PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT (DNA NICKING ASSAY) AND ANTIBACTERIAL ACTIVITIES OF *MORUS NIGRA* L. FRUITS SECONDARY METABOLITES

Najlaa Kadhim Issa [a,*], and Rihan Saadi Abd-Aljabar [b]

[a] Dept. of Chemistry, College Science, University of Duhok, Duhok, Kurdistan Region, Iraq - najlaa.issa@uod.ac
[b] Dept. of Chemistry, Faculty of Science University of Zakho, Zakho, Kurdistan Region, Iraq

ABSTRACT:
The present study was carried out to identify the chemical composition of ethanol, flavonoid and anthocyanin extracts of *Morus nigra* fruits. The results revealed the presence of carbohydrates, glycosides, phenolic compounds and the absence of alkaloids, saponins, free amino acids, proteins and tannins. TLC and column chromatography techniques were used to separate and purify the anthocyanin pigment from anthocyanin extract which is then subjected to acid hydrolysis to obtain the anthocyanidin pigment (a). Many techniques were also employed to confirm pigment (a) chemical structure including (TLC, UV-Visible and IR-Spectroscopy). According to the forgoing analytical results, the proposed structure for the isolated pigment (a) is cyanidin pigment. DNA nicking assay has been used to evaluate the ability of the prepared extracts and pigment (a) to prevent DNA nicking caused by free radicals generated from Fenton's reagent. The highest DNA protective effect was achieved in anthocyanin extract followed by pigment (a) and their effects were identical to the positive control (cyanidin chloride). Antibacterial activities of all extracts and pigment (a) were performed against the growth of two standards and pathogenic G+ and G- bacteria. The results indicated that all plants extracts showed more potent activity against standard bacteria strains than pathogenic ones.

KEYWORDS: Morus nigra, Flavonoid, Anthocyanin, Cyanidin, DNA nicking, Antibacterial activity.

1. INTRODUCTION

It is estimated that there are about 250,000 to 500,000 plant species on earth. Plants produce large and diverse groups of molecules that are involved in the adaptation of plants to their environment. In general, the term secondary metabolites had been used to refer to these groups of compounds (Makkar and Becker, 2007). Plant polyphenols are aromatic hydroxylated compounds, which are considered as natural antioxidants responsible for many colors, aromas and flavors characteristics of vegetables, fruits, herbs and beverages. Many food sources form a significant portion of our diet which is among the most potent and therapeutically useful bioactive substances. Flavonoids are one class of polyphenols present at high concentrations in the epidermis of leaves and the skin of various fruits and have important and varied roles as secondary metabolites. Flavonols are very widely distributed in plants; they act as co-pigment to anthocyanin in petals and leaves of higher plants (Harborne, 1984). On the other hand, anthocyanins are widely dispersed throughout the plant kingdom; responsible for most of the red, pink, purple, and blue pigments found in roots, stems, leaves, flowers, seeds, and fruits (Gould *et al.*, 2009). Anthocyanins consist of an aglycone (anthocyanidin), sugar and, in many cases, acyl group which referred to as acylated anthocyanin. Although light is essential for the biosynthesis of anthocyanins, it is also accelerates their degradation (Rein, 2005).
The antioxidant activity of phenolic compounds is mainly attributed to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Harborne, 1984; Crozier *et al.*, 2006). They can prevent some oxidation-related diseases such as atherosclerosis, cardiovascular, neurodegenerative and cancer diseases (Sun *et al.,* 2009).
The co-pigmentation of anthocyanin can be simple phenols such as catechin, flavanol, chlorogenic acid, caffeic acid and rutin or polyphenols, such as tannins (Castaeda-Ovando *et al.*, 2009). The co-pigmentation of anthocyanin can also be resulted from the interaction with DNA which was illustrated by Sarma and Sharma (1999). They found that calf thymus DNA (ctDNA) and cyanidin can form a cyanidin–DNA co-pigmentation complex as shown in Figure (1), which displayed a 15–20 nm bathochromic shift. Cyanidin-DNA co-pigmentation may be a possible defense mechanism against the oxidative damage of DNA (Sarma and Sharma, 1999).
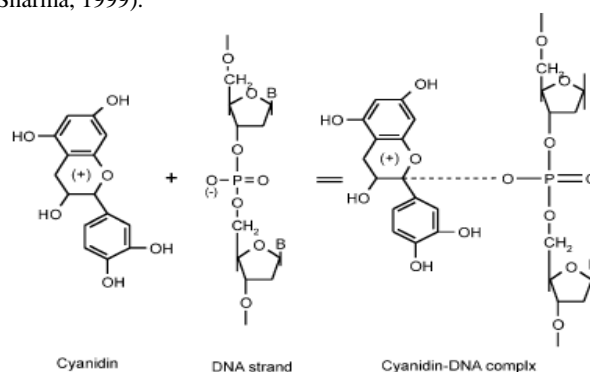

