



Research Article

Studies on Effect of Light Source on the Stability and In Vitro Antioxidant Activity of Dyes and Extracts from Annatto (*Bixa orellana* L.) Seed

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ABSTRACT

Isolation of the oleoresin, total lipid, hexane soluble matter (fat), bixin powder and norbixin formulation from annatto (*Bixa orellana*.L) seed was carried out and the effect of exposure to natural sun light and artificial incandescent light on the stability of bixin, norbixin and their solutions was evaluated. The results could be correlated with data on in-vitro antioxidant activity of the extracts by Ferric reducing power and ABTS assays. Solvent extraction of annatto seed by different methodologies yielded by oleoresin (6.1%), total lipid (5.93%), oil (3.0%) and a natural red dye (1.2%). The stability was higher in dye powders than in solutions when exposed to artificial incandescent light or sunlight. The order of activity as determined by ferric reducing power was, seed oil > oleoresin> total lipid> norbixin> bixin. However, the order of antioxidant activity according to ABTS assay was oleoresin > seed oil > norbixin > total lipid > bixin.

Keywords: bixin, norbixin, annatto extracts, effect of light, antioxidant activity, ferric reducing power, ABTS assay

1. Introduction

Annatto (*Bixa orellana*) plant is a native of central and tropical South America belonging to the Bixaceae family. The natural colour extracted from annatto seed is widely used in dairy products, butter and cheese. In India, annatto is reported to be cultivated in Andhra Pradesh, Assam, Karnataka, Kerala, Orissa, Maharashtra, Tamil Nadu and West Bengal (The wealth of India, 1988). Extraction methods of natural dye from annatto seeds was reviewed by Preston and Rickard (1980).

Analysis of bixin and other degradation products by high pressure liquid chromatography coupled with photodiode array detector was compared with the Uv-visible spectrophotometric methods (Scotteret al., 1998). Annatto dye was reported to be the second most economically important natural colour after saffron in the world and given the colour code as E-160B by the European Union.

World production of annatto seeds is estimated to be 14,500 metric tons per year; and Indian contribution stands at about 500 metric tons. In general, the pigment content of the seeds varies between 5% in hemispheric fruits, 3% - 3.58% in conical type and 1.5-2% in oval fruits (Satyanarayana, Prabhakara Rao, & Rao, 2003). Herzig and Faltis (1923) reported that bixin was the monomethyl ester of an unsaturated dicarboxylic acid. Catalytic hydrogenation studies led them to conclude that bixin contains nine conjugated double bonds. The structures of bixin and norbixin isomers (Fig. 1) were later proposed by Kuhn and Winterstein (1932). It is well known that the polyene chain in carotenoids is responsible for their instability. Susceptibility towards oxidation by various agents such as oxygen and peroxides, addition of electrophiles including H⁺ ion and Lewis acids, temperature and light has been

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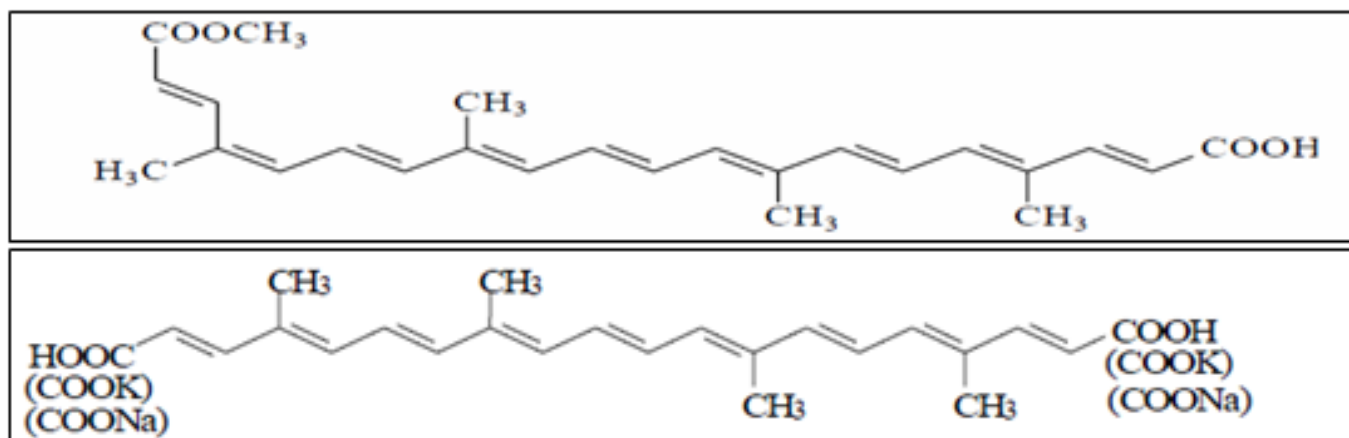


