In Vitro Cytotoxic Study for Purified Resveratrol Extracted from Grape Skin Fruit *Vitis vinifera*

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Abstract

This study was conducted with the aim to extract and purify a polyphenolic compound " Resveratrol" from the skin of black grapes *Vitis vinifera* cultivated in Iraq. The purified resveratrol is obtained after ethanolic extraction with 80% v/v solution for fresh grape skin, followed by acid hydrolysis with 10% HCl solution then the aglycon moiety was taken with organic solvent (chloroform). Using silica gel G60 packed glass column chromatography with mobile phase benzene: methanol: acetic acid 20:4:1 a partial purified resveratrol was obtained then preperative thin layer chromatography technique yielded pure crystals identified as resveratrol (mixture of two isomers cis and trans) in relation to resveratrol standard (35 mg resveratrol crystals / 0.5 kg fresh grape skin was obtained as a result of these processes). The study was also employed an *in vitro* evaluation the cytotoxic effect of pure resveratrol on some cell line including : the murine mammary adenocarcinoma AMN-3 cell line, the human laryngeal carcinoma (Hep-2) cell line and the Rat embryo fibroblast (Ref) cell line, at different concentrations and different expousure time. The cytotoxic effect of the pure resveratrol was studied in comparison with trans- resveratrol standard in concentrations of (12.5, 25, 50 and 100) µg/ml for both purified resveratrol and the standard, also the comparism included methotrexate drug in concentrations (0.05 , 0.1 , 0.2 and 0.4) µg/ml toward the growth effects of the three types of cell lines and at three exposure times (24, 48 and 72) hours. The cytotoxic inhibition effect for the purified extracted resveratrol revealed that the highest significant effect ($P<0.01$) was achieved after 24 hr of exposure on both AMN-3 and Ref cell lines. Hep-2 cell line responded to extracted resveratrol in different manners.

Keywords: Resveratrol , grape skin , polyphenols, cytotoxic study

الخلاصة

هدفت هذه الدراسة استخلاص وتنقية المركب الفينولي Resveratrol من قشور نبات العنب الاسود *Vitis vinifera* المزروع في العراق . تم الحصول على مادة Resveratrol النقية بعد اجراء عملية استخلاص كحولي بمحلول ٨٠% ح/ح لقشور ثمرة العنب الطرية ، تبعتها خطوة تحلل بحامض HCl وبتركيز ١٠% ، ثم اخذ الجزء غير السكري بمذيب عضوي (كلوروفورم) . وباستخدام عمـــود الفصـــل الكروماتوغرافي الزجاجي المعبأ بمادة سيليكا جيل ٦٠ واستخدام الطور المتحرك بنزين : ميثانول : حامض الخليك بنسبة١:٤:٢٠ تم الحصول على مادة Resveratrol منقاة جزئيا ، من ثم وباستخدام تقنية كروموتوغرافي الطبقة الرقيقة التحضيرية نتجت عن بلورات نقية تم التعرف على كونها مادة Resveratrol (كمزيج للنظيرين trans resveratrol و cis resveratrol) بالمقارنة مع المادة القياسية Trans-Resveratrol standard (تم الحصول على ٣٥ ملغم لكل ٢/١ كغم قشور عنب الطرية كنتيجة لهذه الخطوات) . تضمنت الدراسة فضلا عن ذلك تقييم خارج الجسم الحي للفعالية السمية الخلوية للمادة Resveratrol النقية ومدى تأثيرهما في بعض الخطوط الخلوية والتي شـملت : خط سرطان الظهارة للغدة اللبنية الفأرية AMN-3 cell line وخط سرطان ظهارة الحنجرة البشري Hep-2 cell line والخط الخلوي الطبيعي لجنين الفأر Ref cell line عند تعريضها لمختلف التراكيز وفترات العلاج. وقد اجريت دراسة التاثير السمي الخلوي للمادة النقية بالمقارنة مع المادة القياسية trans- resveratrol بالتراكيز (١٢,٥، ٢٥ ، ٥٠ ، ١٠٠) مايكرغرام/مللتر لكل من المادة المستخلصة والمادة القياسية وايضا شملت المقارنة علاج Methotrexate بالتراكيز(٠,٠٥، ٠,١ ، ٠,٢ ، ٠,٤) مايكروغرام/مللتر على الخطوط الثلاث ولفترات تعريض (٢٤، ٤٨ ، ٧٢) ساعة . ان الفعالية التثبيطية السمية للمادة النقية أظهرت فروق معنوية وضمن المستوى ($P < ٠,٠١$) بلغ اقصاه بعد مرور ٢٤ ساعة تعريض بالاخص في خط خلايا سرطان الظهارة للغدة اللبنيـــة الفأرية AMN-3 cell line والخط الخلوي الطبيعي لجنين الفأر Ref cell line . اما خط سرطان ظهارة الحنجرة البشري Hep-2 cell line فكانت استجابته لتأثير المادة النقية المستخلصة بشكل يختلف عن بقية الخطوط الخلوية.

