Isolation and Identification of an Anthocyanin Compound from Cherry Fruit (*Prunus Avium L.*) and Study of its Antibacterial Activity

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(**Received:** 25 / 5 / 2009 ---- **Accepted:** 5 / 1 / 2010)

Abstract
This work includes the isolation and identification of cyanidin-3-glucoside as major anthocyanin in Iraqi Kurdistan cherry fruit (*Prunus Avium L.*). The extracted pigments were purified using physical methods including different chromatographic techniques (thin layer chromatography (TLC), liquid chromatography (LC) and high performance liquid chromatography (HPLC)). The pure extracted pigments were identified using different techniques such as infrared (IR), Ultraviolet (UV), H^1-NMR and C^{13}-NMR. The antibacterial activity of the extracted anthocyanin compound (cyanidin-3-glucoside) was determined against types of standard strains of bacteria which were *Staphylococcus aureus* (gram +ve) and *Escherichia coli* (gram –ve) by disk diffusion method the results indicates that the anthocyanin compound has inhibited the growth of *Escherichia coli*.

Keywords: cherry fruit, natural pigment, isolation, identification, anthocyanin, antibacterial activity, gram +ve, gram -ve.

Introduction
North of Iraq is a very important area for plant diversity. Many fruit species are grown and many different local or native fruit species and varieties are known. One of these is sweet cherry, *Prunus avium L* [1].

Sweet cherries contain anthocyanins has been known since the beginning of the 20th century [2]. Anthocyanins are flavonoid phenolic compounds, widely distributed among fruits, berries and flowers, providing attractive colours, such as orange, red and blue. These pigments are water-soluble and this property facilitates their incorporation into numerous aqueous food systems.

Anthocyanins possess some positive therapeutic effects, mainly associated with their antioxidant properties. They have received increasing attention as natural colorants in food systems, as a consequence of the social trend toward the consumption of natural products instead of synthetic ones. Thus, new sources of pigments, such as anthocyanins, with high colorant power, stability and low cost are desired [3, 4].

Kuang and Wagenknecht [5] have used silicic acid chromatographic columns to isolate and identify of antirrhinin and mecocyanin from sour cherry.

Gao and Mazza [2] were studied anthocyanin content in the sweet cherry by using HPLC and gas chromatography.

This work deals with the isolation and identification of cyanidin-3-glucoside from Iraqi Kurdistan sweet cherry using different chromatographic methods. The antibacterial activity of the extracted anthocyanin compound (cyanidin-3-glucoside) was determined against types of standard strains of bacteria which were *Staphylococcus aureus* (gram +ve) and *Escherichia coli* (gram –ve) by disk diffusion method. The results indicated that the anthocyanin compound has inhibited *Escherichia coli*.

Experimental
Reagents:
All chemicals and reagents were of analytical grade and deionized water was used throughout, the work.

Apparatus:
UV-Visible double beam spectrophotometer Cecil 9000, was used with quartz cells (1 cm).

Pye Unicam SP-3005 automatic and computerized IR-spectroph- otometer was used, in the range of 600-4000 cm^{-1}.

The HPLC system from Knawer Company with smartline UV detector 2500, the ZORBAX C_{18} (25 cm x 4.6 mm i.d.) stainless steel column was used in this study.

Ultrasonic bath (Decon FS200) was used during this work. Rotary Evaporator: Laborota 4000 under vacuum, Heidolph Company. pH-meter (model Jenway 3305), and Electrical grinder (from Thomas).

Sampling:
Samples of cherry fruit were obtained from Hawler city (Haji Omaran) in the Iraqi Kurdistan region in July 2007.

Determination of λ_{max}:
Dilute solution (1:1) (v/v) of cherry was prepared without any treatment from the crude fruit, then absorbance of the pink solution in the range 360-750 nm was taken using uv-visible instrument. Fig. (1) show that 515 nm was the λ_{max} of the solution.