Fig 1. Structures of principle components of annatto dye (a) Cis - Bixin (b) Trans - Norbixin and their potassium or sodium salts

documented in the literature (Mercadante and Pfander, 1998; Mascioet al., 1990). Annatto, especially norbixin, is susceptible to oxidation, particularly when applied in powdered form, although some foods can have a stabilising effect (Berset and Marty, 1986; Collins, 1992; Levy and Rivadeneria, 2000). Loss of bixin was about 10% during the first 2-3 weeks when stored in packaging materials possessing different oxygen transmission rates, which however showed some stabilising effect afterwards and the losses were about 0.04% per day (Carvalho et al., 1993). Bixin degradation followed first-order kinetics and the half-lives showed greater stability in systems of intermediate and high water activity. It was postulated that the ability of water to exclude oxygen from liposoluble materials by surface adsorption, hydrogen bonding with hydroperoxides, inactivate metal catalysts, reduce free radicals and lower the stability of singlet oxygen are some of the possible mechanisms.

The stability of colour in commercial water-soluble annatto solutions was studied by exposing them to temperatures in the range 90-140°C for durations up to 450 min (Ferreria et al., 1999). During heating, increased yellowness and decreased redness was observed. The colour degradation followed first-order reaction kinetics, whereas norbixin degradation was a second-order reaction. The storage stability of water-soluble annatto formulations in the orange RTS model systems was studied by Prabhakara Rao et al. (2002). Annatto oleoresin was found to be more stable than the dye powder during storage for a period of 1 year. Highest loss (60%) was observed for the powder at ambient temperature exposed to diffused daylight compared to ambient temperature conditions in dark (54%) and at refrigerated conditions (5-8°C) in dark (23%) (Balaswamy et al., 2006). It was observed that light was the main degradation factor for bixin (Najar et al., 1988). The effect of light (900 lux intensity) on the stability of a microencapsulated water soluble extract of bixin was studied (Prentice- Hernandez and Rusig, 1999).

Antioxidant activity of annatto extracts was earlier studied by Haila et al. (1996). They studied the formation of hydroperoxide of triglycerides in the presence of extracts of annatto, γ -tocopherol, lutein, and lycopene. Annatto and γ -tocopherol inhibited the formation of hydroperoxide, whereas lutein and lycopene were pro-oxidants. Addition of γ -tocopherol retarded to loss of carotenoid, which in turn was more effective in inhibiting the hydroperoxide formation. Norbixin was the only carotenoid that inhibited the oxidative deterioration of lipids in olive oil and the oil-in-water emulsions stored at 60°C, which was similar to the activity of δ -tocopherol in stored oil (Kiokias and Gordon 2003). Antioxidant capacity of annatto pigment was compared with permitted food antioxidants in lipid peroxidation (Martinez-Tome et al., 2001; Cardarelli et al., 2008). Annatto was reported to have higher antioxidant capacity than either butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) for preventing deoxyribose damage by hydroxyl radicals. In aqueous media, annatto exhibited a lower antioxidant activity than propyl gallate but was more effective at peroxide scavenging than BHA or BHT.

The present work is taken up to carry out a systematic study to determine the stability of annatto dyes during exposure to direct sunlight and simulated conditions of incandescent light and also evaluate the antioxidant activity of annatto dyes and extracts. Antioxidant activity of bixin, norbixin formulation, oleoresin, total lipid and the fat was assessed using DPPH radical scavenging activity, ferric reducing power, and ABTS assays.

