Bull. Pharm. Sci., Assiut University
Vol. 11, Part 2, pp. 185 - 195

INVESTIGATION OF FLOWER ANTHOCYANIN PIGMENTS OF ALTHEA ROSEA CAV. AND MALVA VISCOUS-ARBOREA L. GROWING IN EGYPT

M.S. AFIFI and M. S. KARAWYA
Pharmacognosy Department, Faculty of Pharmacy,
Mansoura University, Egypt.

ABSTRACT

Six anthocyanin pigments were isolated from Malva viscous-arborea L. and five from Althea rosea Cav. (Family : Malvaceae). The identification of these pigments was achieved by paper chromatography, acid and enzymatic hydrolysis and spectral analysis. The study revealed the presence of Cyanidin3B-D-glucoxyloside-5B-D-glucoside, Cyanidin-3,5-B-D-diglucoside, Malvidin3,5B-D-diglucoside, Malvidin3-B-D-glucoside, Delphinidin-3-B-D-glucoside and Pelargonidin-3B-D-glactoside in the petals of Malva viscous-arborea L. and Cyanidin3,7-B-D-diglucoside, Cyanidin-3B-D-glucoxyloside, Delphinidin-3-B-D-galactoside, Petunidin3-B-L-rhamnoside and Pelargonidin-7B-D-glucoside in the petals of Althea rosea Cav.

INTRODUCTION

The colouring matter of the flowers of Althea rosea Cav. has drawn the attention of many workers since 1929. Sobyanin and Saakov[1] stated that this colouring matter acted as an excellent indicator. It acquired a red colour in acid medium and green one in alkaline medium with a sharp transition.

In the same time, Martin[2] isolated the pigment althaein from Althea rosea Cav. On hydrolysis, he obtained the aglycone delphinidin.

After about 44 years, Rakhim Khanov et al[3] is olated gossipicyanin (Cyanidin-3-B-D-glucoside-4-B-D-xyloside) and cannabinin ($C_{26}H_{29}O_{16}Cl$) from the same flowers.

During their work on the flower pigments of many malvaceous plants, Tomas and Stolerin[4] showed by paper chromatography that the flowers of Althea rosea Cav. contained delphinidin, pelargonidin, malvidin and cyanidin pigments.

Reviewing the current literature, nothing could be traced concerning the study of the flower pigments of Malva viscous-arborea L. For this reason and due to the nonsatisfactory informations reported about the pigments of the flowers of Althea rosea Cav. it was deemed of interest to study the pigments of both plants growing in Egypt.

EXPERIMENTAL

Material :

1- The fresh petals of flowers of Malva viscous-arborea L. and Althea rosea Cav. were collected at May 1979 from one year old cultivated plants in Experimental Station, Faculty of Agriculture, Mansoura University. The collected petals of each plant were separately cut to small pieces by hand.

2- Preparation of emulsin enzyme[5] :

10 g of the powdered seeds of sweet almond was triturated with 100 ml of water in mortar. The supernatent solution containing the enzyme was purified by passing over a column of Sephadex G 50 and used in the study.

Reagents[6] :

1- 5 % w/v aluminum chloride in spectroscopic methanol.

Investigation of Flower Anthocyanin Pigments of Althea Rosea Cav. and Malva Viscous-Arborea L. Growing in Egypt.

2- Methanolic HCl :

3.3 ml hydrochloric acid (36.5 %) diluted to 100 ml with spectroscopic methanol.

3- Aniline Phlhalate :

0.99 g of aniline and 1.63 g phthalic acid were dissolved in 100 ml n. butanol.

4- Standard Fehling's Solution[7] :

Solution No. 1 : Dissolve 34.64 g of copper sulphate crystals (A.R.) in water containing a few drops of dil. sulphuric acid and dilute with water to 500 ml.