Extraction of the pink pigment from cherry:
(100 g) of the cherry fruit sample without seeds were taken in plastic bottles, 300ml of each of the following solvents (water, chloroform, acetone, HCl(2M), acetone: HCl (2M), 70% ethanol and 80% methanol) were added separately to the bottles according to their different polarities. All samples were cleaned and crushed with electrical grinder [6], then treated with ultrasound bath for 1 hour and shaked for 24 hours at room temperature (25 °C) [7].

The solutions were filtrated through (S&S No. 598) filter paper neglecting the precipitate. The filtrates were concentrated at 40 °C using vacuum rotary evaporator. Finally the concentrated solutions were dried at room temperature to obtain the pink brown solid material [6]. Table (1) shows the different choices of solvent for extraction according to their

polarities, and 80% methanol was selected as the best solvent for extraction.

After extraction of cherry with 80% methanol, another extraction was done with amyl alcohol to isolate the anthocyanin compounds from the other materials [8]. Scheme (1) shows this process.

Chromatographic techniques for purification:

TLC: Table (2) shows different solvent mixtures used as a mobile phase to test the pink solid material extracted from the fraction A in the scheme (1), on an aluminum plate coated by silica gel.

LC: A glass column of (15-18 mm i.d., 40 cm length) was used with a stopcock at the bottom to control the solvent flow. The paste prepared from 30 g of silica gel (40 mesh) in beaker and adding a mixture of (Butanol: Acetic Acid:Water (4:1:5) top layer) to cover the silica gel. The mixture was shaked to prevent any bubble formed, then added to the column [5]. The pigment was running from the top of the column with 40ml of the same solvent, all fractions were chromatographed with BAW as mobile phase and iodine as developer.

HPLC: Two solvents were used as mobile phase; solvent I (15% Acetic Acid, 1.5% phosphoric acid) and solvent Π (Acetonitrile), with the percentage 90% and 10% respectively. The sample with the concentration of 0.01 g/ml was injected and passes through the column ZORBAX C_{18} (25 cm x 4.6 mm i.d.) with a flow rate of 1ml/min and the λ_{max} 540 nm at 25°C [9].

Acid hydrolysis:

The isolated pigment was heated in (20 ml) 2M HCl in a conical flask for 30 minutes at 100°C in water bath, then equal amount of chloroform was added three times to extract the anthocyanidin (aglycon anthocyanin) compound to the organic phase and the carbohydrates to the aqueous phase[8]. Some tests were carried out for the aqueous phase such as Molish, Benedict, Barfoed, Bial, Siliwanov and Iodine tests to identify the type of glycoside. Table (3) shows these tests.

TLC was also used to identify the type of sugar of the isolated pigment in the aqueous phase using n-Butanol: Acetic Acid: Ethyl ether: Water (9:6:3:1) as mobile phase and silica plates as stationary phase. The developer was ethanol: sulphuric acid: anisaldehyde (18:1:1) [10], while the organic phase was dried and prepared to do spectroscopic tests.

Biological studies:

The sensitivity of the isolated compound against two kinds of bacteria, (*Staphylococcus aureus* and *Escherichia coli*) was tested according to the following steps: 1- Muller – hinton medium was prepared using nutrient agar preservation of pure culture, then sterilized by autoclave, and poured in the petridish to a depth of 4 mm. 2-Activation of each type of bacteria, *Staphylococcus aureus* and *Escherichia coli* before culturing on the nutrient agar in nutrient broth (oxoid) which was used for dilution of bacterial and cultivation of culture isolated, for 24 hours at 37°C, then inoculation of the test plates. 3- Culturing the bacteria on nutrient agar. 4- Application of the compound disks, each disk was prepared by mixing a substance with KBr powder (1:3). The mixture pressed under pressure. KBr was used as blank disk. The prepared disk was placed on the surface of the cultured media with each of the above bacteria. 5- Incubation: The plates were incubated for 24 hours at 37°C [11].