2. Materials and Methods

2.1. Materials

Annatto (*Bixa orellana* L.) seed (25 kg) were procured from M/s. Girijan Cooperative Corporation Ltd., Vishakhapatnam, India. Laboratory grade chemicals and solvents such as hexane, acetone, chloroform, sodium hydroxide, potassium hydroxide, propylene glycol, potassium carbonate and

monoglyceride from M/s Sd Fine Chem Ltd, Mumbai, India were used for extraction of dye and preparation of norbixin formulation. Analytical grade chemicals namely potassium ferricyanide, 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) and 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) ABTS were purchased from M/s Sigma Aldrich Fine Chemical, Bangalore, India.

2.2. Extraction of Oleoresin, Total Lipid, Fat and Annatto Dye

Total lipid of annatto seed was extracted using a solvent mixture of chloroform: methanol (2:1) as stated by Folch, Lees and Stanley (1957). The fat from the seed powder was obtained by Soxhlet extraction method using hexane. Microcrystalline bixin of 88% purity was recovered by a patented process of CFTRI, Mysore (Prabhakara Rao et al., 2004). The oleoresin, total lipid and fat were stored in glass vials under nitrogen and the dye powder (bixin) was packed in aluminium foil laminate pouches with nitrogen flushing.

2.3. Preparation of Water-Soluble Annatto Dye Formulation (Norbixin)

Norbixin (potassium salt) dye was prepared by using crystalline annatto dye by saponifying and solubilising bixin in an aqueous solution of potassium hydroxide applying CFTRI, Mysore, process (Venkateshwara Rao et al., 1980). After complete conversion of bixin into norbixin, the dye solution was subjected to vacuum drying in a small tray. The obtained flakes of norbixin dye were ground to fine powder and packed in aluminium foil laminate pouches with nitrogen flushing. All samples were stored at 3 ± 1 °C for further experiments.

2.4. Effect of Exposure to Artificial Light and Sunlight on Bixin and Norbixin

Bixin dye powder and norbixin, chloroform solutions of bixin and norbixin solutions in 0.1 M NaOH were exposed to artificial light. In case of powders 0.5 g is taken in a petri dish and exposed to artificial light in cardboard chamber (1' x 1' x 1') with all inside walls pasted with white paper for good reflection. A 60 watt bulb (tungsten filament, electric power, 230 V A/c) is arranged at the top of the chamber. For dye solutions, calculated quantities of dyes were weighed and solubilised in chloroform for bixin and 0.1 M NaOH for norbixin respectively, to obtain 0.5 g/100 ml. The temperature of the chamber was 52 ± 2 °C with a relative humidity of 35%. The solution was transferred to 6 test tubes and placed in a test tube stand and exposed. The dye samples were exposed to the artificial light for 15, 30, 60, 120, 180, and 300 min, after which the samples were analysed for bixin/norbixin content. The experiment was repeated by exposing the dyes and dye solutions to direct sunlight between 11.30 am to 4.30 pm for receiving direct and good radiation (temperature, 39 ± 2 °C and relative humidity, 45%).

2.2. Estimation of Bixin and Norbixin in Annatto Dyes

Bixin and norbixin in control formulations and in samples after exposure to light conditions were analysed to assess the effect of light. The estimations were carried out by a spectrophotometric method utilizing the E 1% values of 3230 and 2850 in chloroform and 0.1 M NaOH respectively (Reith and Gielen, 1971). Approximately 0.03 g of bixin and norbixin was dissolved in 50 ml chloroform and 0.1M NaOH respectively from which suitable aliquots were further diluted for obtaining optical density in the range of 0.3 to 0.8 in a UV-visible spectrophotometer (Shimadzu UV-160 A UV-Vis Spectrophotometer, Kyoto, Japan). Similarly, for bixin and norbixin solution samples optical density was measured for suitably diluted aliquots in chloroform and 0.1M NaOH respectively.