Figure 1. Cyanidin-DNA co-pigmentation

Mulberry belongs to the genus Morus of the family Moraceae. Mulberry trees have been used historically for leaf yield in sericulture. In addition, their fruits have been used in folk

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*   Corresponding author

medicine (especially in Chinese medicine) to treat diabetes, hypertension, anemia and arthritis (Kumar and Chauhan, 2008). Black mulberry fruits are used for treating mouth lesions in Turkey. Recently, red and black mulberries have gained an important position in the food industry due to the presence of anthocyanins (Ozgen *et al.*, 2009).

The literature revealed little information on phytochemical constituents of black mulberry fruits *Morus nigra* L. prevailed in Kurdistan Region of Iraq. Thereby, this study was conducted to determine the phytochemical constituents of black mulberry fruit extracts. The work includes the evaluation of flavonoid and anthocyanin profiles using HPLC techniques and identification of cyanidin pigment isolated from anthocyanin extract. Antioxidant activity using DNA nicking assay was performed as well as assessment of antibacterial activity against standard and pathogenic bacterial strains of the prepared extracts and the isolated pigment.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Black mulberry *Morus nigra* L. fruits were collected on June, 2008 from Duhok-dam, Duhok city- Kurdistan Region of Iraq. The ripe fruits were kept in plastic containers at -10 °C until being used (Pantelidis *et al.*, 2007; Spada *et al.*, 2008). The plant was botanically authenticated in Agriculture College –Forestry Department, University of Duhok.

2.2 Chemicals

All chemicals used were of analytical grade. Aluminum chloride hexahydrate, amyl alcohol, ferric chloride and methanol were obtained from Ltd Company. Chlorogenic acid, cyanidin chloride, isoqurecetin dihydrate, kampherol, rutin hydrate and qurecetin dihydrate were obtained from ROTH company and others were obtained either from Fluka or Sigma chemicals companies.

2.3 Preparation of Extracts

2.3.1 Ethanol Extract: Ethanol extract (Eth. ext.) of black mulberry fruits was prepared following the method of Laleh *et al.*, (2006). Thirty grams of black mulberry fruits were mixed with 200 ml (70 %) ethanol using electrical blender and the mixing was assisted with shaker water bath for 4 h at 40 °C. The mixture was filtrated by filter paper (Whattman No.40) and the filtrate was concentrated by evaporation using rotary evaporator to afford 7 grams dark-reddish color extract.

2.3.2 Flavonoid Extract: The Flavonoid extract (F. ext.) of black mulberry fruits was prepared as discussed below (Peach *et al.*, 1955; Harborne, 1984; Andersen and Markham, 2006). Five grams of the alcoholic extract were dissolved in 100 ml of distilled water, then 125 ml of (1%) aqueous lead acetate was added with stirring for 5 min and the solution was left for 2 h to complete precipitation. Filtration was done by (Whattman No. 40) and the violet-pink filtrate was concentrated by rotary evaporator to obtain solid violet-pink material which has been dissolved later in absolute methanol. Fifty ml of (1%) lead acetate in methanol was then added with stirring for few minutes. The solution was left to complete precipitation for 2 h.

The mixture was filtrated using Buchner funnel and the violet precipitate was washed once with 10 ml distilled water, then with 10 ml absolute methanol, and finally 3 times with 10 ml ethyl acetate. The violet precipitate was left to dry in dark at room temperature. The dry precipitate was dissolved in a mixture of [25 ml acetone + 5 ml (2M HCl)] with shaking, and then was left till complete precipitation of lead acetate. The supernatant was separated and evaporated under vacuum at 40 °C using rotary evaporator. Light –reddish viscous extract was obtained (1.56 grams).