Results and discussion

Different solvents used for pigment extraction during a constant time (24 hours) at room temperature (25 °C). Results indicated that (80%) methanol gave the best condition for extraction process, because the total yield percentage was 15.95% which was the highest extract. This reveals that the polar solvent used for the pigments extraction was more suitable than the non polar solvents independently of the plant species [12]. Tables (1 and 4) show the type of solvents and the percentage of methanol used in extraction process, respectively.

Table (5) shows the relation between the different weights of sample with the percentage yield, the yields were not linear because the same solvent during the same time are not enough to extract completely the sample.

Chromatographic techniques:

The extracted and isolated pigment from cherry fruit gave one spot using different chromatographic techniques which indicate that the extracted substance contain just one compound.

Tables (2) and (6) show the TLC results using different solvents and different visualization methods. The R_f value for TLC was 0.15 using n-Butanol: Acetic Acid: Water (4:1:5) top layer as mobile phase system with Iodine indication.

The HPLC technique was used to prove that the extracted pigment gave one spot which done by TLC. Fig. (2) shows the chromatogram of red-pink pigment, which shows only one peak representing one compound. The t_R for the extracted compound was 3.067 min.

Identification of the pigment:

In addition to different chromatographic techniques which were mentioned before and used for identification of the product pigment, other techniques such as I.R., U.V., ^{1}H-NMR and ^{13}C-NMR were also employed for this identification.

IR-spectrum for the extracted compound, showed bands at 1629.97 cm^{-1} for C=C of benzene ring, 1724.92 cm^{-1} for carbonyl group, 2934.98 cm^{-1} for aliphatic hydrogen, and 3429 cm^{-1} for –OH group. The UV spectrum for the isolated pink pigment from the cherry sample; showed absorption band at 271 nm. This is similar to those of anthocyanin compounds.

The ^{1}H-NMR spectrum showed a peak at 9.66 ppm for the O-H protons, peaks in the range (6.18-6.74) ppm corresponds to the protons of the benzene rings, while peaks in the range (3.22-3.77) ppm attributed to

the aliphatic C-H protons of the sugar moiety, in addition to that, the protons of the hydroxyl groups of sugar ring were resonated in the (4.79, 4.91) ppm.

The ^{13}C-NMR spectrum of the same compound shows peaks at the range (91.97-146.539) ppm for the C-H and C-O carbon atoms of the benzene ring, while peaks in the range (61.53-77.19) ppm attributed to the aliphatic carbon atoms of the sugar moiety. Finally, the peak at 60.50 ppm attributed to the aliphatic CH_2 carbon in the form of alcohol of the sugar ring.

The results of hydrolysis of the pink pigment showed two parts, the first was soluble in the organic phase and the second was soluble in the aqueous phase. From the aqueous phase glucose was identified by comparing its R_f of TLC with the standards, the R_f of glucose was 0.732, in addition to some chemical tests as mentioned in Table (3) was proved it.

Structure [1] was selected as the best suggested one for the cyanidin-3-glucoside pigment which was extracted from the cherry sample by the aid of different chromatographic and spectroscopic techniques.

The results of the antibacterial activity indicated that the isolated compound has inhibited only *Escherichia coli*, The action of antibacterial on this microorganism was 16 mm of inhibition zone which tested by disk diffusion method.

Conclusion:

It was concluded from the bacterial activity of the extracted compound that it could be used in both medicinally and in food industry.

