Cold extraction strategy for crude dye extraction from *Cucurbita Pepo* leaves

Nasrullah Shah*[1], Shehla Farman[1], Zahid Hussain[1], Muhammd Balal Arain[1], Sulaiman Shams[2]

[1]Department of Chemistry, Abdul Wali Khan University Mardan, Pakistan

[2]Department of Biochemistry, Abdul Wali Khan University Mardan, Pakistan

*Corresponding author: nasrullah@awkum.edu.pk

ABSTRACT

Cold extraction strategy was used for crude dyes extraction from *Cucurbita Pepo* leaves. 100g and 50g of *C. Pepo* dry mass was taken in 750ml and 500ml of ethanol solvent respectively. The extract obtained quantitatively from cold extraction method was 6.80g and 2.26g respectively. Time optimization study for extraction at ambient temperature was done through spectrophotometric analysis. The fractionation of the obtained dye was done with various organic solvents. Similarly, TLC analyses were done for further evaluation of the dye. The overall study was fruitful for obtaining natural dyes from *C. Pepo* leaves under cold extraction conditions.

Key words: Cold extraction, *Cucurbita Pepo* leaves, crude dye, analysis

1. Introduction

Dyes obtained from natural sources are called natural dyes. The majority of natural dyes are from plant sources – roots, berries, bark, leaves, wood, fungi, and lichens. Throughout history, people have dyed their textiles using common, locally available materials. Plant-based dyes such as woad, indigo, saffron, and madder were raised commercially and were important trade goods in the economies of Asia and Europe. Shahid and Ul-Islam studied recent advancements in natural dye applications. A number of colorants obtained from natural sources like plants, microbes, insect and

animals were investigated for their use in different kinds of applications. A natural dye has application in functional finishing of textiles, coloration of food and dye sensitized solar cells. [1]

Grifoni *et al* described the role of natural dye in UV protection of fabrics made of vegetable fiber. Textiles offered a safest protection from (UV) radiation exposer. The measurements of transmittances were used to calculate the UV Protection Factor. The result showed that the use of natural dyes increase the UV protection of fabrics made of vegetable fibre. [2]

A pumpkin is a gourd-like squash of the genus *Cucurbita* and the family *Cucurbitaceae* (which also includes gourds). It commonly refers to cultivars of any one of the species *Cucurbita pepo*, *Cucurbita mixta*, *Cucurbita maxima*, and *Cucurbitamoschata*. Pumpkins are widely grown for commercial use, and are used both in food and recreation. It is an important family consisting 125 genera and 960 species, mainly in regions tropical and subtropical, all species are sensitive to frost.

Efforts are made since long by various scientists to develop an efficient extraction technique for natural yes extraction. Baliarsingh *et al* [3], extracted natural dye from two plant species (*Saracaasoca* and *Albizia lebbeck*), Using solvent extraction. Dyeing techniques, identification of colour components and their potential antimicrobial properties against common human pathogens were checked. The principal colour compounds such as flavonoids and tannin moieties were isolated from two plant extracts and identified based on chemical and spectroscopic investigations.

Hee-OckBooa *et al* [4], worked on extraction and characterization of some natural plant pigments. To use natural materials for cosmetics, antioxidant activities, total polyphenols and flavonoids, and antimicrobial effects in some plant pigments were determined.

Sinha *et al* [5], extracted natural colorant from the petals of the Flame of forest (Buteamonosperma) flower under different operating conditions such as extraction time, temperature and mass of the petals by conventional extraction technique.

Adamsa *et al* [6] worked on the Extraction, isolation and characterization of oil bodies from pumpkin seeds for therapeutic use. The influence of pH, ionic strength, and temperature on the properties and stability of oil bodies from pumpkin (*C. Pepo*) were determined with a view to patterning oil

body size and structure for future therapeutic intervention.

Krimer-Malešević *et al* [7], worked on the investigation of phenolic acid in pumpkin (*Cucurbita pepo* L.) seeds. Pumpkin (*Cucurbita pepo* L.), a widely cultivated vegetable used for human consumption and in traditional medicine. Its extracts (from different parts of the plant) contain biologically active components which show antidiabetic, antibacterial, hypocholesterolemic, antioxidant, anticancer, antimutagenic, immunomodulatory, antihelmintic, and anti-bladderstone activity, and other miscellaneous effects. Gaudreault and Webb [8], purified galactinol-raffinose galactosyltransferase (EC2.4.1.67), ca 40-fold, an enzyme synthesizing stachyose, from mature leaves of *C. Pepo* using ammonium sulphate precipitation, Sephadex gel filtration and DEAE-Sephadex gel chromatography.