Introduction

Cancer is a complex set of more than 200 diseases with many causes and multiple stages and histological grades of malignancy [1]. result of exogenous environmental, lifestyle and host genetic factor which attributed about 2% of cancer factors [2].

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Cancer treatments including surgical, radiotherapy , chemotherapy, and biotherapy by immunization and gene therapy, are employed as conventional treatments which may not always be satisfactory to relieve sever symptoms or even cure cancer with untolerated side effects and many get resistance [3]. Chemotherapeutic drugs are typically toxic agents to prolong survival without cure in about 50% of cancer patients, but still an important component of multi modal cancer therapy in (25-30)% of cancer patients, due to the early resistance development to chemotherapy in the life cycle of tumour [4] .Complementary and alternative therapy can help relieve symptoms and improve physical and mental well- being [5]. The search for novel and effective cancer chemopreventive agent has led to the identification of various naturally occurring phytocompounds, one of which is resveratrol (trans- 3,4′ ,5 – trihydroxy stilbene) a phytoalexin drived from grape skin and other fruits [6]. Black grape cultivated in Iraq is rich with resveratrol [7]. Resveratrol is shown to have a potent anti-inflammatory effect[8], antioxidant effect [9], anti platelet aggregation and cardiovascular protection[10]; its potential chemopreventive and chemotheraputic activities have been demonstrated in all three stages of carcinogesis in numerous *in vitro* and *in vivo* studies [6,11]. It has the ability to modulate various targets and signaling pathways [12]. As there is drug development; programmes for pre- clinical screening of the vast numbers of chemicals for specific and non- specific cytotoxicity against many types of cells are involved. Both are important for indicating potential therapeutic target and safety evaluation[13]. The use of *in vitro* assay system for screening has been a common practice since the beginning of cancer chemotherapy in 1946, following the discovery of antineoplastic activity of nitrogen mustard. Some phytochemicals have been shown to exhibit cytotoxic effects against cancer cell through cell cycle modulation [14]. The secondary metabolite " Resveratrol" was synthesized by the plant in response to stress , including disease and ultraviolet light , as phytoalexin was first reported in skins of grapes by Creasy and Coffee[15] ,in 1988 later in 1991 was reported by Siemann and Creasy[16] , from wines.

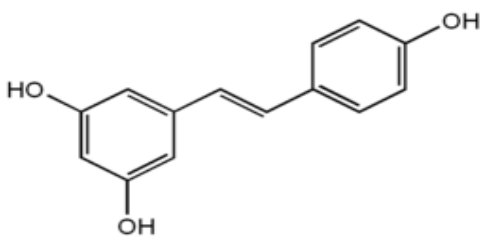

Fig (1) Trans- Resveratrol Structure[17]

Resveratrol [3,5,4′ – trihydroxy stilbene] Fig.(1), is a non Flavonoid polyphenol and it has three phenolic hydroxyl groups and shown to have its biological effects such as; inhibition LDL oxidation[18]. Fresh grape skin contains (50-100) µg/g of trans – resveratrol[19]. The potential for resveratrol to inhibit the development of cancer and extend lifespan in cell culture and animal models have continued to generate scientific interest[20].