Solution No.2 : Dissolve 60 g of sodium hydroxide and 173 g of Rochelle salt (sod. pot, tartarate) in water, filter if neceasary through sintered glass funnel and make up the filtrate and washings to 500 ml.

Keep the two solutions separately in tightly stoppered bottles and mix exactly equal volumes immediately before use.

Apparatus :

Pye- Unicam SP-1800 Ultra-violet Spectrophotomater with a Unicam AR 25 series Linear Recorder, Cambridge, England.

Methods :

Preparation of Extract[8] :

500 g of the fresh petals of Malva viscous-arborea L. and of Althea rosea Cav., was separately macerated in methanol containing 1 % hydrochloric acid for 24 hours with occasional shaking. The procedure of maceration in methanol was repeated several times till the petals became almost colourless (4 x 500 ml, Each). The combined methanolic extract of each sample was concentrated to 20 ml under reduced pressure at 40°C.

Paper Chromatography : Both methanolic concentrates were separately chromatographed on Whatmann 3MM papar using the following solvent systems[9]:

1- n-Butanol-Acetic acid-Water (4 : 1 : 5) (BAW)

2- n-Butanol-2 N HCl (1 : 1) (BH)

3- 1 % hydrochloric acid

4- Acetic acid-HCl-Water (15 : 3 : 82) (AHW)

The results obtained are listed in Table 1.

Isolation of Anthocyanin Pigments :

The anthocyanin pigments of both samples were isolated by preparative paper chromatography on Whatmann, 3MM paper MM, applying the material (methanolic concentrates previously prepared) bandwise. Development was carried out by 1 % HCl and BAW in the case of Malva and Althea, respectively. After development, the bands were located in each chromatogram by their fluorescence under UV light (Table 1) . Each band was cut and extracted with methanol containing 0.1 % HCl. The extract of each band (ca 200 ml) was concentrated under vacuum to about 20 ml at 40°C. Each concentrate was purified by repeated preparative PC as described above. Each pigment was isolated from the respective extract by saturation with ether (80 ml), then allowed to stand overnight in an ice-chest, where a dark reddish-brown crystalline material was obtained, filtered and then washed with ether. Each pigment was subjected to the following study :

Identification of Anthocyanin Pigments :

A- Spectral analysis : About 1 mg of each pigment was dissolved in 5 ml methanol. The resulting solution, in each case, was subjected to spectrophotometric determination before and after the addition of one drop of methanolic Al Cl_3 (Geissman, 1962)[8] The results obtained are recorded in Table 1.

B- Controlled acid hydrolysis[10]:

About 10 mg of each purified pigment was dissolved in 5 ml ethanol and 5 ml 4 N HCl and refluxed on boiling water-bath for 30 minutes. Samples (0.3 ml, each) were removed every 5 minutes, and examined by PC as mentioned before. The results obtained are presented in Table 2.

Investigation of Flower Anthocyanin Pigments of Althea Rosea Cav. and Malva Viscous-Arborea L. Growing in Egypt.

C- Alkaline hydrolysis for acylated pigments [11]:

About 1 mg of each pigment was dissolved in 5 ml 2 N sodium hydroxide and allowed to stand for 2 hours at room temperature. The alkaline solution was then acidified by 2 N HCl and the pigment was extracted by n-butanol and paper chromatography as mentioned before.

Identification of Aglycones and Sugar Moieties :

A- Enzymatic hydrolysis [5]: About 50 mg of each pigment was dissolved in 5 ml water and the resulting solution was mixed with 5 ml emulsin enzyme for 5 hours.

B- Acid hydrolysis : About 10 mg of each pigment was hydrolysed by refluxing with 5 ml 2 N HCl on a water-bath for 30 minutes.

The aglycones resulted in both cases (enzymatic and acid hydrolysis) were extracted by amyl alcohol, evaporated to dryness under reduced pressure and dissolved in 5 ml methanol acidified with 0.1 % HCl. The absorption maxima of each aglycone was determined before and after the addition of one drop of methanolic $AlCl_3$. The methanolic solutions of aglycones were also chromatographed as mentioned before.