2.3. Antioxidant Activity

2.3.1. DPPH Radical Scavenging Activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of annatto seed extracts viz., bixin, nor-bixin, oleoresin, total lipid and seed oil was measured by dispersing varying quantities of 2, 4, 6, 8 and 10 mg in 1ml methanol (Nanjo et al., 1996). Methanolic solution of DPPH (0.004%) (4ml) was added and vortexed (Remi, Mumbai, India) for 30 sec. The contents were incubated at room temperature (RT) 30 ± 2 °C for 30min. The decrease in colour intensity during incubation was measured in terms of optical density at 517nm. The control was prepared without the annatto extract.

2.3.2. ABTS Assay

ABTS (2, 2-azinobis {3-ethyl-benzothiazoline-6-sulfonic acid}) reagent solution, 7μM concentration and the aqueous potassium persulphate (2.45μM) were prepared (Re et al., 1999). ABTS stock solution was prepared by mixing equal quantities (1:1) of above and vortexed for 30 sec. Further, the stock solution was incubated at RT in dark for 12-16 h and stored in refrigerator at 3 ± 1 °C. The ABTS stock solution 1ml was diluted with 40 ml of distilled water to get an optical density (control) of 0.700 (± 0.02) at 734 nm at RT. Annatto extract (0.01-0.6 mg) was added to 3.0 ml of diluted ABTS solution and incubated at RT in dark for 6 min and OD was recorded at 734nm. The % ABTS assay was calculated based on control and sample ODs and compared with BHT.

2.3.3. Ferric Reducing Power (FRP)

The ferric reducing power of the annatto extracts were measured according to the reported method of Yildirim et al. (2001). The extracts (0.1-10 mg) were added to the mixture of 1ml methanol and water in case of norbixin, 2.5 ml of phosphate buffer in different test tubes. Potassium ferricyanide (1%) solution (2.5ml) was added to the test tubes and vortexed for 30 sec. Later, the contents were incubated for 20min in hot water (50°C). After incubation, 2.5ml of trichloro acetic acid

(10%) was added and centrifuged at 8000 rpm for 10min. The aliquot of 2.5ml was mixed with 2.5ml of distilled water and 0.5ml of ferric chloride (0.1%). The absorbance of the solutions was measured by reading optical density at 700nm. The ferric reducing power of annatto extracts was compared with that of BHT.

2.3.4. Statistical Analysis

Annatto dye quality, effect of light source and in vitro antioxidant activity of annatto dyes and extracts based on ABTS assay and Ferric reducing power were determined in triplicate and mean values with standard deviation (SD) were computed using MS Excel 2007. Error bars indicating the standard deviation were depicted in plots drawn for effect of light source on stability of annatto dyes.

3. Result and Discussion

3.1. Yield and Quality of Bixin

The photographs of annatto pods, seed and dye are presented in Fig. 2. Extraction of bixin by using soxhlet apparatus, yielded bixin dye 9 g (1.2%) on average of per batch with a bixin content of 88% as observed from spectrophotometric analysis.

