

Flower pigment analysis of *mirabilis jalapa*

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ABSTRACT

The betacyanin pigments were extracted from the *Mirabilis jalapa* (dark pink) flowers, of *Nyctaginaceae* family, available in Berhampur University campus of south eastern Odisha, using aquash acidified methanol (0.01 % HCl). The extracted pigments were exposed to different pH (1,4,7,10) at different temperature (4°C,25°C,35 °C & 45 °C) under dark conditions. The result showed that increase in pH, temperature or exposure to light destruct the betacyanin pigments. The antioxidant activity of methanolic extract of the test flower and the standard antioxidant ascorbic acid was assessed on the basis of the radical scavenging effect of the stable 2, 2- diphenyl-1-picryl hydrazyl (DPPH) free radical activity according to the method described by (Sadhu et al., 2003).

Key words: Betacyanin, pH, stability, temperature ,antioxidant ,DPPH. ascorbic acid, methanolic extract

1 Introduction

Mirabilis jalapa belongs to the family *Nyctaginaceae*. It is a large herbaceous plant grown in the gardens throughout south-eastern Odisha. This plant is 50-100 cm high. *Mirabilis jalapa* can be grown very easily from flower seed. It needs warm soil to germinate. It germinates in just 10-14 days. The flower seeds should be kept moist. It grows best in full sun, but can tolerate light shade if necessary.

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It needs to be watered regularly and should ideally have a fertile soil. Four O' Clock plants bloom all summer long. They have antifungal, antimicrobial, antiviral, antispasmodic and antibacterial properties (Dimayuga, 1998; Yang, 2001).

The betalain are yellow-to-red nitrogen-containing compounds, derived from the amino acid tyrosine. It comprises the largest group of water soluble pigments in the plant kingdom, only in the order Caryophyllales giving the natural colour yellow, magenta or dark pink to the flower, fruits and coloured leaves. They are included in

flavonoids or phenolic compounds (Malian-aubert et al, 2001). They are synthesized in the plant cell cytoplasm and actively transported across the tonoplast into the vacuole, where they accumulate (leng et al, 2000). Betalains are classified into red (crimson) betacyanins and yellow betaxanthins (Strack et al., 2003).

Beside the colour attraction, they are also beneficial to health by having antioxidants, antimicrobial and pharmacological properties. They are found to be a perfect alternative of the synthetic food colourant and synthetic dye. The intensity and stability of flower colour depends on the concentration of the betacyanin. Which in turn depends on various factors like structure, pigment concentration, pH, temperature, light intensity, co-pigment, metal ions, enzymes, oxygen, ascorbic acid, sugar etc. (lee, 2002, Dimayuga, 1998; Yang SW, 2001).

This paper investigated the relation of flower colour to betalain conc. in different pH level, at different temperature and its antioxidant properties.

2 Materials and methods

2.1 Collection and selection of the test flowers

Fresh blossomed flowers were collected in the early morning and placed in clear domed trays overnight under refrigerated condition. After washing them thoroughly in distilled water, they were dried by soaking them on blotting paper towel under shade in laboratory at $25^{\circ}\text{C} \pm 2$. When they were fully dried, they were homogenised into fine powder using a mortar and pestle, then stored in air tight bottles in cold at 4°C in the laboratory, and were used when required.

2.2 Extraction of pigment:

1 gm of fresh petals were taken & kept for 2/3 hours at room temperature mixed with 10ml of 0.01% HCl (v/v) in Methanol (80%), in (1:1w/v) in darkness then mixed thoroughly with a clean mortar & pestle. The mixture then filtered through whatman 110 no. filter paper and the remaining solids were washed with 0.01% HCl in methanol(80%) until a clear solution were obtained. The combined filtrates were dried using a rotary evaporator at 30°C . The aqueous concentrate was dissolved in 0.01% HCl (V/v) in DW and the solution obtained were made up to 100 ml. & stored at 4°C , For further use. (method from a guide to modern technique of plant analysis by J.B. Harborne).