2.3.3 Anthocyanin Extract: Twenty five grams of black mulberry fruits were mixed with 50 ml of (1% HCl in methanol) using electrical blender for 5 min. The mixture was transferred to dark conical flask and placed in an ice bath with stirring for 12 h. The mixture was filtrated by a Buchner funnel and the filtrate was taken to dryness in dark at room temperature. The obtained extract was washed twice with 25 ml acidified methanol; filtrated through filter paper (Whattman No. 40) and the filtrate was dried in dark at room temperature to obtain 2.44 grams reddish-brown viscous (A. ext.) (Harborne, 1984; Schofs, 2004; Andersen and Markham, 2006).

2.4 Phytochemical Analysis

Preliminary qualitative tests were carried out on the foregoing prepared extracts following the method of Harbone (1984) for the presence of various classes of compounds such as alkaloids, flavanoids, tannins, phenolic compounds, saponins, glycosides, carbohydrates, amino acids and proteins.

2.5 Chromatographic Techniques for Isolation of Anthocyanidin Pigment (Pig. A) from Anthocyanin Extract:

Different solvent systems were tested as mobile phase in TLC technique using silica gel TLC plates to identify the number of components and their R_f values in A. ext. (Harborne, 1984; Mingwei *et al.*, 2006). The best solvent system was (n-amyl alcohol: formic acid) by the ratio (5:1). The open column was filled up with a slurry of silica gel (200-400 mesh size) as a stationary phase and (n-amyl alcohol: formic acid) by the ratio (5:1) as a mobile phase was used for the separation of anthocyanin pigment from A. ext. (Schofs, 2004). The fractions on the top of column was collected and tested by TLC, the tubes sample which have the same R_f value were collected and left to dry at dark room temperature to get a violet viscous anthocyanin pigment.

Anthocyanin pigment was subjected to acid hydrolysis by 2 M HCl for 40 min at a boiling water bath, and then the colored extract was cooled and extracted with ethyl acetate (2x10 ml). The aqueous layer was extracted with amyl alcohol (2x10 ml). The amyl alcohol layer was separated and dried in dark at room temperature to obtain pink-violet anthocyanidin pigment which is referred to as Pig. (a) (Harborne, 1984; Zhang *et al.*, 2005; Andersen and Markham, 2006).

2.6 High Performance Liquid Chromatography (Hplc)

HPLC technique (Smart line manager 5000, smart line column the most at 4000, smart line HPLC pumps 1000, Knauer advanced scientific instrument, Germany) was applied to identify the flavonoid components that present in F. ext. Acetonitrile: phosphoric acid (0.1%) by the ratio (25: 75) was used as a mobile phase. Flavonoid extract and standards were first filtered through a 0.22 µm sterile polytetrafluroethylene filter (Millipore filter), then injected through the Eurospher C18 (250 cm x 4.6 mm i. d) with a flow rate of 1 ml /min. The samples were detected using UV detector 2500 at λ max =368 nm.

HPLC ((Shimadzu, SPD-20A, supelco column RP18, LC20AT HPLC pump, Japan) was also performed for A. ext. and Pig. (a) using 2 solvent systems; solvent I (15% acetic acid and 1.5% phosphoric acid) in water and solvent II ((100% acetonitrile). The solvent mixture was established at 90% solvent I and 10% solvent II. The samples and cyanidin chloride were injected through the

Supelco C18 (25 cm x 4.6 mm i. d) with a flow rate of 1.5 ml /min and detected by UV-VS detector at λ max =540 nm.

2.7 Spectrophotometric Analysis

Ultra Violet-Visible Spectra for Pig. (a) was recorded at (200-800 nm) using UV-VS Spectrophotometer. Visible Spectra was also used to identify catecol nucleus in Pig. (a) by noticing a bathochromic shift in its visible spectrum in the presence of aluminum chloride (Harborne, 1984). IR-Spectrum was recorded for Pig. (a) with the range of (600-4000 cm^{-1}) using potassium bromide disc (Ming-wei *et al.,* 2006).