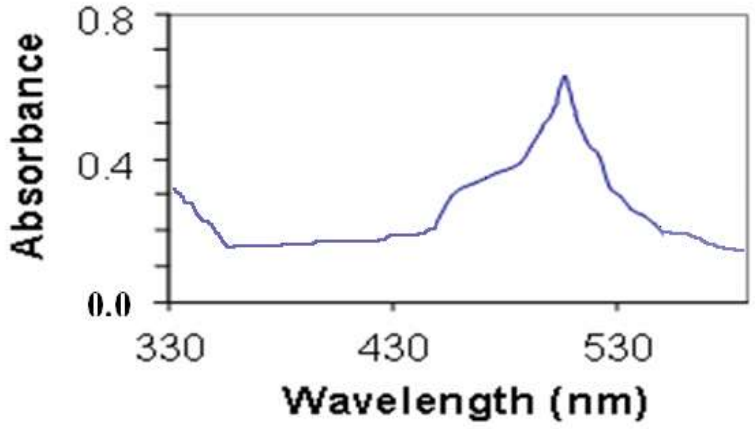
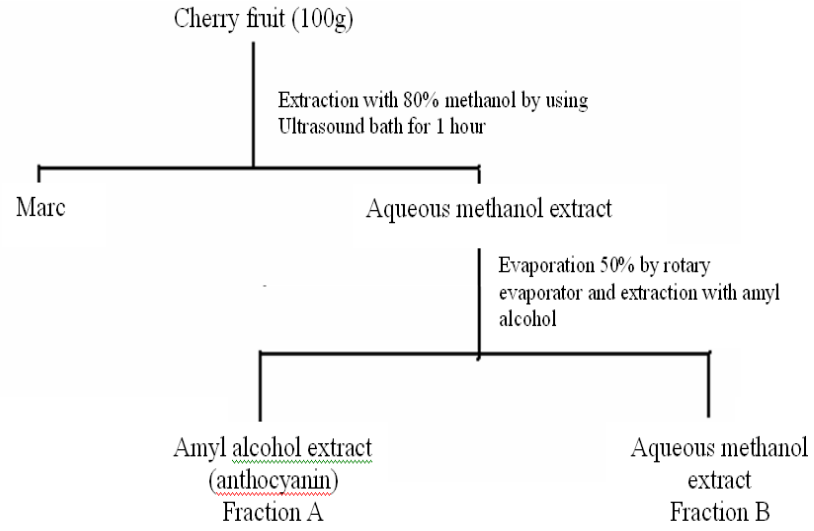


Fig. (1): The absorption spectrum of the pink pigment of cherry fruit



Scheme (1): extraction and isolation of anthocyanin compounds

Table (1): Different solvents used for the extraction process.

solvent	Total yield percentages%
Water	2.56
Acetone	13.89
80% methanol(v/v)	15.95
chloroform	No precipitation
(2M) HCl	15.31
Acetone:HCl(2M)(5:1)	15.59
70% ethanol(v/v)	15.68

Table(2): Solvent mixtures used as a mobile phase in TLC.

Solvent mixture	ratio
Chloroform	100%
n-butanol: acetic acid: water (BAW)	4:1:5
Ethyl acetate: formic acid: HCl(2M)	85:6:9
conc. HCl: acetic acid:water(forestel)	3:30:10
conc. HCl: formic acid: water	2:5:3

Table (3): Some chemical tests for aqueous phase.

Chemical test	results
Molish	+ve
Benedict	+ve
Barfoed	+ve
Bial	-ve
Siliwanov	-ve
Iodine	-ve

Table (4): Different percentages of methanol used for the extraction.

Methanol%	Total yield percentages %
70	14.22
80	15.90
90	13.21
absolute	2.56

Table (5): Different weights of the sample used for the extraction with methanol (80%).

Weight of sample(g)	Total yield percentage %
50	10.20
70	12.31
100	15.91
120	16.95
150	18.32

Table (6): TLC results for extracted pigment from cherry using BAW* as mobile phase.