2.3 Concentration of pigments of the test flowers at different developmental stages

The flowers of different developmental stages were collected i.e. S-2, S-3, S-4 and S5. The S-1 stage i.e. the bud stage was avoided. The total betalain content of each flower was estimated following the methods of Janna and Khairul et al., 2006.



Figure -1 (S-2, S-3 and S-4 stage of *Mirabilis jalapa*)

Total betacyanin content was determined by measuring the absorbance of the extract at 510nm by ph differential method using two buffer solutions one is highly acidic & other one a weak acid i.e. pH 1 and pH 4.5, using a UV-Vis spectro- photo meter. The standard solution used was cyanidin-3-glucoside and 0.1% Hcl (V/v) in methanol solution (500 mg/ml). The molecular wt. of cy-3-gl. i.e. (449.2 gm/mol).The

betacyanin was calculated using the following formulae-

$$A \times mw \times DF \times 10^3 / \sum X L$$

$$A = A_{max} - A_{min}$$

{ Mw=mol.wt , DF=dilution factor (0.2ml. sample in 2 ml.=10)

{ For Cyanidin 3 glucoside , \sum =coefficient =L X $CM^{-1} \times MOL^{-1} = 26900$ }

The concentration of betacyanin was determined by applying the Beer-lambert Law. The spectra recorded in a spectrophotometer were measured at 25 °C, against the solvent (d. w). The two buffer solution used are 1.86 gm kcl + 980 ml distilled water (pH is adjusted to 1 by adding conc.Hcl & the vol. was made up to 1 lt. by adding d.w), 54.43 gm. Of $CH_3CO_2Na.3H_2O$ in ~ 960 ml of distilled water (pH is adjusted to 4.5 by adding conc.Hcl & the vol. was made upto 1 lt by adding d.w).

The mixture of buffer & samples were homogenised & cetrifuged twice at 5000 rpm for 15 minutes. The supernatant was collected & its absorbance were measured at 510 nm by spectrophoto meter.

So total anthocyanin or betacyanin content (mg/lt) = $(A \times mw \times DF \times 1000) / (\sum \times l)$.

2.4 Phytochemical screening

For phytochemical screening the sample solutions of the test flowers were taken and tested by Benedict's reagent for testing sugar, FeCl_2 for phenol and tannins, for flavonoid test the shinoda's test was done. The respective colour was observed by adding the prescribed chemicals shown in the above methods

2.5 The effect of pH and temperature on colour stability

The effect of pH on colour stability was studied with 4 different buffer solutions at the same temperatures i.e strong acid (pH 1.0), weak acid (PH 4.0), neutral (pH 7.0) and base (pH 10.0). Buffer solution of pH 1 is prepared by mixing 50 ml of 0.2 M KCl and 134 ml of 0.2 M HCl, buffer solution of pH 4.0 is prepared by mixing 847.0 ml of 0.1 m acetic acid and 153.0 ml of 0.1 m sodium acetate, buffer solution of pH 7.0 is prepared by mixing 100 ml of 0.1 M KH_2PO_4 and 58.2 ml of 0.1 M NaOH; buffer solution of pH 10.0 is prepared by mixing 100 ml of 0.025 M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 36.6 ml of 0.1 M NaOH. pH of the solution was measured and adjusted to 1, 4, 7, 10 using 1N sodium hydroxide solution or 10% citric acid solution drop wise (a drop was measured as 1/20 of a ml. each time before use). 10 drops of each extract were added to each buffer solution and the colour of the sample solution was measured.