2.8 Antioxidant Activity (DNA Nicking Assay)

Genomic DNA of Escherichia coli HB101 bacteria which harbor the plasmid pBR322 was isolated according to the method reported by (Lema *et al.,* 1994). The optical density (O.D.) of DNA solution (20 µl DNA sample, 1980 µl Tis EDTA buffer) was measured at wave length of 260 and 280 nm. The ratio between the reading at 260nm /280nm provides an estimation of the purity of nucleic acid (Sambrook *et al.,* 1989).The DNA concentration in the solution was calculated according to the following formula:
DNA (µg/µl)

$$= \frac{O.D260 \times dilution\ factor \times 50\ \mu\frac{g}{ml}}{100} \quad (1)$$

DNA nicking assay as described by (Yenug *et al.,* 2002; Lee *et al.,* 2002) was performed using pBR322 plasmid DNA. The reaction was conducted in an eppendrof tube containing 1µL of 0.5 µg genomic DNA, and 10 µL of Fenton's reagent (3 µL of 2 mM $FeSO_4$, 3 µL of (30%) H_2O_2, 2 µL of 50 mM ascorbic acid and 2 µL of phosphate buffer 7.4) was mixed. Two µL of each of the samples (500 µg/ml) Eth. ext., F. ext., A. ext. and Pig. (a) as well as the positive controls (rutin, tannic acid and cyanidin chloride) were added. Sample containing 0.5 µg genomic DNA (without Fenton's reagent) in 15 µL TE buffer was also used as a control. Incubation at 37 ºC for 1 h was done, and then 1 µL of 0.5 EDTA (pH 8.0) was added to stop the reaction. Five µL of loading buffer were mixed with each tested sample, and then samples were loaded carefully to the individual wells onto 0.8% agarose gel and electrophoresed followed by ethidium bromide staining. Photographing was done using digital camera (Maniatis *et al.,* 1982).

2.9 Antibacterial Activity

The antibacterial activity of black mulberry fruits extracts [Eth., F., A. ext. and Pig. (a)] were tested against 2 strains of standard bacteria namely (Staphylococcus aureus NCTC 6571 and Escherichia coli NCTC 5933) and 2 strains of pathogenic bacteria namely(Staphylococcus aureus and Escherichia coli) using agar -well diffusion method (Park *et al.,* 1997; Turkoglu *et al.,* 2007).

3. RESULTS AND DISCUSSION

3.1 Results of Phytochemical Tests

Phytochemical screenings of the prepared extracts are shown in Table 1. Preliminary phytochemical screening for all extracts revealed the presence of carbohydrates, glycosides, flavonoids and phenolic compounds and the absence of alkaloids, saponins, free amino acids and proteins except Eth. ext. which poses saponins and tannins. These results are consistent with the fact that species of Moreacea family are a well-recognized source of secondary metabolites like flavonoids and anthocyanin which impart sweaty flavor to their fruits (Ozgen *et al.,* 2009).

Table 1. Phytochemical analysis of *Morus nigra* Eth., F. and A. Extracts

Phytochemical test	Eth. ext.	F. ext.	A. ext.	Notes
Drageendroff's reagent	-	-	-	No alkaloids
Wagner's reagent	-	-	-	No alkaloids
Hager's reagent	-	-	-	No alkaloids
Mercury chloride	+	-	-	Presence of saponins
Ninhydrin test	-	-	-	Absence of free amino acids
Biuret test	-	-	-	Absence of proteins.
Molish test	+	+	+	Presence of carbohydrates.
Benedict's reagent (before hydrolysis)	+	+	+	Presence of reducing sugars.
Benedict's reagent (after hydrolysis)	+	+	+	Presence of glycosides.
Alcoholic KOH (5N)	+	+	+	Presence of flavonoids.

3.2 TLC and Column Chromatography

TLC technique used for A. ext. gave 5 spots which indicated the presence of 5 components that could be separated by column chromatography technique. The best selected solvent system was [n-amyl alcohol: formic acid (5:1)]. Column chromatography was the best technique used for isolation of pure pigments from A. ext. due to its ability to isolate different anthocyanins from A. ext. silica gel (230-400 mesh size) columns which were applied to obtain different pigments (Johnson and Morris, 1996).
Column chromatography which was used for isolation of anthocyanidin pigment from A. ext. gave 5 separated colored layers. According to the color and the R_f value of the final layer on the top of column; this layer was eluted, separated, dried and subjected to acid hydrolysis to obtain anthocyanin pigment Pig. (a). TLC technique for Pig. (a) using the same mobile phase gave one spot with R_f value of 0.068 which was almost the same R_f value of standard cyanidin chloride indicating that Pig. (a) was cyanidin pigment.