Material and Methods

Collection of samples

Local black grapes cultivated in Iraq were collected from the local market and classified as *Vitis vinifera* by the herbarium of the Biology Department, College of Science, Baghdad University . The skin was separated from the fruit to be then kept in a dark cool place , till the following steps.

Preparation of grape skin extract

Preparation of grape skin extract was according to Harborne [21]. All steps were done away from direct light and extensive stress that led to oxidation of the plant extract. About 500 grams of fresh skin grapes was shaken with 2.5 litters 80% ethanol in cool dark place for 72 hours. The extract was filtered and the filtrate was dried at 30-40 °C by a rotary evaporator to get 1/10 (one tenth) its original volume to be stored at –20°C till the following steps.

Acid Hydrolysis

Acid hydrolysis was done using 10% V/V conc. HCl for (10-30) min at 60°C . This step led to the hydrolysis of the glycosidic linkage and got the aglycone moiety, cool and filter[21]. The filtrate was transferred to seperatory funnel. An organic solvent like chloroform was added in a quantity equal to the aqueous phase , with gently shaking and repeating the process three times . The chloroform layers were collected together and washed from the access acid with distilled water . The collected chloroform layers were evaporated to dryness under vacuum with a rotary evaporator at 30°C . The residue was green viscous aliquots stored in dark umber vessels at–20°C untill use [21] .

Column Chromatography (Partial Purification) by Solid – Liquid Adsorption Chromatography

A partial purification of the residue was proceeding using open glass column (2.5 x 21) cm filled with silca gel G60 special for column chromatography . The residue was dissolved in 1-2 ml methanol and the mobile phase is benzene: methanol : acetic acid , 20:4:1[21]. The elutions were collected in 100 separated tube each filled with 3ml eluent. All fractions were tested for $FeCl_3$ 1% solution as a colourimeteric method for polyphenols identification[21,22]. Only the positive results elutions were collected and dried under

vacuum by a rotary evaporator. The resveratrol spots were detected on a TLC aluminum sheet silica gel $60F_{254}$ in comparison with the standard spot using the same mobile phase in the column chromatography [21] .

Preparative Thin Layer Chromatography (P.T.L.C.)

The procedure was running on in a dark cool place away from light and heat . When the solvent system reached to solvent front, removing the plate from the chamber to dry for few minutes, the pure resveratrol line appeared as dark straight line visually (in day light). This line was scratched and eluted with methanol 10ml three times and stored at –20 for 2 days . An amorphous off white crystals formed , to be collected rapidly in cool, dark place and kept in umber reservoir at –20°C. These crystalles were referred as " pure resveratrol ". The crystals were examined by the following tests.

A) U.V. absorption.

B) Thin layer chromatography: using TLC plate of silic gel 60 with flourecence .The mobile phase is benzen : methanol : acetic acid , 20:4:1

C) HPLC method using the following system[23] Column : C18 – reverse phase. Mobile phase : acetonitrile : water , 60:40 .Flow rate : 0.6 ml/min Standard concentration : 0.6 mg / ml (exposed to sun light)Sample concentration : 0.6 mg / ml . Wave length : 307 nm for trans and 280 mm for cis isomer

D) Fourier Transform Infra - Red (FTIR) assay: To detect the functional groups in resveratrol structure.

E) Specific Reaction for (Aromatic Ring) Aluminium chloride ($AlCl_3$)test(Friedle graft) :

F) Specific test for double bond include [22].
(I) Bromine decolourisation test :
(II) The Baeyer test :

G) Melting point: using (Glascoo,U.K.) apparatus.