The sugars were extracted from the aglycone-free mother liquor (neutralised in the case of acid hydrolysis) by evaporation nearly to dryness and dissolving in pyridine (2 x 30 ml each). The concentrated pyridine extract (10 ml), in each case, was subjected to PC using the solvent system : Ethyl acetate-Pyridine-Water, 2 : 1 : 2 (EPW) [12] and adopting the descending technique. The spots were located with aniline phthalate reagent. The results obtained are presented in Table 1.

Determination of Sugar Molecules in Pigment

10 mg of each pigment was acid hydrolysed (as previously mentioned) and after separation of their aglycones by amyl alcohol, the sugar part was estimated by titration against standard Fehling's solution. The sugar moiety in each pigment was then determined, taking in consideration that 10 ml standard Fehling solution is equivalent to 50 mg glucose. The results obtained are recorded in Table 1.

RESULTS AND DISCUSSION

A good separation was achieved on papaper chromatography of the pigment extracts. The petals of Malva viscous-arborea L. contain at least six pigments while those of Althea rosea Cav.are five.This was confirmed by employing four solvent systems, whereequal number of spots was obtained in the case of each investigated species. Successful isolation of fair pure individual pigments in good yields (10 to 15 mg) was done by preparative paper chromatography.

None of the isolated pigments was found acylated because neither hydrolysis nor absorption at 308-312 nm took place on treating with alkali[11].

The pigments could be identified (Table 1 & 2) by means of their colour, fluorescence under UV light, paper chromatographic behaviourusing several solvent systems, enzymatic and controlled acid hydrolysis, absorbance shift ($\Delta\lambda$) resulted on measuring λmax of the pigment or aglycone before and after the addition of methanolic $AlCl_3$ and by comparing the results with those reported in the literature about pigments of some related species[9,13].

On the above basis, the isolated pigments could be divided into cyanidin group (Malva i & ii, Althea vii & viii), delphinidin group (Malva v ; Althea ix), malvidin group (Malva iii & iv), petunidin group (Althea x) and pelargonidin group(Malva vi& Althea xi).

The identified petal anthocyanins of Malva viscous-arborea L. are : i-cyanidin-3-BD-glucoxyloside-5-BD-glucoside, ii-cyanidin-3,5-BD-diglucoside, iii-malvidin-3,5-BD-diglucoside, iv-malvidin-3-BD-glucoside, v-delphinidin-3-BD-glucoside and vi-pelargonidin-3-BD-galactoside. Those of Althea rosea Cav. are :vii-cyanidin-3,7-BD-diglucoside, viii-cyanidin-3-BD-glucoxyloside, ix-delphinidin-3-BD-galactoside, x-petunidin-3-BL-rhamnoside and xi-pelargonidin-7-BD-glucoside.

Investigation of Flower Anthocyanin Pigments of Althea Rosea Cav. and Malva Viscous-Arborea L. Growing in Egypt.

