

**The Effect of Ellagic Acid Extract
from *Hibiscus Sabdariffa* on Sera of Leukemic Patients**

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Abstract:-

Recent clinical studies have confirmed that ellagic acid (EA) , a naturally occurring plant polyphenol found in various fruits and vegetable, has exhibited anticarcinogenic activity in both *in vitro* and *in vivo* systems.

In the present study a light on oxidant- antioxidant status was shed in 45 patients with two types of acute lymphocytic and myelocytic leukemia (ALL and AML) in addition to twenty healthy as control group .The work consist of two parts:

1- Extraction Part which included extraction and identification of EA from *Hibiscus Sabdariffa* (HS) the terrestrial plants. The results were proved by thin layer chromatography (TLC) and UV-VIS spectrophotometry.

2- Clinical Part which included evaluation of some parameters *in vitro* studies in all leukemic patients compared to control group:

I- Evalution of malondialdehyde (MDA), lipid peroxidation marker, and caeruloplasmin (CP): The results illustrated significant elevation in both malondialdehyde (MDA), and caeruloplasmin (CP) in all leukemic patients compared to control . These elevation decreased to about normal values by treatment with 10 μL (10^{-5} M) ethanolic ellagic acid which extracted from HS. The effect of ethanol which used as solvent was found to be negligible.

II-Evalution of serum transferrin . A decreased in transferrin levels was found in leukemic patients compared to healthy subjects. The levels of TF elevated into normal values by treatment with 10 μL ethanolic ellagic acid.

Introduction :-

Leukemia is a type of cancer group, which is usually called blood cancer. It is classified into several types. Acute and chronic could consider as the essential type that include many types under each term such as lymphocytic, granulocytic and monocytic leukemia [1]. Generally all blood cells are produced by bone marrow are include three main types : Erythrocytes (RBCs), leukocytes (WBCs), and platelets. During leukemia, a disorder in most blood components may be observed and lead to disorder in their biological function[2].

Lipid peroxidation is a well known marker of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures[3].

The production of high amounts of free radicals has been well recognized as important process in biological system and as a primary cause of cell injury and tissue damages in a variety of pathophysiologic processes [4].

In Iraq there are a biodiversity contain many food plants used as herbs, health foods and for therapeutic purpose. This is due to geographical characteristics of Iraq. This makes researchers get a wide works in studying the medicinal role of these plants[5].

Ellagic acid is a naturally occurring polyphenolic constituent found in various fruits, nuts and vegetables. Approximately forty six of plants had been found to contain ellagic acid such as all types of berries, grapes, tomatoes, broccoli, walnuts and pomegranate[6].

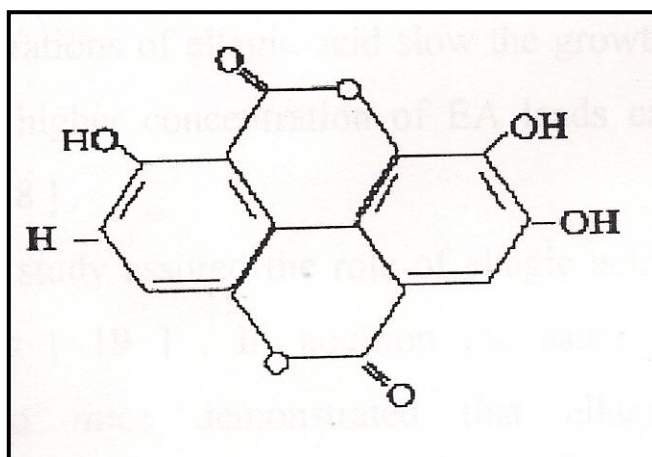


Figure (1):The Structure of Ellagic Acid[6]

Recently ellagic acid is astounding the researchers worldwide with its bioactivity in wide range of diseases which gave it a highest value in latest pharmacological researches, as anticarcinogenesis [7,8]. On the other hand several studies proved that ellagic acid has the ability to inhibit any mutagenesis “ the causes of the alteration in DNA structure (mutation) “ by several different mechanisms [9].

Caeruloplasmin (Cp) the primary antioxidant is an alpha -2- glycoprotein with enzymatic activity . Caeruloplasmin scavenges superoxide anion radical (O_2^-) and protect the cells against oxidation damage [10].