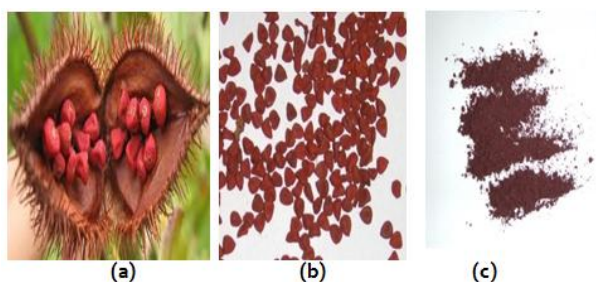


Fig 2. Annatto (*Bixa orellana*) (a) pods (b) seeds and (c) dye

3.2. Effect of Source of Light (Artificial light/Sunlight)

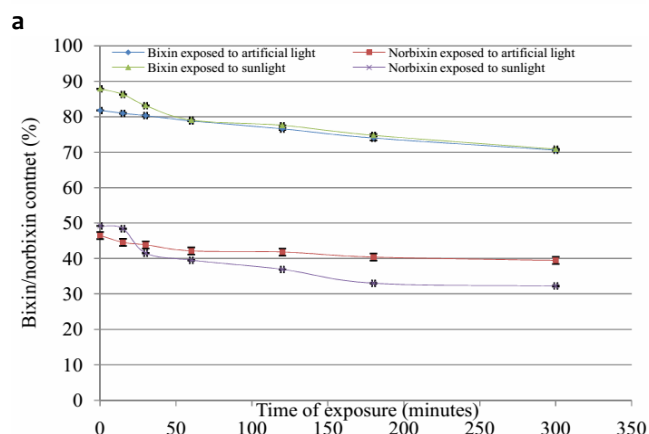
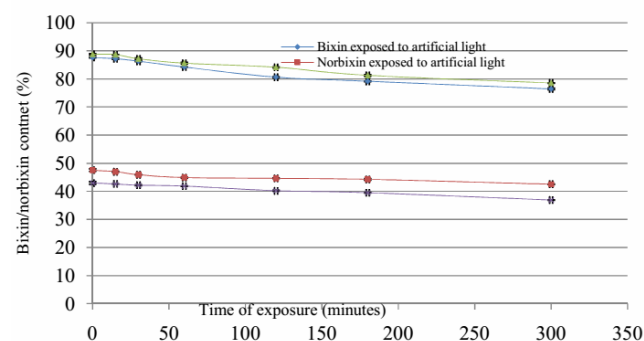
3.2.1. Effect of Artificial Light and Sunlight on Stability of Bixin and Norbixin in Dye Powders

During the experiment the loss of bixin and norbixin was high in sunlight when compared to artificial light. The results are in good agreement with earlier studies of Najar et al. (1988). The effect of sunlight and artificial light on bixin/norbixin content in dye powders during the exposure period of 5 h is presented in Figure 3a. During the initial period of 15 minutes, the loss was very minimal. Losses were higher in norbixin content (14.13%) when exposed to sunlight than artificial light. Bixin content decreased to similar levels in both type of exposures. The percent retention dye in dye powders was 87.19, 89.48% and 88.47, 85.86% when exposed to artificial incandescent light and sunlight respectively for bixin and norbixin respectively.

3.2.2. Effect of Artificial Light and Sunlight on Stability of Bixin and Norbixin Solutions

Changes in bixin and norbixin contents in dye solutions during exposure to light conditions are presented in Fig. 3b. Maximum percent loss in norbixin

content (32.9%) was observed in dye solution after exposure to sunlight for 5 h. Similarly, the values were higher (19.35%) for bixin in sunlight when compared to artificial light (13.73%). In general, light (sunlight and artificial) had destructive effect on bixin or norbixin content. Colour loss was about 10% after 3 h when exposed to artificial light and similar losses were observed with in 1 h when exposed to sunlight. The percent retention of dye in solutions was 86.26, 84.92% and 81.64, 65.52% when exposed to artificial incandescent light and sunlight for bixin and norbixin respectively.



B

Fig 3. Effect of light source on the principal colouring components of annatto a) dye powders; b) dye solutions

3.3. Antioxidant Activity

The dyes and extracts were observed to possess good antioxidant activity. However, it was found that DPPH method was not suitable for measurement of antioxidant activity in case of bixin, norbixin, oleoresin and total lipid as the extracts were also contributing to absorbance at 517 nm and the optical density was increasing with increase in concentration. Hence the data was not considered for presentation. The data obtained by other assay methods was comparable and hence taken for analysis.

3.3.1. Antioxidant Activity by Ferric Reducing Power (FRP)

Activity was measured for bixin and norbixin dye powders at different concentrations. 10 mg bixin showed OD (activity) of 0.630 (Table 1) whereas for norbixin 1.03 OD was observed with 0.8 mg. Similarly the activity (OD) was 0.174 and 0.111 with 2 mg and 0.1

mg bixin and norbixin respectively. Antioxidant activity assayed by ferric reducing power (FRP) method in dye powders was similar with minimum activity, when bixin and norbixin was used 2 mg and 0.1 mg respectively.