In fact, controlled acid and enzymatic hydrolysis and measuring HR_f and $\Delta\lambda$ offered good means for the identification of the isolated pigments. For instance, pigment (i) was identified as cyanidin-3-BD-glucoxyloside-5-BD-glucoside (furfural test + ve) through controlled acid hydrolysis when 3 intermediates (HR_f 28, 40 & 45 with BAW) were obtained within 20 minutes. These intermediates may correspond to cyanidin-3-BD-glucoxyloside (sambubioside) (HR_f 34, furural test + ve), cyanidin-3-glucoside (HR_f 40, given also by intermediate of pigment ii, vii, viii) and cyanidin-5-glucoside (HR_f 45, given also by intermediate of pigment ii). The end hydrolysates of pigment (i) were cyanidin (HR_f 69, $\Delta\lambda$ 15), glucose ($HR_{f_{rh}}$ 0.62) and xylose ($HR_{f_{rh}}$ 0.85, furfural test + ve). Each of pigment (ii), cyanidin 3,5 BD diglucoside ($\Delta\lambda$5, HR_f different from i), and pigment (iii), malvidin 3,5B-D-diglucoside ($\Delta\lambda$8) yielded two intermediates. Their acid hydrolysates comprised 2 mols of glucose (10 mg reduced 2.5 & 2.7 ml Fehling solution respectively) as wall as cyanidin and malvidin (HR_f 60; $\Delta\lambda$ 0) respectively. One of the intermediates of pigment (iii) was chromatographically identical with pigment (iv) malvidin 3 BD glucoside (HR_f 40). Pigments iv, v and vi, however, yielded no intermediates, but malvidin, delphinidin (HR_f 30, $\Delta\lambda$25) and pelargonidin (HR_f 81, $\Delta\lambda$ 0) respectively as well as one mol. of glucose ($HR_{f_{rh}}$ 0.62 & osazone m.p. 204°C) for pigments iv & v and one mol of galactose ($HR_{f_{rh}}$ 0.58; galactosazone, m.p. 196°C) for pigment vi. Ten mg of each pigment on hydrolysis, reduced 1.7 ml Fehling solution proving that the sugar was one mol of glucose or galactose . This indicates that these 3 pigments are malvidin 3 BD glucoside ($\Delta\lambda$ 8), delphinidin 3 BD glucoside ($\Delta\lambda$ 10) and pelargonidin 3 BD galactoside ($\Delta\lambda$ 0) respectively.

On the same basis, the identification of the petal anthocyanins of Althea rosea Cav. was achieved. On controlled acid hydrolysis, pigment vii, cyanidin-3,7-BD-diglucoside($\Delta\lambda$ 16),

produced after 15 minutes two intermediates which may correspond to cyanidin-3-BD-glucoside (HR_f 40, produced by pigments i & ii) and cyanidin 7-BD-glucoside (HR_f 43) and then yielded cyanidin and two mols of glucose(Fehling solution as above) as end products. Pigment viii, cyanidin-3-BD-glucoxyloside (sambubioside or gossipicyanin ($\Delta\lambda$ 8) , gave one intermediate, cyanidin-3-BD-glucoside (produced by pigments i, ii & vii). Pigments ix, x & xi were identified as delphinidin-3-BD-galactoside ($\Delta\lambda$ 18), petunidin-3-BD-rhamnoside ($\Delta\lambda$0) and pelargonidin-7-BD-glucoside. On hydrolysis, they gave no intermediates but yielded delphinidin and galactose, petunidin ($\Delta\lambda$ 20 ; HR_f40) and rhamnose ($HR_{f\,glu}$ 1.2 and methyl furfural test + ve) and pelargonidin and glucose respectively.

The fact that the forementioned pigments (i to xi) yielded to enzymatic hydrolysis with emulsin, indicates that the glucoside linkage is of the B type.

In conclusion, our results concerning the flower anthocyanins of Althea rosea Cav. agree in some respect with those of Martin[2], (presence of delphinidin pigment), to those of Rakhimkhanov et al[3], (presence of gossipicyanin i,e. cyanidin 3 BD glucoxyloside, in which glucose attached to xylose through 1---- 4 linkage) and to those of Tomas and Stolerin[4], in another (presence of delphinidin, pelargonidin, malvidin and cyanidin pigments). Petunidin-3-B-L-rhamnoside was not mentioned by the above authors as well as the last authors confined the identification to the aglycone moiety only and not to the pigments.

As regards the anthocyanin content of the petals of Malva viscous-arborea L. the results obtained are reported for the first time.