Visualization methods	TLC results	R_f
UV-light	+ve	0.15
Naked eye	+ve	0.15
Sulfuric acid (4%)	-ve	--
Iodine	+ve	0.15
Ammonia vapor	+ve	0.15
Alcoholic aluminum chloride (1%)	-ve	--

* BAW: (n-butanol: Acetic acid: Water) (4:1:5) (top layer)

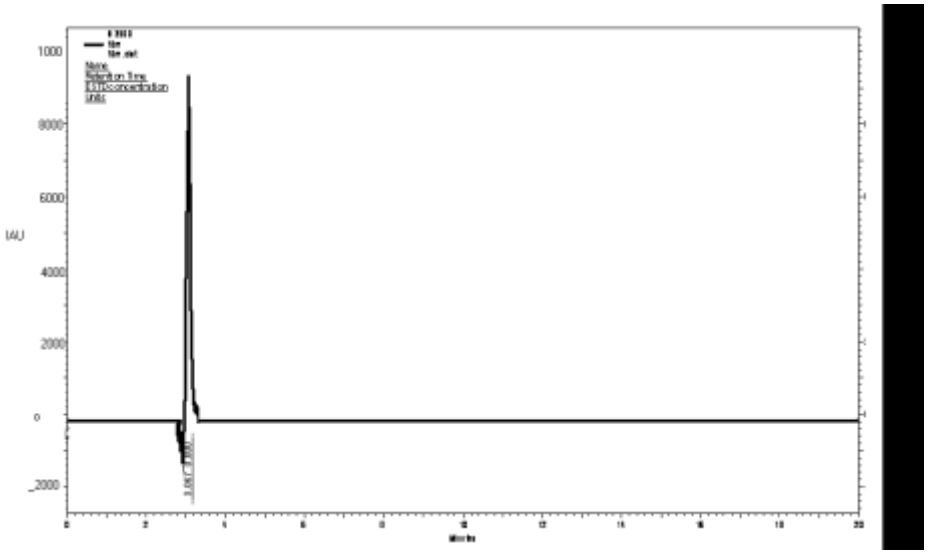
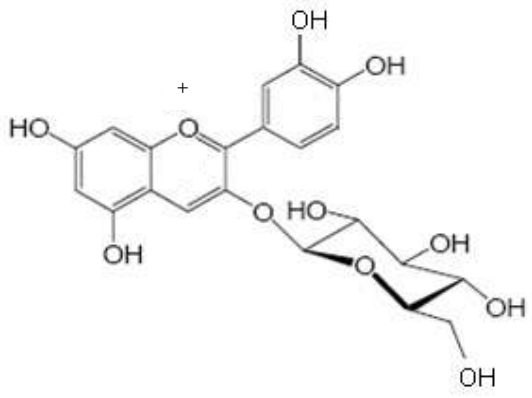


Fig. (2): HPLC chromatogram for the isolated pigment.



[1] (cyanidin-3-glucoside)

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عزل وتشخيص مركب انثوسيانين من فاكهة الكرز ودراسة فعاليته البكتيريه

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(تاريخ الاستلام: ٢٥ / ٥ / ٢٠٠٩ ---- **تاريخ القبول:** ٥ / ١ / ٢٠١٠ **)**

الملخص

هذا البحث يتضمن استخلاص وعزل (cyanidin-3-glucoside) من فاكهة الكرز (*Prunus Avium L.*) الموجود في كوردستان العراق. الصبغة المستخلصة تم تنقيتها باستخدام طرق فيزيائية بضمنها مختلف التقنيات الكروماتوغرافية مثل (LC،HPLC وTLC). تم تشخيص الصبغة ايضاً باستخدام الأشعة تحت الحمراء (IR) والأشعة الفوق البنفسجية(UV) و طيف الرنين النووي المغناطيسي للهيدروجين والكاربون (^{1}H-NMR و^{13}C-NMR). كذلك تم تقدير الفعالية البكتيرية للمركب المستخلص ضد نوعين من انواع البكتريا (*Staphylococcus aureus*) و(*Escherichia coli*) . وقد تبين ان المستخلص لها فعاليه بكتيريه ضد (*Escherichia coli*) فقط.