Thomas and Webb [9], purified α-D-Galactosidase from mature leaves of *C. Pepo* using pH and ammonium sulphate fractionation, Sephadex gel filtration and DEAE Sephadex gel chromatography.

In the present study cold extraction strategy was utilized for obtaining crude dyes from *C. Pepo* leaves. The fractionation of the dye stuff was done with various solvents and analysed by simple chromatographic and spectrophotometric techniques.

2. Experimental

2.1. Materials and methods

All experimental work was performed at Analytical Chemistry lab, Abdul Wali Khan University Mardan, Pakistan. First of all the distillation of all commercially available solvents were carried out. The distilled solvents were used for extraction and thin layer chromatography (TLC). Pre-coated silica gel G-25-UV254 was used for TLC and visualized under ultra violet at 254 nm. Spectrophotometer was used for spectroscopic studies.

2.2. Collection of *C. Pepo* leaves

The leaves of *Cucurbita Pepo* (*C. Pepo*) (1kg dry weight) were collected from Hathyan, Mardan, Pakistan in November 2012. Identification of *C. Pepo* leaves was verified by Dr. Mohib Shah Department of Botany, Abdul Wali Khan University, Mardan, Pakistan.

2.3. Preparation of sample

The *C. Pepo* leaves were washed with tap water and then with distilled water to remove the surface impurities/dust. The leaves were then dried in shade and powdered/grind in Biochemistry Lab of Abdul Wali Khan University Mardan, Pakistan.

2.4. Cold Extraction

100g of sample material was taken and soaked in 500ml of ethanol solvent for two weeks. 1st layer was removed and 250 ml was added to the soaked sample material. After a week the 2nd layer was removed and a third layer (250 ml) was introduced to the sample flask at room temperature, the layers were then mixed and filtered. In Rotary evaporator, the Ethanol filtrate was evaporated at reduced pressure to get dark green residue. After this the Residue was placed in the water bath by keeping the temperature at 70 ^{o}C for 4 hours, by doing this ethanol content evaporated and we got ethanol extract which was collected in a vial and stored for fractionation, antimicrobial activity and other analytical tests.

2.5. Concentration of *C. Pepo* leaves cold extract with rotary evaporator

An Rb flask was taken and makes cleaned. *C. Pepo* (leaves) extract was then taken in Rb flask. Temperature of rotary evaporator was set at 78^{o}C according to the boiling point of ethanol used for extraction. The extract was then treated in rotary evaporator. When all the solvent was removed, the concentrated extract was then taken out from Rb flask. The concentrated extract was stored for Fractionation and other analytical test.

2.6. Fractionation of *C. Pepo* (leaves) cold extract

For fractionation the concentrated cold extract was taken. The order of solvents used for fractionation was from Polar to non-polar. The solvent used were n-hexane, chloroform, ethyl acetate and acetone. First the n-hexane solvent was added to conc. Cold extract of *C. Pepo* leaves and fraction was removed until further addition of n-hexane not dissolved more extract and clear layer of n-hexane formed. A solvent of polarity higher than n-hexane like chloroform was added and fraction was removed until further addition of chloroform not dissolved more extract and clear layer of chloroform was formed. Ethyl acetate and Acetone fractions were then removed using the same procedure. Fractions were stored in voiles for TLC and antimicrobial activity.

3.6. Determination of protein and glucose content of *C. Pepo* (leaves) cold extract

Ethanolic cold extract was taken in a beaker. The protein and glucose sensitive strip was dip in that extract. Change in color of the strip was observed and calibrated with colored chart for protein and glucose content. From calibration the amount of protein and glucose content was noted.