Table 1- Chromatographic and Spectral Data of Anthocyanin Pigments, Aglycone and Sugar Moieties

Name	Anthocyanin Pigments Colour		HR_f			Aglycone					Sugar Moiety							
							λ max(nm)						HR_f	λ max(nm)			HR_f	Fe mle 10mg Pi.	Mols No.
	D.L	UV	BAW	BH	1%HCL	AHW	Me	Me+ $AlCl_3$	$\Delta\lambda$	Name	DL	UV	BAW	ME	Me+ $AlCl_3$	$\Delta\lambda$	Name	EPW		
Malva viscous arborea L.																				
i-Cyanidin 3BD glucoxyloside 5BD glucoside	P	R	18	10	41	55	528	533	5	Cyan	Bl	P	69	535	550	15	GL+ Xy	62 85	-	-
ii-Cyanidin-3,5-BDdiglucoside	P	R	28	4	16	42	530	535	5	Cyan	Bl	P	69	535	550	15	Gl	62	2.5	2
iii-Malvidin-3,5-BD-diglucoside	P	Pu	30	2	12	45	535	543	8	Malv	Pu	Pu	60	542	542	0	Gl	62	2.7	2
iv-Malvidin-3-BD-glucoside	P	Pu	40	16	8	30	537	545	8	Malv	Pu	Pu	60	542	542	0	Gl	62	1.7	1
v-Delphinidin-3-BD-glucoside	P		26	12	5	21	530	540	10	Del	Pu	Pu	30	545	570	25	Gl	62	1.6	1
vi-Pelargonidin-3-BD-galactoside	RR	OR	45	38	14	35	508	508	0	Pel	R	OR	81	520	520	0	Ga	58	1.6	1
Althea rosea Cav.																				
i-Cyanidin-3,7-BD diglucoside	I	R	20	6	18	50	587	543	16	Cyan	Bl	P	69	535	550	15	Gl	62	2.5	2
ii-Cyanidin-3-BD-glucoxyloside	P	R	34	24	25	52	525	533	8	Cyan	Bl	P	69	535	550	15	Gl+ Xy	62 85	-	-
iii-Delphinidin-3-BD-galactoside	P	Pu	23	12	5	20	530	548	18	Del	Pu	Pu	30	545	570	25	Ga	58	-	-
iv-Petunidin-3-BD rhamnoside	P	Pu	42	24	12	36	530	530	0	pet	Pu	Pu	40	545	565	20	Rh	100	-	-
v-Pelargonidin-7-BD-glucoside	OR	OR	48	52	14	0	508	508	0	Pel	R	OR	81	520	520	0	Gl	62	1.6	1

Cyan., cyanidin; Del., delphenidin; DL, day light; Fe., Fehling; Ga., D-galactose; Gl., D-glucose; Me., methanolic H CL; Malv., malvidin; OR., orange; P., pink; Pel., pelargonidin; pet., petunidin; Pu., purple; Pi., pigment; R., red; RR, rose red; Rh., l-rhamnose; Xy., D-xylose.

Table 2

Results of Controlled Acid Hydrolysis of Anthocyanin Pigments

Hydrolysates of Pigment / Time of Hydrolysis in min.	HR_f in BAW					
	i	ii	iii	iv	v	vi
Malva viscous-arborea L						
---	18	28	30	40	26	45
5	18+28	28+40+45	30+40+45	40+60	26+30	45+81
10	28+40+45	40+45	40+45	60	26+30	81
15	40+45	40+45	40+45	60	30	81
20	40+45+69	40+45+69	40+45+60	60	30	81
25	69	69	60	60	30	81
30	69	69	60	60	30	81
Aglycone	Cyanidin	Cyanidin	Malvidin	Malvidin	Delphinidin	Pelargonidin
Althea rosea Cav.	vii	viii	ix	x	xi	
---	20	34	23	42	48	
5	20+40+43	30+40	23+30	42+40	48+81	
10	40+43	30+40	30	40	81	
15	40+43+69	30+40+69	30	40	81	
20	69	69	30	40	81	
25	69	69	30	40	81	
30	69	69	30	40	81	
Aglycone	Cyanidin	Cyanidin	Delphenidin	Petunidin	Pelargonidin	

REFERENCES

1- N.P. Bobyanin and S.G. Saakov; J.Chem. Ind., 6, 735-6 (1929).