Samples were taken right after preparation i.e. (day 0) and after day 1, 2, 4, 6, 8, 10, 15, 20, 25 and 30. It was centrifuged at 300 rpm (956 xg) for 5 minutes and

analysed using UV-visible spectrophotometer. The absorbance at 537, 573, 627 and 628 nm was observed for the extract at pH 1, 4, 7 and 10 respectively. (data shown in Fig.II)

Secondly aliquots of the sample were placed in 10 ml capped glass vials and kept in incubator for the study of effect of different temperatures i.e (4°C, 25°C, 35°C & 45 °C), on colour stability. data shown in figure-III

2.6 Antioxidant activity (DPPH assay : free radical scavenging activity)

2.6-1 Preparation of standard solution

3 mg of ascorbic acid was dissolved in 3 ml of distilled water in 1:1 ratio. Then, it was diluted to 05, 10, 15, 20, and 25 $\mu\text{g}/\text{ml}$ by adding distilled water.

2.6-2 Preparation of test sample:

Stock solutions of samples were prepared by dissolving 10 mg of dried hydro methanolic extract of the test flower in 10 ml of methanol to give concentration of 1mg/ml. Then different sample concentrations (05, 10, 25, 20 and 25 $\mu\text{g}/\text{ml}$) were prepared by diluting it in distilled water.

2.6-3 Preparation of DPPH solution (2, 2-diphenyl-1-picryl hydrazyl)

DPPH solution was prepared by dissolving 3.4 mg of DPPH in 3 ml methanol.

Procedures

5 ml of freshly prepared DPPH solution was mixed with 5 ml of the extract solution and standard solution

(05, 10, 25, 20, and 25 µg/ml) thoroughly. These solution mixtures were kept in the dark for 30 min. The change in the Purple colour of DPPH to yellow indicated the effectiveness of scavenging activity of the extract. The absorbance of the combination was measured at 517 nm using UV-Visible Spectrophotometer and ascorbic acid was served as a positive control. The antioxidant activity of methanolic extract and the standard antioxidant ascorbic acid was assessed on the basis of the radical scavenging effect of the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH) free radical activity according to the method described by(Sadhu et al., 2003). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage of scavenging was denoted by using the formulae given below:

% DPPH radical-scavenging =

$(A_{\text{control}} - A_{\text{test Sample}}) / A_{\text{control}} \times 100$

The treatments like DPPH were applied in pigment extract of *Mirabilis jalapa* flowers to verify their antioxidant activity.

2.7-1 The effect of pH

The effect of pH on stability of the petal extracts of flowers of dark pink (*Mirabilis jalapa*) was studied with different buffers at the same temperatures, strong acid (pH 1-3), weak acid (pH 4-6), natural effluent (pH 6-7) and base (pH 10.0). The extracts produced pinkish red solutions at pH 1.0 to 5.0 range, while at pH from 5.5 to 10, the extracts produced brown to

All the data were analysed and graphically presented using Microsoft excel. The data were computed to obtain the average and standard error (\pm S.E.) with the significant level at 95% and were subjected to ANOVA to detect significant differences among seasons and among treatments ($P < 0.05$) and significant difference between means were detected following the method of (Gomez and Gomez, 1984

2.7 Result & discussion

The spectral analysis showed that In *Mirabilis jalapa*, the A_{max} were at 535-537 nm i.e. the blue-green region, showing the presence of betacyanins (J.B Harborne, 1984).

Here, maximum pigments are in S4 (230mg/gm) and minimum are in S-2(130mg/gm).The total betalain content was in the following order \rightarrow S-4 > S-3 > S-5 > S-2.Data in Figure-II

yellowish-brown solutions. Beyond pH 10, the solution was yellow or colourless. (Shown in Figure III and Table I).

Betacyanins are not as susceptible to hydrolytic cleavage as the anthocyanins studied in *Clitorea ternatea*, *Rosa indica*, *Hibiscus rosasinensis*. Betacyanins are relatively stable over the broad pH range from 3 to 7 (Jackman & Smith, 1996). Betalains

present in *Mirabilis jalapa* also stable in pH range from 3-7 supporting the view of Jackman and Smith, 1996 and Attoe & von Elbe, 1981. So, these were applied to low acidity foods as food colourants. Below pH 3.5, the A_{\max} shifted towards lower wavelength and above pH 7, it changed towards upper wavelength the betacyanin to give reddish colour shades to low acid foods. Moreover, Betacyanins can effectively be stabilised by adding ascorbic acid, which on the other hand, impairs anthocyanin stability (Shenoy, 1993).