3. Results and Discussions

3.1. Extraction of crude dye from *C. Pepo* leaves

Two methods of extraction, cold extraction and hot extraction were used. In cold extraction method 100g and 50g of *C. Pepo* dry mass was taken in 750ml and 500ml of ethanol solvent respectively. In hot extraction method 50g of *C. Pepo* dry mass was taken in 500ml of n-hexane solvent. The extract obtained quantitatively from cold extraction method is 6.80g and 2.26g respectively while the extract obtained quantitatively from hot extraction method is 2.47g as clear from the Table 1. It is concluded from the tables that hot extraction method is better than cold extraction method. The reason behind is the influence of temperature on the process of extraction. It is noted from the literature that by increasing temperature the extract concentration also increases. Hot extraction is better than cold extraction because it`s a rapid process of extraction, extraction is good due to the involvement of temperature, no filtration is required due to the use of filter paper in hot extraction. Usually a low boiling solvent is used like n-hexane in hot extraction. Though hot extraction is better than cold extraction in the sense of quantitative yield but in the sense of coast and availability cold extraction is better than hot extraction, because cold extraction can be carried out in a simple pot but for hot extraction a heating source, condenser, round bottom flask and the most important electricity is required.

3.2. Thin layer chromatography (TLC) for *C. Pepo* (leaves) cold extract fractions

Thin layer chromatography was performed for separation and identification purposes. A strip of TLC was taken. Base and solvent front lines were drawn. A narrow spot of sample (fraction) was putted on the base line with a capillary tube. The TLC strip was then kept in TLC tank having mobile phase, the mobile phase was allowed to move up on TLC strip till the solvent front line reached. The TLC was then studied under UV light,

the spots were marked with pencil and RF values for the spots were determined. The m.phase used was a mixture of n-hexane and ethyl acetate. The polarity of mobile phase was changed from low to high.

Table-1: Quantitative production of crude extract of the leaves of *C. Pepo* using ethanol solvent. The experiment was performed at ambient temperature using 100g of *C. Pepo* dry mass in 750ml of ethanol.

S.no	Sample weight (g)	Solvent volume (ml)	Weight of beaker (g)	Weight of beaker +extract (g)	Weight of extract (g)
1	100	750	161.83	168.63	6.80
2	50	500	161.83	164.09	2.26

Table-2: Quantitative production of crude extract of the leaves of *C. Pepo* using n-hexane solvent. The experiment was performed at ambient temperature using 50g of *C. Pepo* dry mass in 500ml of n-hexane.

Sample weight (g)	Solvent volume (ml)	Weight of beaker (g)	Weight of beaker +extract (g)	Weight of extract (g)
50	500	35.18	37.65	2.47

4.2-A. Time optimization for extraction of crude dye at ambient temperature

Time was optimized for crude dye extraction at ambient temperature using 5g of *C. Pepo* dry mass in 200ml of ethanol. The effect of time on extraction was determined using spectrophotometric studies. The absorbance of extract was determined at various wave lengths (nm). It is shown in the Table 3 that a linear increase in absorption was observed with increasing time. The absorbance is directly related to extract concentration. Although a linear increase in concentration was observed but still there are some fluctuation in result. As shown in the table at 690nm a linear increase in absorbance was up to 240min but at 400min a decrease in absorption was observed similarly, at 700nm there is no linearity in absorbance. The

maximum absorbance at various time intervals was observed at 660nm. It is concluded that with increasing time the extract concentration also increased.

Table-3: time optimization study for extraction of dye in ethanol under cold extraction strategy. The UV/Vis spectrophotometric study was done for determining the absorbance.

Wave length (nm)	Absorbance					
	30min	60min	120min	180min	240min	400min
600	0.155	0.284	0.354	0.401	0.463	0.502
610	0.185	0.344	0.430	0.491	0.563	0.615
620	0.196	0.358	0.453	0.515	0.606	0.658
630	0.192	0.350	0.440	0.501	0.605	0.652
640	0.247	0.441	0.568	0.651	0.742	0.803
650	0.402	0.718	0.931	1.067	1.142	1.277
660	0.663	1.159	1.504	1.668	1.729	1.863
670	0.534	0.936	1.252	1.421	1.444	1.573
680	0.174	0.299	0.382	0.427	0.496	0.514
690	0.051	0.101	0.106	0.110	0.144	0.173
700	0.029	0.063	0.075	0.097	0.0104	0.109