3.3 High Performance Liquid Chromatography (HPLC)

The flavonoid constituents of F. ext. from black mulberry fruits were determined by HPLC. The chromatogram of F. ext. and all standards acquired at 368 nm are presented in Figure (2) which shows many peaks. Five of these peaks had R_t values approximately identical to the R_t of corresponding standards which indicated the presence of chlorogenic acid (R_t=3.15 min), rutin (R_t=3.96 min), isoquerctine (R_t=4.43 min), querctine (R_t=12.10 min) and kaempferol (Rt=22.21 min) in F. ext. The obtained results were in agreement with reports of Apak *et al.,* (2007) who found that black mulberries were rich source of phenolics such as rutin, kaempferol, quercetin, isoquercetin and chlorogenic acid. The anthocyanin constituents of A. ext. and Pig. (a) were also determined by HPLC. The chromatogram of A. ext. and Pig. (a) recorded at 540 nm are presented in Figure (3) which revealed the presence of a peak with R_t values approximately identical to the Rt of cyanidin chloride standard.
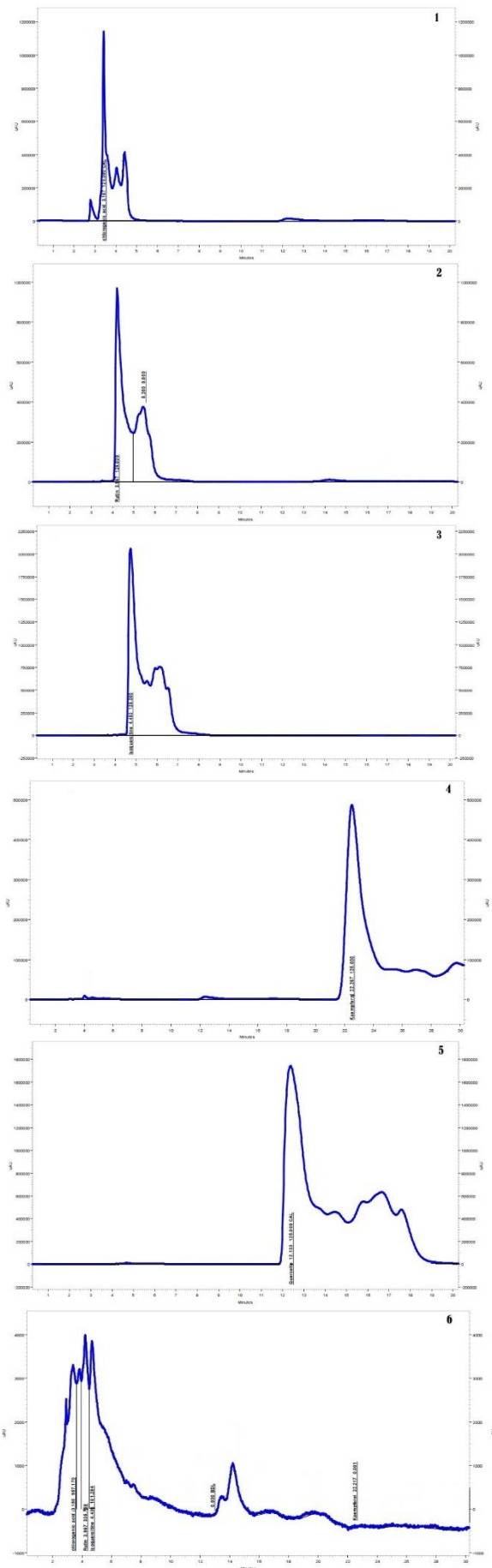
Figure 2. HPLC chromatogram for: (1) Chlorogenic acid, (2) Rutin, (3) Isoquerctine, (4) Kaempferol, (5) Querctine and (6) F. ext.
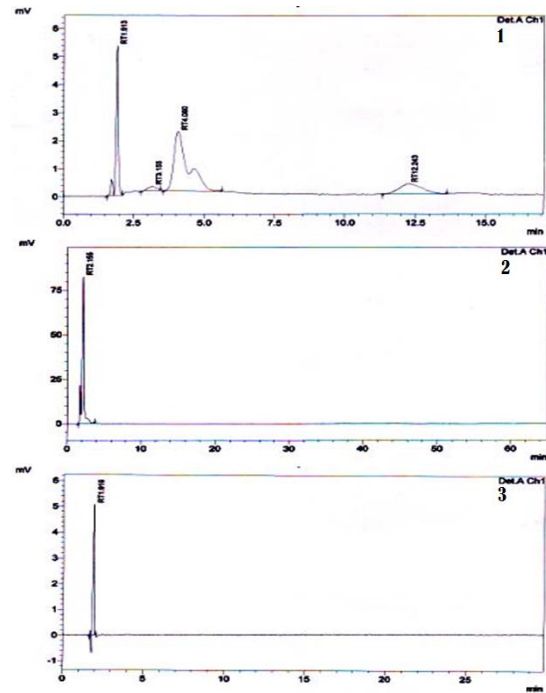