Transferrin (TF) has a complementary role of Cp work . TF, beta 2- glycoprotein, carry ferric ions (Fe^{+3}) and prevent formation of free redicals by Fenton reaction. Both CP and TF prevent free radicals generation [10].

The aim of this work is to clarify at lest in part the effect of ellagic acid from a known wide spread plant *Hibiscus Sabdarilfa* (HS) in Iraq on lipid peroxidation marker MDA and some antioxidant parameters in sera of leukemic patients.

Materials &Methods :-

Extraction part :-

Preparation of plant Extracts :-

Hibiscus Sabdariffa (HS) was collected from south of Iraq. The extracts of HS species has been prepared using ethanol in soxhlet extractor for 16 hrs. Dried flower of HS (50 gm) was used with 300 mL of ethanol. The extract was concentrated by evaporation process. The extract was stored in cold dark bottles until use.

Indentification of Ellagic Acid From Ethanolic Extract :

Two known methods were used to investigate ellagic acid in the extract qualitative and quantitative methods[11]:

1- Thin Layer Chromatography (TLC):

TLC was performed on aluminum sheets with 0.2 mm layer of silica gel (wahtman). Ellagic acid was detected by iodine.Two types of mobile phases were used as shown below:

Ethanol : Distilled water 80:20

Ethanol : Chloroform 80:20

2- UV –Visible Spectra :-

A double beam UV-VIS spectrophotometer was used to identify ellagic acid in the extract of HS.The spectrum of authentic sample of ellagic acid has been recorded for the range of scan from 200 nm to 800 nm. The spectrum for the prepared extract was recorded for comparison with that of authentic sample from minstry of science and technology.

The concentration of ellagic acid in extract was determined using calibration curve made at 365 nm. The absorbance of the extract was recorded in the same wave length (365 nm) consequently, the concentration of ellagic acid in extract was found according to the mentioned calibration curve.

Biological Part :

Selection of Subjects :

The study conducted in Medical City Hospital Baghdad. The work consist of three groups included sixty five subjects aged from (5-18) years . They have been classified into three groups as the following:

- ALL group : included twenty patients (18 male, 2 female) with acute lymphocytic leukemia.
- AML group : included twenty five patients (19 male , 6 female) with acute myelocytic leukemia.
- Control group : included twenty healthy subject (15 male ,5 female)

- Collection of Blood Samples :-

Six ml of blood samples were collected by venipuncture between (9-12 A..M.) which transferred into disposable tube and centrifuged (750 g ,10 min) within 15 min. after collection. The serum that obtained was stored at -20°C until used .

The effects of ellagic acid (EA) was investigated *in vitro* by addition of different concentrations of polyphenolic compound to human serum taken from patients of heart disease.

The concentration of EA was chosen to be 10^{-5} M among other concentrations studied for its effectiveness and this was compatible with previous study [12].

- Determination of Malondialdehyde (MDA):

MDA was determined according to the Fong method [13], by monitoring the thio barbituric acid(TBA). Reactive complex formed in the incubation mixture containing plasma, TBA and trichloroacetic acid in a water bath at 60°C for 90 min.

The absorbance of complex was measured at 532 nm.

Determination of Caeruloplasmin (Cp) :

The method for Cp determination in serum based on the catalytic ability of Cp to oxidize the colorless P-phenylene diamine to a blue- violet oxidized form which has maximum absorbent at 525 nm, using molar absorbtivity coefficient of $0.68\text{ mol}^{-1} \cdot \text{cm}^{-1}$ for the base [14].

Determination of Transferrin (Tf) :

The concentration of the Tf was determined indirectly as the ability of plasma protein to bind iron, the so called total iron-binding capacity (TIBC), where the unsaturated iron binding capacity (UIBC) is determined by the addition of a known ferrous salt concentration to the serum sample, so the added iron will bind to the

unsaturated sites on transferrin. The excess (unbound) iron are reacted with ferine to form the colored complex and determined as in the method of serum iron determination using the ready kit TECO Dignostic France. The difference between the added iron and the amount of iron measured represents the unsaturated iron binding capacity; therefore the (TIBC) is determined by adding the serum iron value to the UIBC value, Then transferrin was estimated as the following equation [15]:

$$\text{Transferrin (mg/dl)} = 0.7 \times \text{TIBC (mg/dl)}.$$

Statistical analysis :

The results were expressed as mean $\pm$ SD , using t-Test. A significant variation is considered when P- values is ≤ 0.05 [16].