4.5. TLC and UV-Vis study of various fractions of *C. Pepo* cold extract

A good TLC was developed at ambient temperature and pressure in 30% of m.phase (n-hexane 7ml + ethyl acetate 3ml) system for various fraction of *C. Pepo* cold extract. The solvents used for fractionation were n-hexane, chloroform and acetone. It was observed from the developed TLC of n-hexane, chloroform and acetone fractions that the extracted dye consist of six components while the developed TLC of acetone fraction show that the extracted dye consist of

four components. All components have distinct R_f values. The Rf values for various fractions are shown in the Tables 4-7. The λmax values were also determined for the above fractions and

it is clear from the tables (4.5 E-H) that the n-hexane, chloroform and ethyl acetate fractions have six values of λmax. It is

shown from the table that the λmax values were observed at 360nm, 420nm, 500, 530nm, 610nm and 660nm. A little bite deviation from these values was because of the solvent used in fractionation. The UV studies of fractions confirming that the extracted crude dye consist of 6 components/functional groups except the acetone fraction having 5 λmax values.

Maximum R_f values were observed for chloroform, like Rf_1 for chloroform is 0.91cm. For n-hexane, ethyl acetate and acetone were 0.97cm, 0.84cm and 0.55cm respectively. All fractions except acetone, 6 spots were observed on developed TLC while acetone has 5 spots on developed TLC. Similarly the maximum UV-Vis absorbance was shown by n-hexane fraction like at 360nm the UV-Vis absorbance for n-hexane, chloroform, ethyl acetate and acetone were 1.231, 1.210, 1.205 and 1.184 respectively. The same trend was observed at other λmax.

Table-4: n-hexane fraction. TLC was developed at ambient temperature and pressure in 30% of m.phase (n-hexane 7ml + ethylacetate 3ml) system.

No. of spots	Distance trveled by solvent front	Distance traveled by spots	Rf value
1	3.5cm	3.4cm	Rf_1 = 0.97
2	3.5cm	3.2cm	Rf_2 = 0.91
3	3.5cm	2.9cm	Rf_3 = 0.82
4	3.5cm	2.2cm	Rf_4 = 0.62
5	3.5cm	1.1cm	Rf_5 = 0.31
6	3.5cm	0.7cm	Rf_6 = 0.20

Table-5: Chloroform fraction: TLC was developed at ambient temperature and pressure in 30% of m.phase (n-hexane 7ml+ ethylacetate 3ml) system

No of spots	Distance traveled by solvent front	Distance traveled by spots	Rf values
1	3.2cm	3.1cm	Rf_1 = 0.98
2	3.2cm	2.5cm	Rf_2 = 0.78
3	3.2cm	2.2cm	Rf_3 = 0.68
4	3.2cm	1.6cm	RF_4 = 0.50
5	3.2cm	1.2cm	Rf_5 = 0.37
6	3.2cm	0.8cm	Rf_6 = 0.25

Table-6: Ethyl acetate fraction. TLC was developed at ambient temperature and pressure in 30% of m.phase (n-hexane 7ml + ethylacetate 3ml) system

No of spots	Distance traveled by solvent front	Distance traveled by spots	Rf value
1	3.8cm	3.2cm	Rf_1= 0.84
2	3.8cm	2.9cm	Rf_2 = 0.76
3	3.8cm	2.1cm	Rf_3 = 0.55
4	3.8cm	1.5cm	Rf_4 = 0.39
5	3.8cm	1cm	Rf_5 = 0.26
6	3.8cm	0.6cm	Rf_6 = 0.15

Table-7: Acetone fraction. TLC was developed at ambient temperature and pressure in 30% of M.phase (N-hexane 7ml + Ethylacetate 3ml) system

No of spots	Distance traveled by solvent front	Distance traveled by spots	Rf value
1	3.4cm	1.9cm	Rf_1 = 0.55
2	3.4cm	1.4cm	Rf_2 = 0.41
3	3.4cm	0.9cm	Rf_3 = 0.26
4	3.4cm	0.6cm	Rf_4 = 0.17

3.8.1. Percent yield/ Percent recovery of cold extract of *C. Pepo*

Mass of plant leaves: 100g

S. No	Fractions	Percent Yield (w/w)
1.	ethanolic extract	6.80%
2.	n-hexane	3.41%
3.	chloroform	0.76%
4.	ethyl acetate	0.16%
5.	acetone	2.47%

Table 8: Determination of λmax for n-hexane, chloroform, ethylacetate and acetone fractions.