Figure 3. HPLC chromatogram for: (1) A. ext. (2) Pig. (a), (3) Cyanidin chloride standard

3.4 Spectrophotometric Analysis

Pigments are able to absorb visible light due to the presence of the chromospheres (Schofs, 2004). The UV spectrum of Pig. (a) as shown in Figure 4 revealed the appearance of 2 absorbance bands at (228 nm) and (315 nm) which corresponded to $\pi \rightarrow \pi^*$ electron transition of conjugated double bond system and $n \rightarrow \pi^*$ from the non-bonding electrons of the oxygen atom to the aromatic π system of the B-ring (Vermerris and Nicholoson, 2006).

Visible spectrum of Pig. (a) as shown in Figure 5 revealed absorption band at (535 nm) which corresponds to $n \rightarrow \pi^*$ transition of the non- bonding electron of the oxygen atom (Silverstien and Morril, 1981). These spectral bands usually appear in similar anthocyanin compounds (Harborne, 1984; Andersen and Markham, 2006). Visible spectrum of Pig. (a) undergoes a bathochromic shift about 15 nm after the addition of aluminum chloride solution as shown in Figure 6. This shift indicated the presence of vicinal free hydroxyl groups of anthocyanidin pigment in the proposed structure which contained catechol nucleus (Harborne, 1984).
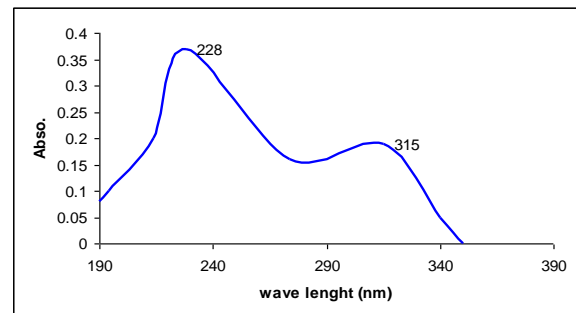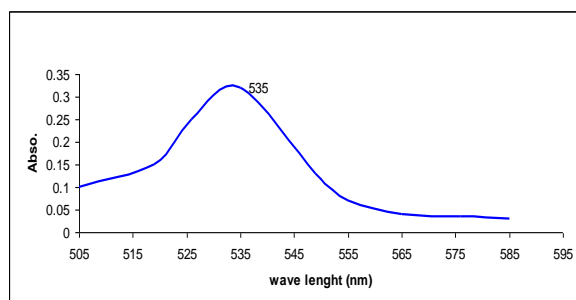

Figure 4. UV-spectrum of Pig. (a)

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Figure 5. VS spectrum of Pig. (a)

Infrared spectroscopy is useful to determine what functional groups are attached to the basic skeletal structure of the flavonoid molecule. The Infrared spectrum of Pig. (a) as shown in Figure 7 indicated the appearance of strong bands at 3100-3600 cm-1 related to the vibration of hydroxyl bond which indicated the presence of polyphenolic compound.
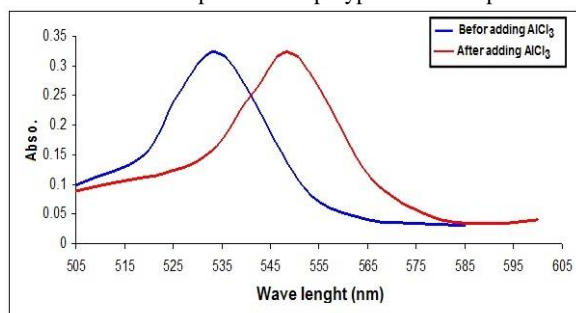