Table 1 . Antioxidant activity of dye powders and annatto extracts by FRP^a

Quantity of dye/extract (mg)	Antioxidant activity by Ferric reducing power (Optical density)				
	Bixin	Norbixin	Oleoresin	Total lipids	Seed oil
0.1		0.111 ± 0.003	0.312 ± 0.002		
0.2		0.307 ± 0.005	0.326 ± 0.007	0.442 ± 0.003	
0.4		0.545 ± 0.004	0.779 ± 0.013	0.632 ± 0.002	0.839 ± 0.003
0.5					1.336 ± 0.010
0.6		0.754 ± 0.005	0.882 ± 0.003		1.409 ± 0.004
0.8		1.014 ± 0.003	1.034 ± 0.004	0.803 ± 0.004	
1.0					
1.2				1.111 ± 0.003	
1.6				1.368 ± 0.003	
2	0.174 ± 0.003				
4	0.189 ± 0.001				
6	0.249 ± 0.002				
8	0.440 ± 0.004				
10	0.630 ± 0.006				

^a Values are mean of triplicate analyses ± SD

Table 2 . Antioxidant activity of dye powders and annatto extracts by ABTS assay^a

Quantity of dye/extract (mg)	Antioxidant activity by ABTS Assay (%)				
	Bixin	Norbixin	Oleoresin	Total lipids	Seed oil
0.010					39.84 ± 0.04
0.012			31.50 ± 0.056		
0.016			39.17 ± 0.035		
0.02			54.34 ± 0.066	22.32 ± 0.06	48.37 ± 0.08
0.04		56.57 ± 0.07	81.04 ± 0.061	28.83 ± 0.04	70.07 ± 0.05
0.06		73.47 ± 0.06		36.74 ± 0.07	79.37 ± 0.03
0.08		90.61 ± 0.10		42.01 ± 0.04	86.66 ± 0.10
0.1	28.10 ± 0.11	98.82 ± 0.08		42.94 ± 0.04	91.00 ± 0.19
0.2	52.73 ± 0.15				
0.4	84.95 ± 0.06				
0.6	90.17 ± 0.06				

^a Values are mean of triplicate analyses ± SD

The activity was around 1.0 in oleoresin and total lipids at 1 mg concentration. Maximum activity of 1.4 was observed in total lipids and seed oil when applied with 1.6 and 0.6 mg concentration respectively. Ferric reducing power indicated activity in the order seed oil > oleoresin > total lipid > norbixin > bixin. In general, seed oil showed higher antioxidant activity when measured by FRP, which may be due to the presence of natural antioxidants such as tocopherols (mainly unsaturated δ -tocotrienol) which is present in the lipid fraction of annatto seeds (Frega, Mozzon, & Bocci, 1998). In an earlier study (Balaswamy et al., 2006), annatto oleoresin was found to be more stable than the dye powder during storage.

In this study, the dye powder could be prepared from the oleoresin by removal of the fatty oil.

3.3.2. Antioxidant Activity by ABTS Assay

Oleoresin and norbixin dye powder showed higher antioxidant activity when ABTS assay was performed (Table 2). Among all samples tested, oleoresin showed

highest antioxidant activity at a very low concentration of 0.04 mg (81.04%) as measured by ABTS assay. Norbixin at 0.1 mg concentration showed inhibition of 98.82% whereas, bixin showed 28.10% for a similar concentration. Seed fat and norbixin dye powders shown almost similar and higher antioxidant activity of 98.82% and 91.0%, respectively at a concentration 0.1 mg. Bixin dye powder and total lipid were found to possess the minimum antioxidant activity of 28.1% and 42.94% respectively, at a concentration of 0.1 mg. The order of antioxidant activity of annatto extracts according to ABTS assay was oleoresin > seed oil > norbixin > total lipid > bixin. In both methods oleoresin and seed oils were the extracts with higher antioxidant capacity, whereas bixin possessed the lowest activity.

Annatto dyes and extracts possessed considerable antioxidant activity. Both the studies of exposure to light and antioxidant activity can be positively correlated that norbixin showed higher activity and showed lower stability and the converse is true for bixin, which showed higher stability and lower activity.

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