Cytotoxic Effect of Resveratrol on Cell Line: In vitro Study

The *in vitro* method was used to investigate the effect of pure resveratrol on two types of tumor cell lines (human laryngeal carcinoma Hep-2 and murine mammary adenocarcinoma AMN-3 tumor cell lines) and to compare the results with resveratrol effect on normal cell line Ref-2 cell line at different concentrations and different exposure times. The study involved a comparison between the cytotoxic effects of the extracted resveratrol and a standard (Resveratrol – sigma) and a traditional cytotoxic drug Methotrexat at different concentrations and different times of exposure.

Purified Resveratrol dilutions

Pure resveratrol 4 mg was dissolved in 20ml phosphate buffer saline (PBS) and 0.02 ml dimethylsulfoxide (DMSO) as organic solvent for dissolving the substance. The stock was kept in a dark container at –20°C after sterilization with 0.22µm Millipore filter. Immediate serial dilutions were prepared starting with 100µg/ml and to end with 12.5 µg/ml).The dilution was done with a serum free medium (medium without serum).

Standard resveratrol solution :

Trans- resveratrol standard 2mg was dissolved in 0.01ml DMSO and complete the volume to 10 ml with PBS , sterilized and kept in a dark container at –20°C . An immediate dilution was made starting with the resveratrol concentration 100µg/ml, 50µg/ml, 25µg/ml, and 12.5 µg/ml , using sterile serum free medium.

Methotrexate solution

Methotrexate vial (10mg/ml) was used as a traditional drug in comparison with the effect of standard and pure resveratrol . A stock solution (0.1mg/ml) was prepared. Serial sterile dilutions were made with the following methotrexate concentrations 0.4 µg/ml, 0.2 µg/ml, 0.1 µg/ml and 0.05 µg/ml [24].

Maintenance of the Cell Lines:

When the cells in the flask formed a confluent monolayer, a Freshney, [25] protocol was performed for cell line maintenance

Cytotoxicity Assay

It is also called a cell growth inhibition assay. In this assay, the three types of cell lines were treated with pure resveratrol extract and standard concentrations ranging from 12.5 µg/ml to 100 µg/ml, at the same time the lines were exposed to methotrexate drug in concentrations ranging from 0.05 µg/ml to 0.4 µg/ml using a microtiteration plate (96 wells) cell culture technique. The protocol assay, which included the following steps [25]:

(a) Seeding : The tryptinized and suspended cells were seeded in a microtiter plate by taking 0.2 ml cell suspension into each well that might contain (10^4–10^5) cells/well, growth medium used for seeding

(b) Incubation : All plates were incubated in CO_2 incubator at 37°C for full cells attachment .

(c) The treatment (or cells exposure):
Using the maintenance medium (serum free medium) as a negative control and serum free medium with 0.1%DMSO as positive control , the microtiter plates after cells attachment were exposed to serial dilutions of pure resveratrol , standard resveratrol and methotrexate in the concentrations rang mentioned before.The exposure times were (24,48 and 72) hours. Each plate was designed to contain three replications of each concentration and 12 wells for negative control and 12 wells for positive control .

(d) Recovering times and reading the results:
At the end of the exposure times the medium was decanted off , the cells in the wells were gently washed by the addition of sterile PBS twice , finally 50 µl of crystal violate stain was added to

the wells and the plates were incubated for 30 minutes at 37°C, then the plates were washed gently with distilled water and left to dry.The plates of different cell culture at the end of the assay were examined by ELISA reader at 492 nm transmitting wave length.Only viable cells were able to take the stain , the dead cells were not. The proliferation rate was measured according to [26] and as follows:

Proliferation rate % =

$$\frac{\text{Absorbance at 492 nm of test} \times 100}{\text{Absorbance at 492 nm of control}}$$

While the inhibition rate was measured according to[27] as follows:

