMnO_4$ and $K_2Cr_2O_7$) and metal ions (Mg^{++} and Zn^{++}) for a specified period. The stability of the melanin was assessed by measuring changes in the absorbance at 285 nm. The results revealed that the fruit melanin was more stable at 50 ºC while the seed melanin was quite stable at 25 ºC. However, their stability was markedly affected by oxidants and metal ions.

Keywords: Melanin; *Nyctanthes arbor-tristis*; UV-visible spectroscopy; IR-spectroscopy; Oxidant.

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1. Introduction

Melanins are the most common pigments studied extensively [1, 2]. It is an endogenous, non-hemoglobin derived, brown-black pigment that is widely produced by animals, plants and microorganisms [3]. Previously, several reports have been published describing biological properties of melanins from chestnut, sunflower seeds, black beans and grapes [1]. Melanins play several important biological roles, such as thermoregulation,

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chemoprotection, camouflage and sexual display [4]. In animals and plants coloration of seeds, berries, flowers, human skin or hair is essentially due to melanin [1]. Melanin is used commercially as a component of photo protective creams, for anti-melanoma therapy and also reported to possess immunopharmacological properties [5].

Melanins have high commercial value in food industries as natural additives and colorants. Craze for natural colorants and food additives have been increased tremendously among consumers as synthetic colorants are frequently perceived as undesirable or harmful. Plants are always considered to be a best source of natural pigments such as melanin and others. Wang *et al.* [6] have recently isolated and characterized melanins from seeds of *Osmanthus fragrans* and studied its biological properties to be used as food colorants [6]. The aim of the present study is to explore *Nyctanthes arbor-tristis* (Oleaceae) fruits and seeds as a source of melanins. *N. arbor-tristis* commonly known as Night Jasmine or Coral Jasmine is used in traditional medicine [7, 8]. Previously we reported antibacterial properties of different parts [9], but this is for the first time we report here the isolation of melanins from this plant. Both fruits and seeds yielded melanins in sufficient amounts. Melanins were characterized by UV and IR spectroscopy and tested for stability at different temperature and in presence of oxidants and metal ions.

2. Materials and Methods

2.1. *Plant materials*

Fresh fruits and seeds of *N. arbor-tristis* were collected from Arcot in Vellore district of Tamil Nadu during the month of December and February and immediately brought to the laboratory, School of Biotechnology, Chemical and Biomedical Engineering, VIT University, Vellore, Tamil Nadu, India. Melanin was isolated from dried seeds and fruits during the month of March, 2007.

2.2. *Isolation and partial purification of melanin*

Melanin was isolated and purified according to the procedures published previously [3, 6, 10]. Dried seed and fruit powder (10 g each) were mixed with 75 ml of 2M sodium hydroxide, pH 10.5 for 24 hours. Then, the mixture was centrifuged at 8000 rpm for 15 minutes. The supernatant was acidified with 2M hydrochloric acid to pH 2.5 and incubated at room temperature for 2 hours, and centrifuged at 4000 rpm for 15 minutes. The precipitate, thus obtained was purified by acid hydrolysis using 6M hydrochloric acid at 100˚C for 2 hours to remove carbohydrates and proteins. The precipitate was then treated subsequently with chloroform, ethyl acetate and ethanol to wash away lipids. The precipitate was dried at room temperature and re-dissolved in 2M sodium hydroxide and again centrifuged at 8000 rpm for 15 minutes. The supernatant thus obtained was precipitated by adding 1 M hydrochloric acid. The precipitate containing melanin was washed with distilled water and stored at room temperature.

2.3. *UV-visible and IR spectra of melanin*

50 mg of partially purified melanin was 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. For infrared spectroscopic analysis, 50mg of dried melanin was mixed with KBr prior to spectral analysis.

2.4. *Effect of temperature, oxidants and metal ions on the stability of melanin*

Heat stability of melanin was determined following the 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.

The effects of oxidants and metal ions were evaluated according to Wang *et al.* [6] except minor modification in their concentration used. An aliquot of melanin (50 mg/15 ml) 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 a final volume of 3 ml. The absorbance of the homogenate was measured at λ max.