Results & Discussion :-

Identification of EA in the HS Extract by TLC:

Ellagic acid has a highest importance for its biological activities such as free radical scavenging , anticoagulation reducing risk of heart disease and wide range of cancer types [6].

Table (1) shows R_f values of ellagic acid in thin layer chromatography for standard and HS ethanolic extract in ethanol:water and ethanol:chloroform solvents.

It is obvious that HS ethanolic extracts contain ellagic acid. one additional unidentified spot was found on the TLC sheet of HS extract when ethanol: water mixture was used as eluent.The presence of unidentified spot indicate that extract of HS can contain another phenolic compounds or any other organic compounds.When recrystallized extract was eluted on TLC sheets, only one single spot was obtained with the R_f values comparable to that of standard ellagic acid .

Table(1):R_f values of Ellagic acid in thin layer chromatography for standard and HS ethanolic extract .

Samples	RF values	
	Ethanol : Water (80 : 20)	Ethanol : Chloroform (80 : 20)
Standard EA	0.91	0.72
HS Extract	0.83	0.70

Identification of EA in the HS Extract by UV.VIS Spectra :-

UV.VIS spectrum of standard ellagic acid shows three bands with λ_{max} at 255, 295 and 365 nm as shown in figure (2). In fact , the same three bands can be recognized in UV.VIS spectra of HS ethanolic extracts as shown in figure (3).

Quantization of Ellagic Acid in HS by UV-VIS Spectrophotometer :-

The content of ellagic acid in HS ethanolic extracts was determined using UV-VIS spectrophotometer.The absorbance of extract at λ_{max} = 365 nm, was compared with calibration curve of standard ellagic and between concentration and absorbance at the same λ_{max} (365 nm) as shown in figure (4).

The EA concentration in HS ethanolic extract was 98 $\mu\text{g/g}$ of dry weight. Malondialdehyde (MDA), Caeruloplasma (Cp), and Transferrin (TF) levels in sera leukemic patients:-

The levels of MDA, Cp , TF before and after addition of EA from HS extract were reported in table (2). The effect of ethanol which used as solvent was found to be negligible.

Lipid peroxidation can be considered as one of several processes which is initiated by oxygen free radicals attack to cell membrane in whole human body [17]. The test for malondialdehyde (MDA), an end product for LPO, has been used for year to measure the lipid peroxidation[18].

The results of the present study were revealed a significant elevation in serum MDA levels in the two patient groups (ALL , AML) compared to control group.

The increase in leucocytes count during leukemia as well as stimulation of phagocytes, lead to further free radicals generation by normal respiration of these cells consequently, membrane lipid peroxidation process increases rapidly and causes elevation in MDA concentration [19].

The most considerable studies have been reported that ceruloplasmin levels rise during the most types of cancer. This rising was found to be as a result of the increase in rates of its synthesis and secretion by the liver cell[20].

Table (2) shown a significant elevation in serum Cp levels in the two types of leukemic patients (ALL, AML) compared to control group. The results are in agreement with Senra study [21], which demonstrated that the concentration of Cp increased significantly in the advanced stage of solid malignant tumors and during several types of cancer .

Transferrin, could be considered as an important preventive antioxidant in wide range of diseases, which apotransferrin (unsaturated transferrin) binds to free iron to prevent its participation in highly reactive hydroxyl radical production [20].

The results of the present study showed slight (non significant) decrease in serum transferrin in two types of leukemia patients (ALL, AML) in comparison with control group. Whereas a significant decrease in serum UTF can be observed in the two patient groups compared to control group as shown table (2).

The results shows also a significant decrease in serum of each MDA and Cp levels in the two leukemia patient groups when sera treated with 10 μ L ellagic acid of HS ethanolic extract, while TF revealed elevation in the levels.