Inhibition rate % =

$$\frac{\text{Abs. at 492 nm of control} - \text{Abs. at 492 nm of test} \times 100}{\text{Abs. at 492 nm of control}}$$

Abs = Absorbance
The –ve results referred to the inhibition rate %
While the+ve results referred to proliferation rate %
All values were analyzed statically

Results and Discussion

Partial purification

The resultant fractions from silica gelG60 glass column chromatography technique were eluted according to their affinity to the mobile phase benzene: methanol: acetic acid 20:4:1.Only fractions that gave positive ferric chloride test were collected and detected by T.L.C silica gel $60F_{254}$ plate with same mobile phase. Both the positive fractions and the standard gave violet fluorescence spots with R_F value=0.43. While the negative fractions were not. The dried collection was designated as "Partial purified resveratrol" [28].

Cytotoxic Effect "Growth Inhibitory Assay" of the Purified Resveratrol

Three cell lines were studied (AMN-3, Hep-2 and Ref cell line) at three exposure time 24,48 and 72 hours using two fold dilutions to get concentration ranging from 12.5 µg/ml to 100 µg/ml for both the purified extracted resveratrol and the trans- resveratrol standard, while for methotrexate drug the concentrations included (0.05 , 0.1 , 0.2 and 0.4) µg/ml according to Al-Shemary study [24]. Table(1) showed the results of the significant effect at (P<0.01) level on AMN-3 cell line.The highest concentration 100 µg/ml of the purified extracted resveratrol gave highest cytotoxic inhibitory effect (–37%) after 24 hours of exposure. While trans- resveratrol standard gave the highest cytotoxic inhibitory effect (–16.3%) after 72 hours of exposure on AMN-3 cell line at concentration 100 µg/ml.Methotrexate inhibitory effect at 0.4µg/ml concentration and after 48 hours of exposure gave the highest significant effect (P<0.01). Among three types of treatment (extract, standard and the drug) at all concentrations and for different intervals of exposure; the extracted purified resveratrol had the best efficiency in inhibiting AMN-3 cell growth within (100 µg/ml) at the first 24 hours. These results were declared in a published study [29] on MDA-MB468 breast cancer cell line intended cytotoxic effect of the polyphenol fraction from grape seeds. Resveratrol was considered to be a phytoestrogen, based on its structural similarity to diethyl stilbestrol , a synthetic estrogen. It can bind to both alpha and beta – estrogen receptors and activates estrogen – dependent transcription in human breast cancer cells [6]. The cytotoxic effect of the purified extracted resveratrol on Hep-2 cell line was shown in table(2) . The best concentration with the significant differences (P<0.01) was 100 µg/ml after 24 hour of exposure. While for the standard 100 µg/ml concentration gave the highest inhibitory effect (–27.5%) after 48 hours of exposure. Methotrexate treated cell exhibited significant (p<0.01) inhibitory effect after 48 hour at 0.4 µg/ml concentration. The differences in Hep-2 responsing toward different treatments might indicate a presence or absence of specific cellular receptors in each type of cell lines; making the cells interacts at same concentration in different manners. Moreover the metabolic pathways in response to each treatment differed from one line to another. This fact was mentioned in different studies which investigated at different plant extracts in treating several types of cell lines [30, 31]. In the current study the rat embryo fibroblast cell line (Ref) was treated as well as other cell lines with the extracted resveratrol , the resveratrol standard and the drug in consideration to establish their cytotoxic effect on a normal cell line as a control line. The Ref cell culture passages number 74 and 75 might undergo a transformation**.** Table(3) showed the growth was inhibited significantly (p<0.01) at concentration 50 µg/ml after 24 hours of cell exposure to the purified extracted resveratrol. While for the standard the concentration 100 µg/ml gave the highest inhibitory effect (–61%) after 48 hour of exposure. Methotrexate concentration range (0.1-0.12) µg/ml gave highest inhibitory effect (–57% – 58%) after 48 hour of exposure. The extracted pure resveratrol showed the highest cytotoxic growth inhibition effect in almost all cell lines treated at the concentration range (50 – 100) µg/ml specially at the first 24 hours of exposure. The most sensitive cell line was AMN-3 cell line , the lowest effect was for Hep-2 cell line which was more resistant to resveratrol cytotoxic effect.