3. Results and Discussion

3.1. *Seed and fruit melanin*

After purification with organic solvents (chloroform, ethyl acetate and ethanol), acid hydrolysis and repeated precipitation the yield of melanin isolated from fruits and seeds were 50mg/100g dry weights. The isolated melanins were subjected to ultraviolet-visible (UV) and infra red (IR) spectroscopy for their characterization. Their UV spectra are presented in Fig. 1. UV spectrum of fruit melanin did not match with seed melanin, likewise their IR-spectra also slightly differs from each other. While the fruit melanin did not show any absorption at 285 nm, seed melanin showed a sharp absorption peak at 285 nm. Like UV spectra of the two melanins studied here their IR spectra were also differs from each other. Both fruit and seed melanin showed absorption below and above 1600 cm^{-1} in IR region, however number of peaks appeared below 1600 cm^{-1} were more than the peaks appeared above 1600 cm^{-1}. The peaks at 2926 and 2927 cm^{-1} appeared in fruit and seed melanin's IR spectra which indicate the presence of saturated carbons, while signals at 3046 (fruit) and 3452 (seed) shows the presence of alcohol or phenol groups. The signals from 2925 to 1700 cm^{-1} indicated the presence of alkynes. Fruit melanin had a signal at 1124 cm^{-1} corresponding to the carbonyl, alcoholic and phenolic groups. In contrast, seed melanin had no peak at 1124 cm^{-1}. Spectral properties of the two melanins reported here are found to be very similar to the melanins reported from *Osmanthus fragrans*, black tea, *E. coli* and fungi [3, 6, 11, 12]. All these earlier studies have revealed

that melanins exhibited absorption spectra between 200-800 nm with a characteristic λ285 nm.

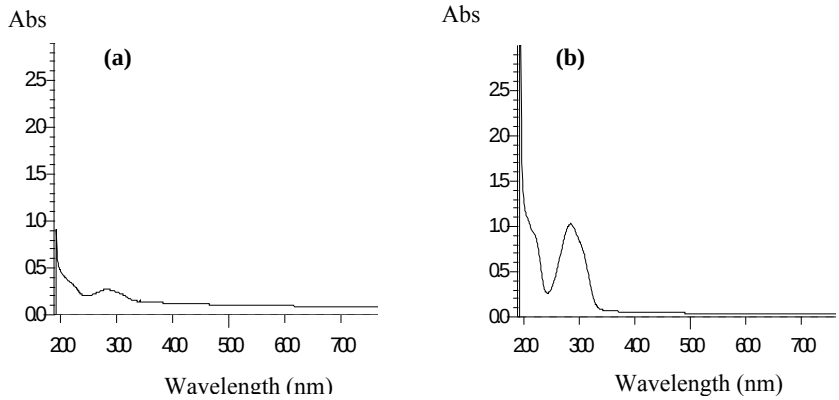

Fig. 1. Ultraviolet-visible spectrogram of *N. arbor-tristis* (a) fruit and (b) seed melanins.

3.2. IR Spectra of seed and fruit melanin

Table 1 summarizes the data of IR spectral analysis of fruit and seed melanins. IR spectra of fruit and seed melanins have an informative character. For instance, the bands 1651

Table 1. Infra red spectral analysis of *N. arbor-tristis* fruit and seed melanin.

	Fruit melanin		Seed melanin
Signals wave length (cm^{-1})	Groups	Signals wave length (cm^{-1})	Groups
3405.92	Alcohol/Phenol	3451.94	Alcohol/Phenol
2925.99	Saturated carbon	2927.01	Saturated carbon
		2062.06	Alkynes
1650.80	Carboxylic acid C=O stretch Amide C=O stretch	1635.04	Carboxylic acid C=O stretch Amide C=O stretch
1513.73	Carboxylic acid	1512.32	Carboxylic acid
1455.63	Aldehydes and ketones	1455.85	Aldehydes and ketones
1123.76	Carbonyl, alcohol phenolic		
1032.64	Amines	1033.45	Amines

cm^{-1} and 1635 cm^{-1} observed near 1700 cm^{-1} in IR spectra of fruit and seed melanins can be ascribed to the free carboxylic group COOH and the band 1456 cm^{-1} to the ionized carboxylic group COO^-. The bands in the spectral regions 1100 to 1050 and 1240 to 1200 cm^{-1} were assigned to the carboxylic, alcoholic and phenolic groups, respectively. The bands in the region 1600 cm^{-1} appeared due to the vibrations on the plane of the CH=CH bonds. The results of the IR spectral analysis of fruit and seed melanins presented here

completely matches with those reported previously by Liu *et al.* [16]. They have broadly studied the properties of melanins by spectrophotometers.

3.3. *Effect of temperature, oxidants and metal ions on stability of melanin*

The effects of temperature, oxidants and metal ions on stability of melanins were assessed by measuring the changes in the rate of absorption at 285 nm. Figs. 2a and 2b depict the effects of temperature (0 to 75ºC) on stability of fruit and seed melanins. In general, seed melanins showed higher absorption values than fruit melanin at all the temperature used. At 25ºC, rate of absorption for fruit melanin increased significantly while for seed melanin it remained more or less similar during the first 2h of incubation period. At 50ºC, rate of absorption for fruit melanin increased considerably till 1 h, thereafter declined linearly. In contrast, rate of absorption for seed melanin markedly fluctuates during the incubation period (3h). At 75ºC, the rate of absorption for both the melanins studied increased significantly at the end of incubation period after rise and fall in absorption rate during early incubation period. These results suggests that fruit melanin is more stable at 50ºC whereas seed melanin at lower temperature 25 ºC.