2.7-2 Effect of temperature

Here the maximum stability was at 4°C and least at 35 °C. The colour degradation was in the order of 4°C > 45°C > 25 °C > 35 °C. Some studies reported that the rate of betalain degradation increases with increasing temperatures (Saguy et al., 1978; Havli'kova' et al., 1983; Garcí'a Barrera et al., 1998). Thermal degradation of betacyanin in betanin solutions as well as in red beet and purple pitaya juices was reported, which follows the first-order kinetic reaction (Von Elbe et al., 1974; Saguy et al., 1978; Saguy, 1979; Herbach et al., 2004b). During heat processing, betanin may be degraded by isomerisation, decarboxylation or cleavage (by heats or acids), resulting in a gradual reduction of red colour and eventually the appearance of a light brown colour (Huang & von Elbe, 1985). Brown colour shifted to yellow colour, when dehydrogenation of betanin occurs. It leads to form neobetainin. Further, cleavage

It was found that the optimum pH range for betalain stability in *Mirabilis jalapa* was at pH 5-6 confirming the view given by Von Elbe and Amundson, 1985; However, as the anthocyanins are less stable at pH values above 3 (Stintzing & Carle, 2004) makes

Hence, application of betacyanins instead of anthocyanins for colouring foods with high ascorbic acid contents may be possible. (Herbach et al., 2006).

of betanin and isobetainin, generates the bright yellow betalamic acid and the colourless glycoside (cyclo-Dopa-5-O-glycoside). It can also be induced by bases (Schwartz & von Elbe, 1983; Schliemann et al., 1999). Herbach et al. (2004) proposed that while betanin colour is maintained upon C15-isomerisation or decarboxylation, C17-decarboxylation causes a hypsochromic shift of the absorption peak from 538 to 505 nm, resulting in an orange-red colour. Betanin may be regenerated from their primary degradation products, when the extracts are kept for some time under temperature below 10 °C and pH around 5.0 (Huang & von Elbe, 1985, 1987). Betanin regeneration, which consists in a partial resynthesis of betanin from its hydrolysis products, involves a condensation of the amine group of cyclo-Dopa-5-O-glycoside with the aldehyde group of betalamic acid. Betanin is rapidly formed when both compounds are mixed in solution (Huang & von Elbe, 1985). The

activation energy for betacyanin degradation decreased with pH. This did not impair pigment application to most foods undergoing ordinary thermal treatment. For example, it lost less than 10% when the extract was acidified to pH 4 at 80°C for 5 mins (Vaillant et al, 2005). Havlikova et al, 1983 reported

2.7-3 The antioxidant effect

The decrease in absorbance of DPPH radical was caused by antioxidants, because of the progress of the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation. Data is mentioned in Figure-V.

The present results suggest that the tested plant extracts have moderate to potent antioxidant activity. A high correlation was demonstrated between colour concentration and antioxidant capacities. It was observed that flower pigments with high antioxidant properties tend to be brightly coloured data in *Figure IV*. The *Mirabilis jalapa* showed fast scavenging activity than the other flowers like *Clitoria*, *Rosa indica* and *Hibiscus rosasinensis* (Vankar and Srivastava, 2010).