Figure 6. Visible spectrum of catechol nucleus using alcoholic AlCl₃

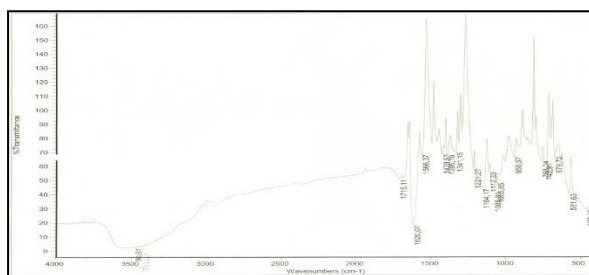Figure 7. IR-Spectrum for the isolated Pig. (a)

The bond at 1620 cm-1 related to the vibrational bond stretching of (C=C) conjugated double system in aromatic compound. The band at 1117 cm-1 related to dialkyl ether group (C-O-C) bridge indicated the presence of aromatic ether compound (Silverstien and Morril, 1981). Consequently, according to the available data of chromatographic and spectroscopic applied on Pig. (a), one can conclude that the isolated pigment could be cyanidin. This conclusion was confirmed by Lee *et al.,* (2004) who found that mulberries had cyanidin-based anthocyanins, particularly cyanidin-3-glucoside (79%) and cyanidin-3-rutinoside (19%).
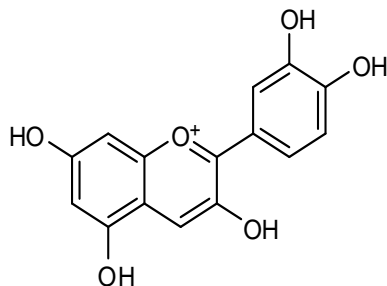

Figure 8. The proposed structure for Pig. (a)

3.5 Antioxidant Activity Using DNA Nicking Assay:

Good yields of genomic DNA were obtained by DNA isolation method, (Lema *et al.,* 1994). The average yields were 18.00 µg/30ml broth culture with purity about (1.64) measured by spectrophotometer ratio A260/A280, which is considered as a high purity. Ratios above 2.0 correspond to RNA contamination, while ratios below 1.6 suggest protein contamination (Sinha *et al.,* 2001). The effects of Eth., F., A. ext. and Pig. (a) as antioxidants on the free radical-induced DNA damage were investigated as shown in Figure 9.

The protective effect can be investigated in both non site – specific assay using ascorbic acid –iron-H_2O_2-EDTA system and site –specific scavenging assay without EDTA. Lane 2, represent the interaction of HO˙with DNA which was continuously (without addition of EDTA) resulting in a complete DNA damage and showed the presence of a detectable smear of DNA fragments but lane 3 showed that the interaction of HO˙ with DNA after incubation for 1 h was stopped by addition of 0.5 M EDTA as chelating agent causing single strand breaks of super coiled DNA into relaxed open circular state or converted super coiled DNA to linear form by double strand breaks (Larson *et al.,* 1992; Yenug *et al.,* 2002).
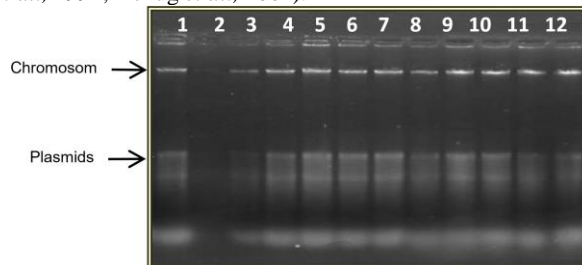

Figure 9. 0.8% Agarose gel electrophoresis at 70 volts for 1.5 h.

Lanes 1, 6 and 12, genomic DNA + TE buffer
Lane 2, Fenton reagent+ genomic DNA
Lane 3, Fenton reagent+ genomic DNA + 0.5M EDTA
Lane 4, Fenton reagent+ genomic DNA + A. ext. + 0.5M EDTA
Lane 5, Fenton reagent+ genomic DNA+ cyanidin chloride + 0.5M EDTA
Lane 7, Fenton reagent+ genomic DNA+ tannic acid + 0.5M EDTA
Lane 8, Fenton reagent+ genomic DNA+ Eth. ext. + 0.5M EDTA
Lane 9, Fenton reagent+ genomic DNA+ rutin + 0.5M EDTA
Lane 10, Fenton reagent+ genomic DNA+ F. ext. + 0.5M EDTA
Lane 11, Fenton reagent+ genomic DNA+ Pig. (a) + 0.5M EDTA.