Table 1: The cytotoxic effect as inhibition rate percent (%IR) of extracted pure resveratrol and trans-resveratrol standard and methotrexate drug on AMN-3 at (24,48 and 72) hours of exposure .
SE= Standard Error

Treatments	Concentration µg/ml	(%IR)		
		After 24 hour	**After 48 hour**	**After 72 hour**
pure Extracted Resveratrol	100	-37.00 ± 1.73 ^{g}	-5.40 ± 0.23 ^{c}	-16.30 ± 1.15 ^{e}
	50	-6.00 ± 0.28 ^{d}	-4.30 ± 0.17 ^{bc}	-15.00 ± 1.73 ^{e}
	25	-.20 ± 0.25 ^{c}	-2.20 ± 0.11 ^{b}	-15.00 ± 1.73 ^{e}
	12.5	20.00 ± 1.15 ^{a}	1.10 ± 0.05 ^{a}	4.20 ± 0.11 ^{c}
Standard Trans-resveratrol	100	-9.00 ± 0.57 ^{d}	-11.00 ± 1.15 ^{d}	-16.30 ± 1.15 ^{e}
	50	-6.00 ± 0.57 ^{d}	-11.00± 1.15 ^{d}	20.00 ± 1.15 ^{a}
	25	-8.80 ± 1.15 ^{d}	-11.40 ± 0.23 ^{d}	14.00 ± 0.57 ^{b}
	12.5	12.00 ± 1.15 ^{b}	-13.60 ± 1.15 ^{d}	20.00 ± 1.15 ^{a}
Methotrexate Drug	0.4	-0.80 ± 0.05 ^{c}	-28.00 ± 1.73 ^{f}	17.50 ± 0.86 ^{a}
	0.2	-22.00 ± 1.73 ^{e}	-23.00 ± 1.73 ^{e}	5.00 ± 0.57 ^{c}
	0.1	-28.00 ± 2.30 ^{f}	-25.00 ± 1.15 ^{e}	-6.00 ± 0.57 ^{d}
	0.05	-36.00 ± 2.88 ^{g}	-11.00 ± 1.15 ^{d}	-8.00 ± 1.15 ^{d}
LSD		4.169**	2.896 **	3.200 **
Probability level		0.0001	0.0001	0.0001

** (P<0.01).

The means within any column with different letters are of significant differences , using ANOVA test , then the least significant difference test (LSD) to compare significant between columns.

Table 2: The cytotoxic effect as inhibition rate percent (%IR) of extracted pure resveratrol and trans-resveratrol standard and methotrexate drug on Hep-2 cell line at (24,48 and 72) hours of exposure
SE= Standard Error

