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Research Article

PRODUCTION OF BETALAINS FROM HAIRY ROOT CULTURE OF *BETA VULGARIS* AND ITS USE IN PARACETAMOL SYRUP AS A NATURAL COLOURANT

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ABSTRACT

Objective: The present study was aimed to extract betalains from hairy root culture of *Beta vulgaris* and its use in pharmaceutical formulations as a colorant.

Methods: Hairy roots were initiated using different strains of *Agrobacterium rhizogenes* such as A.2/83, A.20/83, A.4, and LMG 150; LMG 150 was found to initiate a large number of hairy roots, and betalain content was estimated. Paracetamol syrup was prepared using extracted betalains as a colorant at different concentrations of 10 and 30 mg/150 ml. Stability studies were carried out at a different temperatures such as 25°C, 30°C, and 40°C) and light (dark, 1000 and 2000 lux) for 45 days.

Results: In case of a concentration of 10 mg/150 ml syrup, the effects of temperature such as 25°C, 30°C, and 40°C and dark condition on the degradation of betalains were found to be 48%, 88%, and 100% in 45 days, respectively. The effects of temperature such as 25°C, 30°C, and 40°C at light 1000 lux on degradations of betalains were found to be 81% and 98% at 25°C and 30°C in 45 days, respectively, and 100% at 40°C in 30 days and at 2000 lux were found to be 100% at 25°C and 30°C in 30 days and 100% at 40°C in 10 days. The similar levels of degradation rate were observed with a concentration of 30 mg/150 ml.

Conclusion: Experimental data demonstrated that formulation with betalains exhibited better stability at the dark condition and lower temperature.

Keywords: *Beta vulgaris*, Betalains, *Agrobacterium Rhizogenes,* Paracetamol syrup.

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INTRODUCTION

Color is one of the first characteristics perceived by the senses and is indispensable for the identification and acceptance of the product. Today color has become one of the major constituents of food and pharmaceutical products [1]. The main objective of adding colour to food and pharmaceutical products is to improve marketability and aestheticity [2]. Colors are of two different origins such as synthetic and natural. Synthetic colors mainly belong to xanthene, azopyrazolone, triarylmethane, and indigoid class of compounds, which are highly water soluble and most of them have high stability toward light, temperature, acids, alkalis, etc [3]. Natural colors are materials extracted, isolated, or otherwise derived from plants, animals, or minerals that are capable of imparting a color when added to the formulations. Important classes of natural colors are carotenoids, tetrapyrrole, phycocyanin, anthocyanin, and indolic biochromes [4]. Natural colors provide more a more natural look to products with respect to the glossy and brightness of synthetic colors [5].

Hairy root culture is one of the techniques of plant tissue culture remain unsurpassed as the choice for model root system due to their fast growth rate and biochemical stability [6]. It is a genetic transformation of *Agrobacterium rhizogenes* and involved the transformation of t-DNA to the plant cells [7]. In certain cases, the level of secondary metabolite production has been observed higher than the nontransformed roots and hence, one of the widely used methods for the production of root-derived secondary metabolites [8].

Red beet is native to the coasts of Mediterranean, Europe, America, and India which is the major producers [9]. *Beta vulgaris* has been well known for centuries as an attractive food color composed of major red pigment betacyanin and minor yellow pigment betaxanthin belonging to Chenopodiaceae family. Roots of the *B. vulgaris* used as sedative and emmenagogue leaves known to possess diuretic, anti-inflammatory and seeds are used as diuretic, expectorant, aphrodisiac, and emmenagogue [10,11]. The major advantages of betalains as dietary antioxidants are their bioavailability, which is greater than most flavonoids, and their superior stability in comparison to anthocyanin [12].

Artificial dyes tend to produce free radicals responsible for carcinogenesis whereas natural color eliminates free radicals. For these reasons, synthetic dyes have been progressively banned for use in pharmaceutical products is being replaced by natural colors [13]. This prompts us to take up this study to evaluate the effect and stability of betalains as a colorant in paracetamol syrup.