In the presence of extracts, the extent of DNA damage could be significantly reduced and the protective effects is clearly appeared (lanes 4, 8, 10 and 11) because antioxidant compounds present in the extract which is considered as good electron donors, may accelerate the conversion of H_2O_2 to H_2O (Shon *et al.,* 2007). These results are due to the interaction of anthocyanins and DNA forming cyanidin-DNA co-pigmentation complex (Sarma and Sharma, 1999). It is concluded that the antioxidant activity is observed in the non- site -specific assay rather than site-specific assay when the same concentration (500 µg/ml) of several extracts were used which implying that the extracts scavenging hydroxyl radical directly rather than chelates metal ion (Duan *et al.,* 2008 and Singh *et al.,* 2009).

3.6 Antibacterial Activity of Mulberry Fruit Extracts and Pig. (A)

Black mulberry extracts and Pig. (a) could be used as food preservative, that it can prevent food spoilage due to their antibacterial activity (Radovanovic *et al.,* 2009). The

antibacterial activity of black mulberry fruit extracts and Pig. (a) are shown in Figure (10) and the inhibition zone diameters are shown in Table 2. The obtained results revealed that all plant extracts and Pig. (a) had a pronounced effect against the growth of G+ and G- standard strains compared with the pathogenic ones. This could be reasoned to the fact that pathogenic bacteria undergo many mutations and consequently become more resistant to antibiotics. These antibiotics have more potency against bacterial growth than plants extracts.
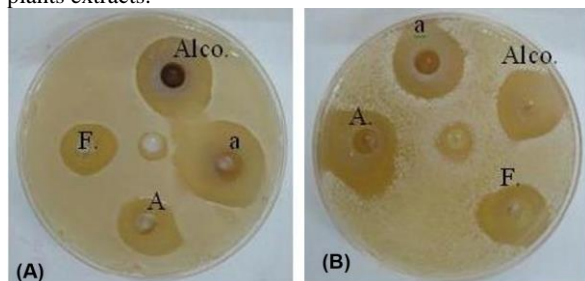

Figure 10. Effect of Eth., F.A. ext. and Pig. (a) at the conc. 100 mg/ml (A): against *S. aureus* (G+) standard bacteria (B): against *E. coli* (G-) standard bacteria

All plants extracts showed more potent activity against G+ than G- bacteria due to presence of many condensed fat bilayers lipid in the cell membrane of G- bacteria compared with gram positive cell membrane (Radovanovic *et al.,* 2009). Regarding to standard bacterial strains, all plants extracts exhibited various reduction in growth of microorganisms, depending on the extract concentration and the chemical compositions present in the studied plants extracts.

Table 2. Antibacterial activity of Eth., F., A. ext. and Pig. (a) against standard and pathogenic (G+) and (G-) bacterial strains

Bacteria Extracts & conc.(mg/ml)		*S. aureus* (NCTC6571)	*S. aureus* (Pathogenic)	*E. coli* (NCTC5933)	*E. coli* (Pathogenic)
Eth.	25	11	---	8	---
	50	14	---	12	---
	75	16	---	16	---
	100	18	14	16	---
F.	25	9	---	10	---
	50	10	---	11	---
	75	11	---	12	---
	100	12	---	14	---
A.	25	10	---	8	---
	50	12	---	16	---
	75	14	---	15	---
	100	13	---	17	---
Pig. (a)	25	8	---	8	---
	50	12	---	13	---
	75	6	---	14	---
	100	18	---	16	---

4. CONCLUSIONS

It can be concluded that (F. ext.) is a rich source of flavonoids including chlorogenic acid, rutin, isoquerctine, querctine and kaempferol. Also, A. ex. is a rich source of anthocyanins including cyanidin pigment. The proposed structure for the isolated pigment (a) is cyanidin pigment which was approved by HPLC and UV-Visible techniques. The black mulberry fruits had the potential protective effect to prevent DNA damage, the highest DNA protective effect was achieved in anthocyanin extract followed by Pig. (a) where their effects were identical to the positive control (cyanidin chloride). All plants extracts showed more potent activity against standard bacteria strains than pathogenic ones.

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