Ellagic acid , a naturally occurring plant polyphenol, was exhibited anticancer activity which act as free radical scavenger led to fall serum MDA level [22,23].

Table (2) : The levels of MDA , Cp and TF in sera of two types of leukemic patients (ALL, AML) and control group before(B)and after(A) treatment with 10 μ L ellagic acid from HS ethanolic extract.

Subject	No	MDA (nmol/L)		Cp (mg/L)		TF(gm/L)		UTF(gm/L)		t-Test
		B	A	B	A	B	A	B	A	
Contro l	20	12.4 $\pm$ 0.61	-----	208 $\pm$ 19.7	-----	2.35 $\pm$ 0.3 1	-----	1.714 $\pm$ 0.31	-----	P $\leq$ 0.0 5
ALL	20	193.5 $\pm$ 22. 2	13.2 $\pm$ 0. 3	315 $\pm$ 18.2 1	205 $\pm$ 13.1	1.72 $\pm$ 0.1 1	0.153 $\pm$ 0.01	1.003 $\pm$ 0.23	0.13 $\pm$ 0.009	P $\leq$ 0.0 5
AML	25	186.8 $\pm$ 19. 9	15.3 $\pm$ 0. 6	350 $\pm$ 15.1 9	209 $\pm$ 10.1 2	1.83 $\pm$ 0.1 7	0.114 $\pm$ 0.00 9	1.012 $\pm$ 0.01 9	0.165 $\pm$ 0.01 1	P $\leq$ 0.0 5