MATERIALS AND METHODS

Plant material

Seeds of *B. vulgaris* were obtained from Indo-American hybrid seeds, Bengaluru. It was identified and authenticated by Dr. Madhava Chetty, Assistant Professor, Botany Department, Sri Venkateswara University, Tirupati. A voucher specimen was deposited in the Department of Pharmacognosy (BV-54/2017).

Preparation and sterilization of culture media

Murashige and Skoogs medium was prepared as per the standard procedure using stock solutions of nutrients followed by the addition of supplements. pH of the medium was adjusted to 5.8 using 1N HCl/1N NaOH before the addition of 1% agar. The solution was poured into flasks, plugged with nonabsorbent cotton, and sterilized in an autoclave.

Maintenance of *A. rhizogens* culture

Four different strains of *A. rhizogens* such as A.4, A.2/83, A.20/83, and LMG 150 were maintained in yeast mannitol broth medium.

Development of seedlings
Seeds of beetroot were washed in running tap water to remove surface particles. Seeds were treated with 70% ethanol for 15 s and then with 0.1% mercuric chloride for 5 min and finally washed with sterile distilled water. The sterilized seeds were transferred onto MS basal media for the development of aseptic seedlings.

Initiation of hairy roots
The leaves along with the petiole portion from seedlings were removed under aseptic conditions; an injury was made at different points using a sterile needle. The injured parts were treated with a smear of *Agrobacterium* culture and subsequently incubated in the dark on MS gelled medium. After the initiation of hairy roots, the cultures were made free of the bacterium by adding the antibiotic carbenicillin at 500 mg/l and then subcultured to MS semi solid medium for maintenance. Subsequently, hairy roots grown in MS liquid medium and incubated on the shaker at a speed of 90 rpm at 25°C in the dark.

Extraction of betalains
About 10 g of fresh tissues were added to 100 ml of 0.1% acidic methanol and was blended in vertex mixer. The extract was filtered using nylon membrane and the fresh solvent was added to the biomass residue to recover the remaining pigment. The procedure was repeated until the entire hairy root was whitish and pooled. It is then centrifuged at 10,000 g for 10 min; the supernatant was collected and concentrated under vacuum.

Estimation of betalains
Extracted betalains dissolved in 5 ml of 0.1% acidic water and the absorbance was measured using ultraviolet (UV)-Visible double beam 1710 spectrophotometer at 540 nm for betacyanins and 480 nm for betaxanthins [14]. Estimation was carried out on every 5th day till 30 days. The betalain content was calculated using the formula.

Percentage betacyanin = a/1120 × 5/fresh wt × 100/10 a=absorbance at 540 nm

Percentage betaxanthin = a^1/1120 × 5/fresh wt × 100/10 a^1=absorbance at 480 nm

Content of betalains = betacyanin + betaxanthin

Preparation of paracetamol syrup
Paracetamol syrup is prepared as per the procedure described by Kohli [15]. Briefly, the primary syrup was prepared by dissolving sucrose in 250 ml water, filtered and transferred to the mixing tank fitted with a stirrer. Sodium benzoate, methylparaben sodium, and propylparaben sodium dissolved in 17 ml of water and added to the syrup under stirring. Citric acid and disodium dissolved in 17 ml of water separately and added to the syrup. Paracetamol was dissolved separately in propylene glycol under stirring till completely dissolved and then added to the syrup. Beet color was obtained from hairy roots by extracting with 0.1% acidic methanol, filtered and centrifuged at 10,000 g for 10 min. The supernatant was analyzed spectrophotometrically for betalain content, and the beet extract was then concentrated under vacuum at 20°C to obtain a semi-solid slurry containing 100 mg of betalain per ml. The standard colorless syrup was taken in aliquots of 150 ml and each aliquot received 10 mg, and 30 mg of betalains, respectively. The syrup was mixed to obtain homogenous syrup with the help of magnetic stirrer, and initial betalain content was estimated by a spectrophotometric method as described above.