It has been determined that the antioxidant effect of plant products is mainly due to radical scavenging activity of phenolic compounds such as flavonoids, polyphenols and tannins (Mishra and Srivastava et. al., 2007). The antioxidant activity of phenolic compounds is mainly due to their oxidation reduction properties, which can play an important role in

that high temperature shifted the optimum pH for betacyanin stability towards pH 6. However, anaerobic condition favours betalain stability at lower pH (Von Elbe, 1985).

adsorbing and neutralising free radicals, reducing singlet and triplet oxygen, or decomposing peroxides (Harborne, 1984). The blue coloured flowers showed fast scavenging activity within 5 min than red coloured flowers in 20 min. This may be due to the structure of flavinium ion. Blue anthocyanins like Malvidin, Delphinidin, Petunidin contain higher number of methoxy groups than in red coloured anthocyanins like Cyanidin, pelargonidin and peonidin which have more of hydroxyl groups (Andersen and Jordheim, 2006).

For flavonoids, the major factors that determine the radical-scavenging capability [Sahidi et.al,1992; Bors et.al,2002] are:

- (i) The ortho-dihydroxy structure on the B ring, which has the best electron-donating properties and confers higher stability to the radical form and participates in electron delocalization.
- (ii) The 2, 3-double bond with a 4-oxo function in the C ring, which is responsible for electron delocalisation from the B ring.

(iii) The 3- and 5-hydroxyl groups with the 4-oxo function in A and C rings, which are essential for maximum radical scavenging potential.

(iv) The 3-hydroxyl group is important for antioxidant activity. The 3-glycosylation reduces their activity when compared with corresponding aglycones.

2.7-4 Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of flavonoids, phenols, tannins and reducing

sugars in the studied test flower, showing its involvement in medicinal fields. The anti-bacterial nature of the test flower, will be confirmed by the antimicrobial test which is needed to be done later. Screening and proper evaluation of the test flowers could offer possible alternatives that may be both sustainable and environmentally acceptable

2.8 Figures and Tables

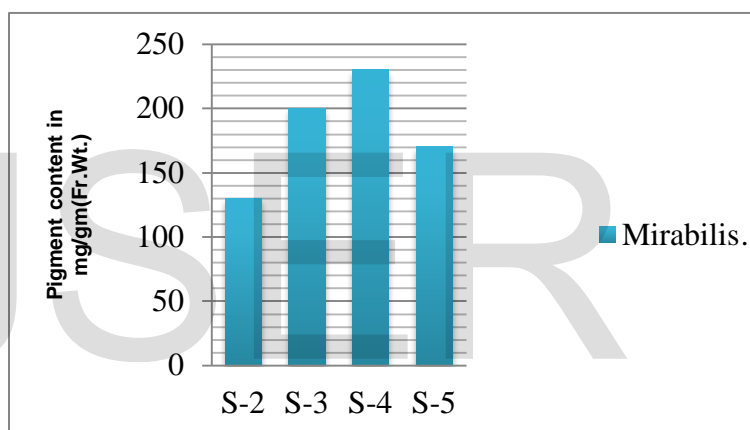


Figure II Total pigment content in different developmental stages in *Mirabilis jalapa*

(S₂-early developmental stage, S₃-petal partly opened but fully developed

S₄-fully opened flower, S₅-petals at the senescence stage)



Figure-III (a) showing effect of pH on colour of the extracts of *Mirabilis jalapa* (Before 30 days)



Figure - III (b) showing effect of pH on colour of the extracts of *Mirabilis jalapa* (after 30 days)

Table-I

Effect of pH on colour stability of the petal extract of *Mirabilis jalapa* flower

% of residual colours in diff.days at diff. pH in *Mirabilis jalapa* flower

Day	pH-1	pH-4	pH-7	pH-10
zero	75	90	80	65
1st day	74	89	77	63
2nd day	73	86	75	60
4th day	71	85	70	58
6th day	71	84	68	55
8th day	65	84.2	65	50
10th day	58	83	63	48
15th day	56	80	64	46
20thday	50	75	62	42
25thday	45	70	60	38
30thday	43	68	59	35

Figure-III Effect of pH on colour stability of the petal extract of *Mirabilis jalapa* flower