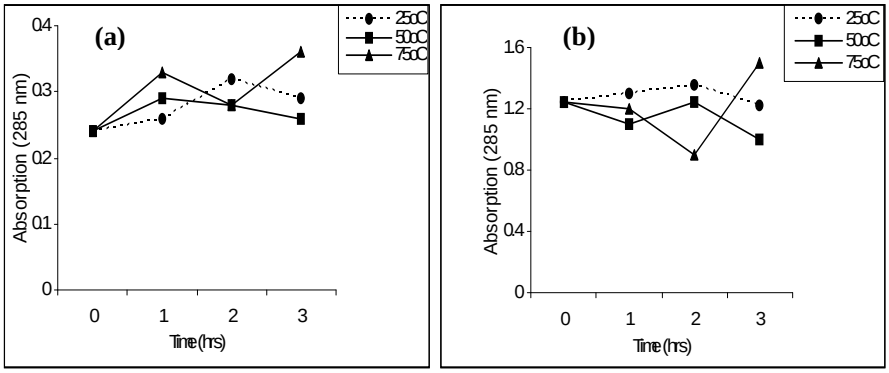

Fig. 2. Effect of temperature on stability of *N. arbor-tristis* (a) fruit and (b) seed melanins. The data are the average of three independent experiments.

Effects of oxidants ($K_2Cr_2O_7$ and $KMnO_4$) and metal ions ($MgCl_2$ $ZnSO_4$) on stability of the two melanins are presented in Table 2 and 3. Fruit melanin that did not show any absorption at 285 nm had shown highest absorption peak at 285 nm following the treatment with antioxidants used. Seed melanin, however, showed usual peak at 285 nm but with higher absorption values after antioxidant treatment. Seed melanin treated with $K_2Cr_2O_7$ and $KMnO_4$ (higher concentration) also showed maximum absorption rate at 207 nm. Besides, an absorption peak at 411 nm was also observed following $KMnO_4$ treatment of the seed melanin. Effects of oxidants on fruit melanin were appeared to be more

complex. Fruit melanin treated with $K_2Cr_2O_7$ showed significant absorption at 223 and 307 nm while that treated with $KMnO_4$ wider absorption at 275, 341 and 398 nm.

Metal ions also affect the spectral properties of the fruit and seed melanins (Table 3). Interestingly metal ions did not affect the rate of absorption at 285 nm. Treatment of melanins with $MgCl_2$ $ZnSO_4$ resulted in increase in absorption properties that is reflected by appearance of additional peaks at 199, 224, 275 and 376 nm. From the results of the study it is clear that *N. arbor-tristis* fruits and seeds yielded comparable amount of melanin to that of *O. fragrans* seeds but their stability could not be defined [6]. *N. arbor-tristis* fruit and seed melanin was dissolved in 2 M sodium hydroxide (pH 7.5) solution as they showed good solubility in neutral and basic aqueous solution, but very poor solubility in acidic aqueous solution and in most organic solvents [1, 13-15].

Table 2. Effects of oxidants on *N. arbor-tristis* fruit and seed melanin*.

Oxidant	Con. (μgmL^{-1})	Absorbance (nm)							
		207	223	275	286	307	341	398	411
Fruit melanin									
$K_2Cr_2O_7$	8.3	-	0.48	-	10.0	0.25	-	-	-
	16.9	-	0.49	-	10.0	0.56	-	-	-
$KMnO_4$	8.3	-	-	1.09	10.0	-	0.43	0.61	-
	16.9	-	-	1.02	1.83	-	0.53	0.68	-
Seed melanin									
$K_2Cr_2O_7$	8.3	10.0	-		10.0	-	-	-	-
	16.9	10. 0	-		10.0	-	-	-	-
$KMnO_4$	8.3	10.0	-		10.0	-	-	-	0.34
	16.9	-	1.04		1.83	-	-	-	0.27

*Data are the average of the three independent experiments.

Table 3. Effect of metal ion on *N. arbor-tristis* fruit and seed melanin*.

Metal ion	Con. (mg/ml)	Absorbance (nm)				
		199	224	275	283	376
Fruit melanin						
$MgCl_2$	5	10.0	-	-	0.136	-
$ZnSO_4$	5	-	10.0	0.29	-	0.252
Seed melanin						
$MgCl_2$	5	-	0.85	-	1.172	-
$ZnSO_4$	5	-	10.0	-	1.214	0.230

*Data are the average of the three independent experiments.

4. Conclusion

N. arbor-tristis fruits and seeds appeared to be a good source of melanins however their stability could not be defined well with respect to the high temperature, oxidants and metal ions. The stability could be achieved by converting natural melanins into new kind

of melanin. Chemical structure of natural melanin has not been established yet, because of its complicated polymeric structure and type diversity. Therefore, extensive analysis of melanins by IR and other advanced analytical techniques such as crystallography, NMR spectroscopy and MALDI are needed in order to understand the structure and type diversity of melanins.

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