Stability studies
Stability of the prepared formulation was evaluated at different temperature and light conditions. 10 ml of stock syrup was dispensed into about 45 glass vials and incubated at a different temperature such as 25°C, 30°C, and 40°C and light (dark, 1000 and 2000 lux) for 45 days. The change in color was measured by periodically measuring the absorbance at 480 nm for betaxanthin and 540 nm for betacyanin up to 45 days [16,17].

RESULTS

Initiation of hairy roots
Hairy roots appeared at the site of infection after about 2 weeks with LMG 150, A.2/83, and A.4 strain and there was no initiation with A.20/83 strain. The morphological characters of the hairy roots of different strains were studied and recorded in Table 1. The average number of hairy roots initiated per explant was found to be greater with LMG 150 strain, and hence, hairy roots of LMG 150 strain were further grown in MS liquid medium and used for further studies (Fig. 1).

Estimation of betalains
MS media were found to support both growth (60 folds) and betalain production (1.15%). The maximum growth and betalain content were observed on the 20th day, and the results were shown in Table 2.

Stability studies
The stability of betalains in the paracetamol syrup was evaluated at different temperatures and light conditions. In case of a concentration of 10 mg/150 ml syrup, the effects of temperature such as 25°C, 30°C, and 40°C and dark condition on the degradation of betalain were found to be 48%, 88%, and 100% in 45 days, respectively. The effects of temperature such as 25°C, 30°C, and 40°C and light 1000 lux on degradations of betalain were found to be 81%, 98% at 25°C, and 30°C in 45 days, respectively, and 100% at 40°C in 30 days. The effects of temperature such as 25°C, 30°C, and 40°C and light 2000 lux on degradations of betalains were found to be 100% at 25°C and 30°C in 30 days and 100% at 40°C in 10 days. Formulation with 10 mg betalains/150 ml syrup exhibited better stability at low temperature and dark condition, whereas color was rapidly degraded at high temp and light conditions. Formulation with 30 mg concentration was also showed a similar level of degradation of betalains. The results are summarized in Tables 3 and 4, respectively.

DISCUSSION

Betalain, highly colored pigments occur abundantly in red beet are important natural colors used in various food products and are recommended to replace synthetic dyes. Some of the synthetic colors have been banned; hence, natural colors have been in great demand, but its production is hampered by several agro-climatic conditions. An alternative method to produce natural pigments is the hairy root culture technique which has several advantages such as rapid growth, increased secondary metabolite production, and genetic stability. Hence, the present investigation was focused on betalains production from hairy root cultures of *B. vulgaris.* In the present study, different strains of *A. rhizogens* such as A.2/83, A.4, A.20/83, and LMG 150 are used as different strains are known to contain variations in the left and right border of T-DNA resulting in variation in the morphological pattern of the transformed hairy root. These variations also reflected in the biochemical functions of the hairy root; thus, it is necessary to screen

Table 1: Nature of hairy roots induced with different strains of *Agrobacterium rhizogens*

Bacterial strain	Number of explants	Number of hairy roots induced	Average hairy root per explant	Morphological observation
A.4	13	27	2.07	Color: Yellowish Length: 0.2-1.25 cm
A.2/83	20	41	2.05	Color: Slight Yellowish length: 0.3-3 cm
LMG 150	15	38	2.53	Color: Reddish yellow length: 0.5-3.4 cm
A.20/83	No hairy roots induced			

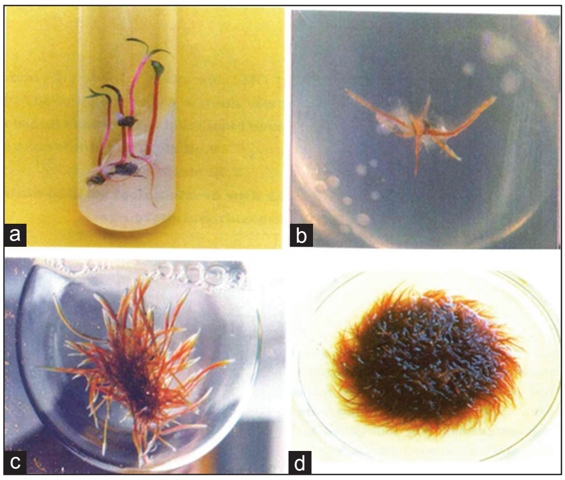

Fig. 1: (a) 2-week-old aseptic seedling (b) initiation of hairy roots (c) 10 days old hairy roots (d) 20-day-old hairy roots. Nature of hairy roots induced with LMG 150 strain