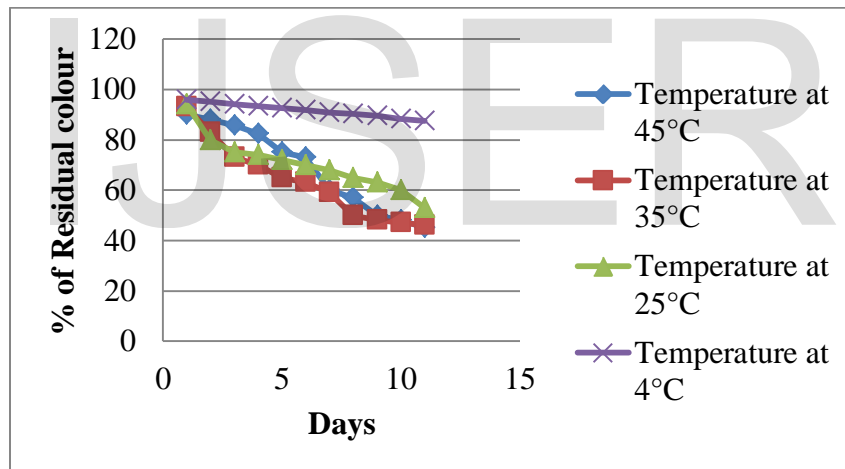
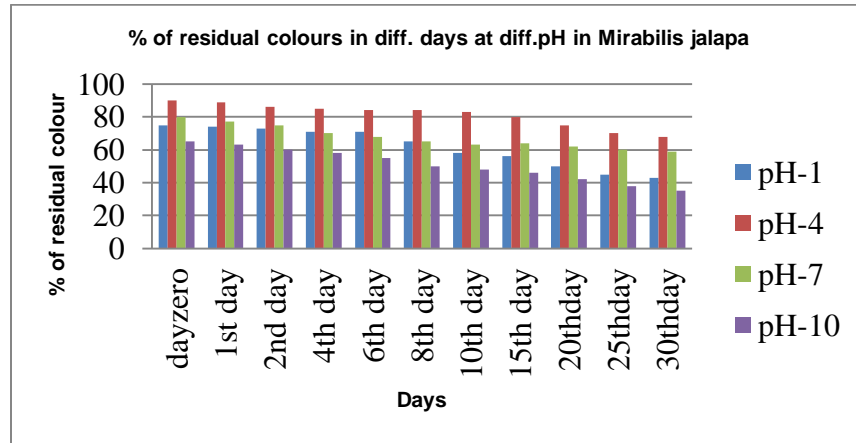


Figure IV Effect of different temperature on colour stability of *M.jalapa* floral extract

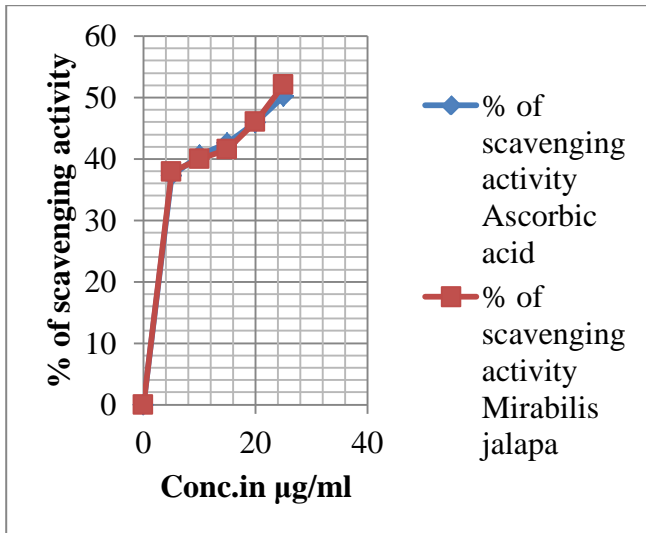


Figure- V Scavenging activity of the *M.jalapa*

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