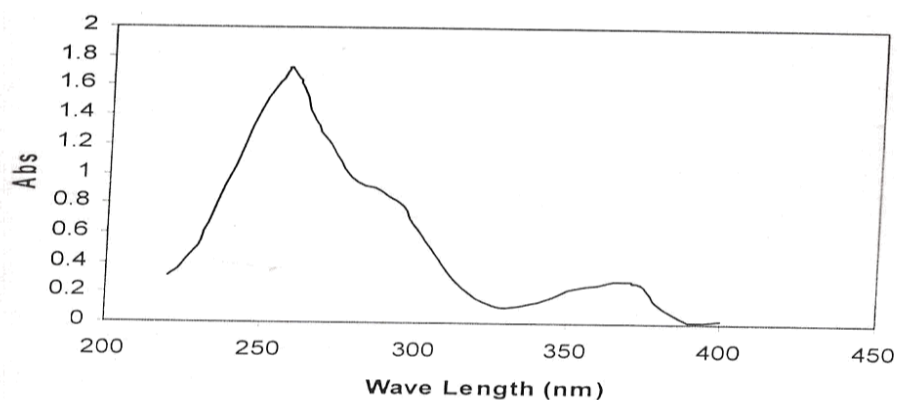


Fig.(3):UV-VIS Spectrum of (4 ppm) Authentic Ellagic Acid Standard

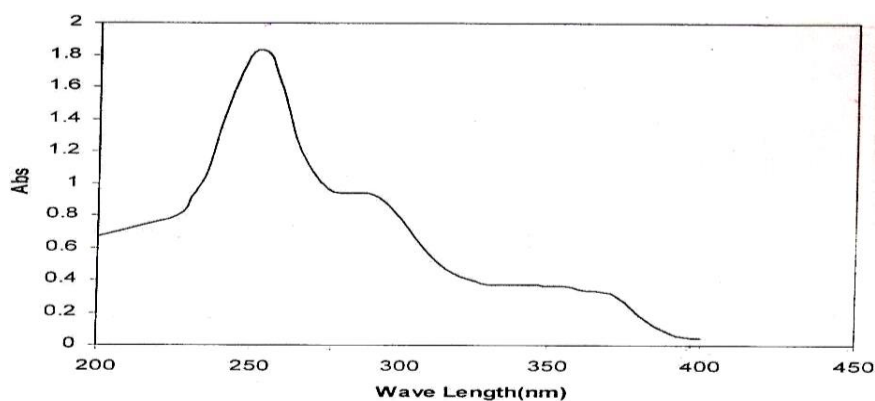


Fig.(4):UV-VIS. Spectrum of HS Ethanolic Extract

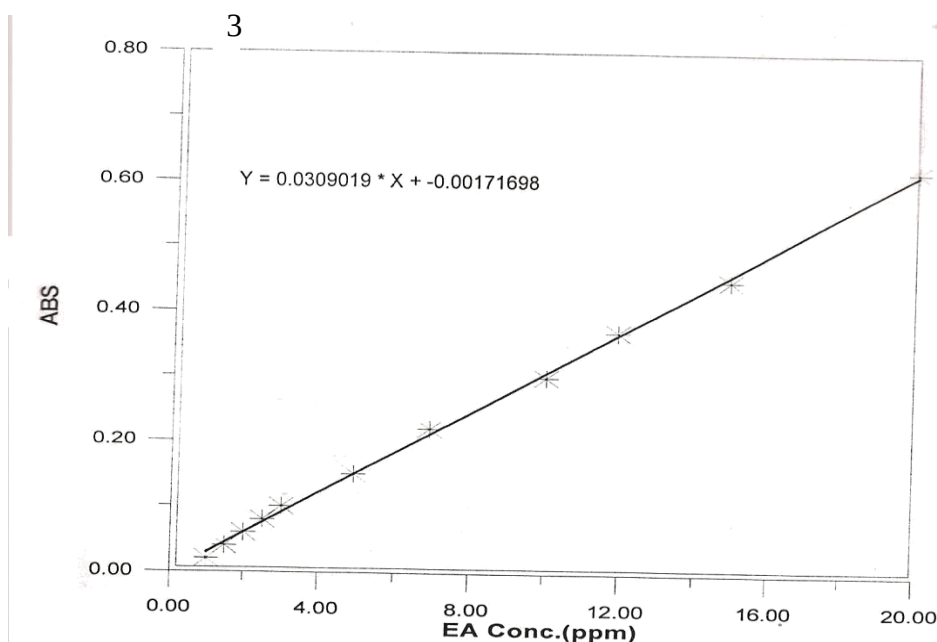


Fig.(5): Calibration Curve of Standard Ellagic Acid

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تأثير حامض اللاجيك المستخلص من نبات الكوجرات على مصل مرضى سرطان ابيضاض الدم

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الخلاصة :-

تضمنت الدراسة الحالية تسليط الضوء على ظاهرة الاكسدة – مضاد الاكسدة في 45 مريضاً يعانون من سرطان ابيضاض الدم من نوع سرطان ابيضاض الدم المايولوجي واللمفاوي الحاد بالاضافة الى 20 شخصاً كمجموعة سيطرة تتراوح اعمارهم بين (4 – 18 سنة).

تم دراسة تأثير حامض اللاجيك المستخلص من الكوجرات على ظاهرة الاكسدة – مضادة الاكسدة في دراسة خارج جسم الكائن الحي. حيث تضمنت الدراسة جزءين :

1- الجزء الاول تضمنت استخلاص وتشخيص حامض اللاجيك المستخلص من الكوجرات ، النبات البري ، أثبتت النتائج بواسطة كروموتوغرافيا الطبقة الرقيقة وجهاز المطياف فوق البنفسجية – المرئية .

2- الجزء الثاني تضمن دراسة سريرية لبعض الدوال في دراسة خارج جسم الكائن الحي:-
اختقدير المألون ثنائي الالديهيد (MDA) كمؤشر للاكسدة الفوقية للدهون ، والسيريلوبلازمين (Cp) في المصل لمرضى سرطان ابيضاض الدم من نوع ALL و AML مقارنة بمجموعة السيطرة.
اظهرت النتائج وجود زيادة معنوية في تركيز كل من MDA و Cp في مرضى ابيضاض الدم من النوعين ALL و AML . تراكيز كل من MDA و Cp تناقصت وكانت ضمن القيم الطبيعية عند استخدام 10 µL من حامض اللاجيك المستخلص من الكوجرات .

ب-تقدير التراسفيرين في المصل :حيث بينت النتائج انخفاض في مستويات التراسفيرين في مرضى ابيضاض الدم مقارنة بالاصحاء. مستويات التراسفيرين كانت ضمن القيم الطبيعية عند استخدام 10 µL من حامض اللاجيك المستخلص من الكوجرات .