Treatments	Concentration µg/ml	%IR		
		After 24 hr.	**After 48 hr.**	**After 72 hr.**
Pure Extracted Resveratrol	100	-21.50± 0.28 ^{g}	-7.50± 0.28 ^{d}	-24.00 ± 1.73 ^{h}
	50	-0.44 ± 0.02 ^{f}	-9.50± 0.57 ^{de}	-17.50± 1.15 ^{g}
	25	14.00 ± 1.15 ^{e}	5.40± 0.23 ^{b}	-8.00± 0.57 ^{e}
	12.5	24.50 ± 1.73 ^{c}	11.60± 1.15 ^{a}	1.60± 0.11 ^{b}
Standard Resveratrol	100	25.70± 1.15 ^{bc}	-27.50± 1.73 ^{i}	-3.00 ± 0.28 ^{d}
	50	26.00± 1.15 ^{bc}	-11.00± 0.57 ^{ef}	0.50 ± 0.05 ^{bc}
	25	26.00± 1.00 ^{bc}	5.00± 0.28 ^{b}	11.00± 1.15 ^{a}
	12.5	28.50± 1.73 ^{b}	0.00± 0.00 ^{c}	10.50± 1.15 ^{a}
Drug Menotrexate	0.4	35.00 ± 2.31 ^{a}	-22.50± 1.15 ^{h}	-3.60± 0.11 ^{d}
	0.2	15.00 ±1.15 ^{de}	-18.20± 1.73 ^{g}	-2.00 ± 0.11 ^{cd}
	0.1	29.00± 1.73 ^{b}	-16.50± 1.15 ^{g}	-13.50 ± 1.15 ^{f}
	0.05	0.00± 0.00 ^{b}	-13.50± 0.57 ^{f}	-18.00± 1.73 ^{g}
LSD		3.744 **	2.822 **	2.893 **
Probability level		0.0001	0.0001	0.0001

** (P<0.01).

The means within any column with different letters are of significant differences , using ANOVA test , then the least significant difference test (LSD) to compare significant between columns.

Table 3: The cytotoxic effect as inhibition rate percent (%IR) of extracted pure resveratrol and trans-resveratrol standard and methotrexate drug on Ref cell line at (24,48 and 72) hours of exposure
SE= Standard Error

Treatments	Concentration µg/ml	%IR		
		After 24 hr.	After 48 hr.	After 72 hr.
Pure Extracted Resveratrol	100	-4.00 ± 0.57 [b]	2.00± 0.11 [a]	-34.00± 1.73 [bc]
	50	-46.00 ± 2.30 [h]	-20.00± 1.73 [b]	-33.00± 1.73 [bc]
	25	-40.00 ± 2.30 [g]	-6.40 ± 0.23 [a]	-30.00± 1.15 [b]
	12.5	-11.00 ± 1.15 [c]	5.00 ± 0.57 [a]	-37.00± 2.30 [c]
Standard Resveratrol	100	-36.00± 1.73 [gh]	-61.00 ± 3.46 [h]	-30.00± 1.74 [b]
	50	-37.00 ± 2.30 [b]	-55.00± 2.88 [gh]	-23.00± 1.15 [a]
	25	-36.00 ± 1.73 [fg]	-44.00 ± 2.30 [a]	-28.00± 1.73 [ab]
	12.5	-32.00 ± 1.74 [f]	-27.00 ± 1.73 [d]	-29.00± 2.30 [ab]
Drug Menotrexate	0.4	5.00 ± 0.57 [a]	-50.00± 2.88 [fg]	-34.00± 2.31 [ab]
	0.2	-21.00 ± 1.15 [e]	-58.00± 3.46 [h]	-38.00± 4.04 [c]
	0.1	-17.00 ± 1.73 [de]	-57.00± 2.88 [h]	-39.00± 2.88 [c]
	0.05	-16.00 ± 1.15 [d]	-40.00± 2.30 [e]	-38.00± 1.73 [bc]
LSD		4.815 **	6.848 **	6.435 **
Probability		0.0001	0.0001	0.0006

** ($P<0.01$).

The means within any column with different letters are of significant differences , using ANOVA test , then the least significant difference test (LSD) to compare significant between columns.

Conclusion

The current results analysed the effect of (cis and trans) resveratrol mixture. The yielded purified crystals were very sensitive and liable through many environmental changes with corresponding protections . In spite of that, the best effect was achieved with the extracted resveratrol among the trans- resveratrol standard and methotrexate drug. The attractiveness in the results for this naturally occurring compounds as a cancer chemopreventive agent has been escalated in recently as an ideal chemopreventive / chemotherapeutic agent acting by modulating aberrant signaling pathways and/or inducing apoptosis, and acting to target the multiple biochemical and physiological pathways involved in tumor development with minimizing toxicity in the normal tissue [32].

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