2- K. Martin; J.Chem. Ind., 6, 110-21 (1929).

3- Z.B. Rakhimkhanov, A.S. Sadykov, A.I. Ismailov and A.K.Karimdzhanov; Khim.Prir. (Soedin), 2 , 180 (1973).

4- M. Tomas, and S. Stolerin; Stud. Cercet. Biochim. 19, 113-19 (1976) Through C. A., 85,119586 r (1976).

5- S.I. Balbaa; Chemistry of Crude Drugs, Laboratory Manual, El-Shaab Printing House (1980), P. 96

6- T.J. Mabry, K.R. Markham, and M.B.Thomas; ; "The Systamatic Identification of Flavoids ", Springer-Verlag, New York, Heidelberg, Berlin (1970), P. 35.

7- A. Vogel; " Practical Organic Chemistry" 3rd Ed., Lowe and Brudon LTD London (1972), P. 330.

8- T.A. Geissmann;"The Chemistry of Flavonoid Compound", The Macmillan Company, New York (1962), P. 76, 269 and 275.

9- J.B. Harborne, "Comparative Biochemistry of the Flavonoid", Academic Press, London and New York (1967), P. 14, 31 and 150.

10- B. H. Koeppen, and D.S. Basson; Phytochemistry.,5, 133 (1966)

11- F.J. Francis, and J. B. Harborne; J. Food Sci., 31 (4), 524 (1966).

12- I. Smith; " Chromatographic and Electrophoretic Techniques, 3rd Ed., William Heinemann Medicinal Books,LITD, London (1969) P. 241.

13- M. S. Karawya, and M.S. Afifi, "Investigation of anthocyanin Content of the Petals of Hibiscus rosea-sinensis L. Growing in Egypt"; Bull. Fac. Pharm. (Cairo), Under Press.

دراسة المواد الملونة لبتلات نباتـــــى خطية الزهور والملفافسكاس اربوريا التى تنمو فى مصـــر

محمد سعد الدين كراوية و محمد صفوت عطا عبدالمعـــز عفـــــــــــــيفى

قســـــــم العقاقير ـ كلية الصيدلة ـ جامعة المنصورة

تم فصل والتعريف على ستة مواد ملونه الموجودة فى بتلات أزهار نبـــــات الملفافسكاس أربوريا وخمس مواد ملونه الموجودة فى بتلات أزهار خطية الزهـــور وقد تم التعرف عليها بفحصها بواسطة كروماتوجرافيا الورق والتحليل الحامضـــى والانزيمى ودراستها بواسطة الاشعة الفوق بنفسجية ـ وأوضحت هذة الدراسة عـــن وجود السيانيدين-٣-جلوكوزايلوزيد-٥ - بيتاجلوكوزيد والسيانيدين ٣ره بيتا داى جلوكوزيد ،مالفيدين-٣ و ٥-بيتا داى جلوكوزيد، مالفيدين-٣-بيتاجلوكوزيد ،دلفندين ٣ بيتا- جلوكوريد، بلارجوندين-٣-بيتا-جالاكتنوزيد فى بتلات أزهار الملفافسكـــــى أربوريا والسيانيدين ٣و٧ بيتا داى جلوكوزيد ،سيانيدين-٣-بيتا-جلوكوزايلوزيد دلفندين-٣-بيتا-جالاكتوزيد ،بتيونيدين-٣-بيتا-رامنوزيد وبلارجوندين ٧ بيتـــــا جلوكوزيد فى بتلات أزهار نبات خطية الزهور .

received in 15/6/1986 & accepted in 25/2/1989