Wave length (nm)	Absorbance			
	n-hexane	Chloroform	Ethylacetate	Acetone
360	1.231	1.210	1.205	1.184
370	1.136	1.106	1.094	1.078
380	0.705	0.674	0.709	0.696
390	0.111	0.099	0.078	0.129
400	0.878	0.889	0.830	1.028
410	1.618	1.659	1.590	1.658
420	1.708	1.689	1.675	1.680
430	1.680	1.661	1.651	1.652
440	1618	1.570	1.590	1.573
450	1.469	1.443	1.399	1.403
460	1.230	1.213	1.100	1.162
470	0.860	0.830	0.776	0.797
480	0.919	1.130	0.759	1.089
490	1.927	2.169	1.642	1.322
500	1.963	2.345	1.994	1.207
510	1.571	2.372	1.547	1.041
520	1.331	2.444	1.316	0.915
530	1.494	2.259	1.505	0.884

540	1.062	2.261	0.890	0.769
550	0.930	2.095	0.729	0.665
560	0.913	1.923	0.701	0.605
570	0.789	1.545	0.514	0.536
580	0.732	1.339	0.455	0.493
590	0.777	1.634	0.635	0.490
600	1.004	2.221	1.093	0.530
610	1.187	2.085	1.213	0.520
620	1.057	2.078	0.960	0.461
630	0.991	1.894	0.890	0.437
640	1.093	2.015	1.203	0.483
650	1.618	2.048	1.940	0.682
660	2.104	2.045	2.024	1.044
670	2.136	2.059	2.021	0.939
680	1.643	2.032	1.505	0.460
690	0.727	0.921	0.536	0.274
700	0.508	0.419	0.262	0.225
710	0.399	0.206	0.169	0.201
720	0.347	0.135	0.118	0.189
730	0.333	0.095	0.090	0.178
740	0.306	0.046	0.071	0.171
750	0.293	0.026	0.061	0.163

760	0.280	0.015	0.049	0.157
770	0.262	0.009	0.025	0.150
780	0.254	0.007	0.009	0.146
790	0.239	0.006	0.001	0.141
800	0.236	0.005	-0.002	0.138

4.8. Glucose and protein determination in *C. Pepo* leaves extract

Glucose and protein content of *C. Pepo* plant extract with n-hexane and ethanol was determined under ambient temperature and pressure using striping method. It was found that both of the n-hexane and ethanol extract change the color of glucose and protein sensitive strip. It is clear from the Table 9 that quantitatively both the extract had the same amount of glucose but different quantity of protein which suggests that the *C. Pepo* leaves contain high percentage of polar protein.

Table-9: Glucose and protein content of *C. Pepo* plant extract with n-hexane and ethanol was determined under ambient temperature and pressure using striping method.

No of samples	Nature of sample	Glucose content	Protein content
1	*C. Pepo* Cold extract with ethanol	250 mg/dl	30 mg/dl
2	*C. Pepo* Hot extract with n-hexane	250 mg/dl	10 mg/dl

4.9. Oil determination of *C. Pepo* leaves

Oil content of *C. Pepo* (leave) n-hexane extract was determined in Rotary evaporator at reduced pressure and at a temperature of 46^{o}C. The table showed that the *C. Pepo* leaves have little percentage of oil also. It is concluded that *C. Pepo* (leaves) can be used

for extraction of essential oils. The oil content data is shown in Table 10.

Table-10: Oil content of *C.Pepo* (leave) n-hexane extract was determined in Rotary evaporator at reduced pressure and at a temperature of 46^{o}C.

Volume of sample taken	Weight of empty Rb flask (w_1)	Weight of Rb +sample after rotary evaporator (w_2)	oil contet (w_2-w_1)	Oil content w/v%
10ml	241.85 g	241.88 g	0.03g/10ml	0.3%

Conclusion

Cold extraction strategy was applied to obtain crude dye which was further fractioned. The spectrophotometric study proved that with increase in time the absorbance increased which indicated the enhancement of extraction of dye with prolonging the time. Under used conditions maximum extraction was observed upto 3 days. The crude dye fractionation was done with different solvents and checked with TLC which indicated that the majorly the dye has six distinct components. Glucose and protein contents showed that appropriate amount is present in the crude dye.

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