Table 2: Effect of MS media on growth rate and betalain content

Days	Fresh wt (g)±SD	Dry wt (g)±SD	Betalain content (%)±SD
0	0.02±0.045	0.0022±0.09	0.16±0.03
5	0.043±0.07	0.0037±0.07	0.21±0.04
10	1.87±0.20	0.192±0.05	0.27±0.016
15	2.82±0.36	0.294±0.03	0.65±0.13
20	5.82±0.44	0.405±0.036	1.15±0.12
25	5.51±0.28	0.520±0.04	1.02±0.27
30	4.7±0.20	0.490±0.06	0.63±0.025

The data were expressed as Mean values±SD. SD: Standard deviation

Table 3: Effect of temperature and light on degradation of betalains (%) at concentration 10 mg/150 ml of syrup

Days	Dark			1000 lux			2000 lux		
	25°C	30°C	40°C	25°C	30°C	40°C	25°C	30°C	40°C
1	0	0	0	0	0	0	0	0	0
5	7	11	23	13	17	35	21	28	63
10	16	24	41	28	36	56	42	57	100
20	23	36	52	47	51	74	78	85	-
30	31	53	86	59	66	100	100	100	-
45	48	81	100	83	98	-	-	-	-

Table 4: Effect of temperature and light on degradation of betalains (%) at concentration 30 mg/150 ml of syrup

Days	Dark			1000 lux			2000 lux		
	25°C	30°C	40°C	25°C	30°C	40°C	25°C	30°C	40°C
1	0	0	0	0	0	0	0	0	0
5	6.7	11	23	12	17	34	20	26	60
10	16	23	40	26	36	55.5	40	56	100
20	22	34	52	47	52	74	77	87	-
30	30	52	87	58	68	100	100	100	-
45	47.5	81	100	81	98	-	-	-	-

different strains to obtain maximum biomass production and secondary metabolites [18]. The hairy roots appeared after about 2 weeks with LMG150, A.2/83, and A.4 strains whereas no initiation with A.20/83 strain. The average hairy roots per explant were found to be better with LMG150, and hence, this strain was chosen for further studies. Betalain comprised two main groups, namely red violet betacyanin group and yellow betaxanthin group. Betanin accounts for approximately 75–90% of total betacyanin and the major yellow pigments are Vulgaxanthine I and Vulgaxanthine II [19]. The total content of betalains was estimated spectrophotometrically by measuring optical density at 540 nm for betacyanins and 480 nm for betaxanthins. Many synthetic colorants are known to cause carcinogenesis due to the production of free radicals, and this led to the progressive ban of synthetic dyes, which are being replaced by natural colors [12]. Paracetamol syrup was prepared using betalain extracted from hairy roots of *B. vulgaris,* but the main problem of these colors is their instability toward pH, temperature, light, radiations, and enzymes. The stability of betalains decreased with an increase in temperature, on heating the red color gradually diminishes and eventually a light brown color appears. Presence of light, air, exposure to gamma, or UV radiation increases the rate of degradation of betalains [20,21]. Stability of the prepared formulation was evaluated at different temperature and light conditions. Formulation with 10 mg betalain/150 ml syrup exhibited better stability at low temperature and dark condition, whereas color was rapidly degraded at high temperature and light conditions. Formulation with 30 mg concentration was also showed a similar level of degradation of betalains.

CONCLUSION

The findings of the present study demonstrated that low temperature and dark conditions favour the stability of betalains in paracetamol syrup. Stability could further be enhanced by encapsulating the betalains with a suitable encapsulating material.

AUTHORS CONTRIBUTIONS

Ashoka Babu VL carried out the experimental work and drafted the manuscript under the guidance of V Madhavan.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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