Study of the Cytotoxic Effect of New Copper(II) Complexes and Aqueous Extract of *Origanum Vulgare L.* Plant on Cancer (Cell Line RD)

Saadiyah A.Dhahir, Jinan H.Murtadha and Iman H.Abdul Razzaq
Department of Chemistry, College of Science for Women, University of Baghdad, Iraq.

Abstract

The new complexes of copper (II) 1,2 where L_1 in complex (1) was 2-Amino-5-[2-amino -5-(3,4,5-trimethoxy-benzyl)-pyrimidinyl-4-azo]-phenol, while L_2 was 2-[2-Amino-5-(3,4,5-trimethoxy-benzyl)-pyrimidinyl-4-azo]-4-bromophenol and aqueous extract of *Origanum Vulgare L.*plant (after the chemical assay)were studied on the growth on of Rhabdomyo sarcomas(RD)cell line in human by using *in vitro* system and compared with anticancer drug cisplatin (cis-pt) as a posative control.The cancer cells were treated with different concentration (31.25, 62.5, 125, 250)µg/ml for each of the three treatments and cis-pt after 72 hour exposure time. The cytotoxic activity was tested by inhibition rate as parameter. The results showed significant differences ($p<0.05$) for each three treatments when the inhibition rates were increased. The inhibition levels was reached to 48.13% , 51.75% and 51.63% respectively at 250µg/ml.There was strong correlation between the three treatments and the different concentrations in comparison with cisplatin.

Keywords: Copper(II) complexes, Cytotoxicity cisplatin , Origanum Vulgare.

Introduction

The earliest report on therapeutic use of metals or metals containing compounds in cancer and leukemia data form the sixteenth and nineteenth centuries ,cis platin ,cis platinum or cis–diaminedichloroplatinum (II) (CDDP) is platinum-based chemotherapy drug used to treat various types of cancers, including sarcomas, some carcinomas, ovarian cancer and lymphomas[1].

Numerous other metal compounds containing platinum, other platinum metals, and even non platinum metals were then shown to be effective against tumors in man and experimental tumors in animals. These compound comprise main-group of gallium , germanium ,early transition metals complexes and late transition of ruthenium, rhodium, platinum and copper [2].Chuan Dong et al [3] studied the anticancer activities of some reported copper complexes of salicylaldhydepyrazolhydrozone (Cu-SPHs) derivatives and they showed antiproliferative on growth inhibition to cell line A549. Glutamine Schiff base copper complexes have potential anticancer treatment and prevention [4]. Azo compound have the potential to act as drug carrier that facilitate the selective release of therapeutic agents to colon cancer [5]. Complexes of nickel (II), copper(II) and zinc(II) with thiosemicarbozone were studied on cell proliferation of human leukemiaU937cell lines and ndicated the inhibiting of cellular growth[6]. The Ortance , necessity and potentiality of medicinal plants in practices of medicine today well established and cannot be looked, *Origanum Vulgare L.* belongs to family lamiacean (*origanum*),oregano(*origanumvulgare* , some time listed with *Majoram* as *Origanum majorana*) is also called wild majoram,thats native to Europe ,the Mediterranean, south and central Asia[7]. The *Origanum vulgare* plant contain many compounds terpinen, thymol, sabinine, linolool,terpinolene and large amounts of poly phenols, namely flavonoids (Quercetin, apigenin) [8]. Abdel Massih et al [9] studied anti- prolifferative activity of plant extracts form *Origanum Vulgare* on human lymphoblastic leukemia cell line. The objective of this study is to detect the chemical compounds in *Origanum Vulgare* plant to determine, efficiency of aqueous extract of this plant and two new copper (II) complexes L(1) and L(2) were compared with anti cancer drug cis-platin.

Experimental Work

1-(Cis -platin) (10mg/20ml)drug was provid by Ebew (Austria)

2- New copper(II) complexes L(1) and L(2) were provide by sanna [10]. 10 mg of copper(II) complexes dissolved in 20 ml of normal saline(stock solution) and were stored at(2-8) C° until processing.

3-Aqueous solution extraction of *Origanum Vulgare* plant which prepared as following method.

The *Origanum Valgare* plant were purchased from the local market in Baghdad city. 15 gm of plant were put in to the thimble of soxhlet apparatus which contain 100ml of distilled water in around flask and boiled at 100 C° for 4 hour and mixture were evaporated by using the distillation apparatus to give total weight of component of *Origanum Valgare* powder, then 10 mg of powder extract was dissolved in 20 ml of normal salin as a stock solution and stored at (2-8) C° until further used[11] .

phytochemical screening of aqueous extract of *origanum vulgare* plant were preformed using standard procedure according to the of salmman[12].Test of phenols, steroids ,resins and test of Terpenoids, Alkaloids,Tannis and Saponins according to the method of Ayoola et al[12].

4-Study of cytotoxic effect on cancer cell line**.**

The method was used to investigated the effect of aqueous extract solution of *Origanum Vulgare* plant and new copper (II) complexes on Rhabdomyo Sarcomase (RD) in human cell line was provided by center of biotechnology research center of Al-Naharin university. All solutions are prepared at the same center and culturing tissues were studied *in vitro* under optimum conditions by the same center.The growth media used in tissue culture technique was MEM (Minimum Essential Media) was provided by Fetal Calf Serum (10%) to form a confluent monolyer, then Subculture to discard the previous growth medium and the cells washed with sterilized phosphate buffer solution(PBS) by autoclave at 121 C° for 15 min and addition 2-3ml of trypsin-versen solution was added for 3-5 min and moving the culture flask kindness. The trypsin-versene solution to discard and cells incubated at 37 C° until the cell separation from ground flask, added new growth media and redistribution of cells at the microtiter and incubated at 37 C°[13].

Cytotoxicity Assay

The it is also called a cell growth inhibition assay. In this assay, the cell line (RD) was treated with aqueous extract, new copper complexes and cisplatin by using four concentrations (31.25, 62.5, 125, 250) µg/ml. Immediately by adding of 25ml trypsin-versene solutions in to culture bottle and 20 ml of culture medium which contains 10% of serum to provide the suspend cells, mixed very well and addition of 0.2ml to each microtiter. The plates were incubated at 37 C° for 24 hour until to form monolayer, then the previous culture medium which present in to the plates to discard 0.2 ml of compounds under study were added and these three Preparation repeated as negative control (cancer cell line RD with buffer solutions)and incubated at 37 C° for 72 hour exposure time. The culture medium to discard from micro liter plates, about 0.2ml of crystal violet solution was added to wells and the plates were incubated for 20 min at 37 C°. The plates were washed gently with distilled water and left to dry. In the end of assay the plates were examined by ELISA reader at 492nm transmitting wave length .Only viable cells were able to take a stain while the dead cells were not. The inhibition rate was measured according to Gao et al [14] and as follows:

$$\text{Inhibition rate\%} = \frac{\text{Absorbance of negative control- Absorbanc of Test}}{\text{Absorbance of negative control}} \times 100$$

Statistical Analysis

Data were analyzed by analysis of variance ANOVA. Investigation of differences between cis-platin and the relation with other groups by toward susing the statistical program (SPSS) within significant level ($p<0.05$) [15].

Results and Discussion

-Phytochemical (screening of plant materials) of the *Origanum Vulga*re studied the result are presented in Table (1).

Table (1)
The phytochemical screening of the Origanum Vulgare.

Active compounds	*Reagents*	*Indicators*	*Results*
Tannins	Lead acetate,Ferric chloride	Gelatinous ppt. Green – blue solution	+
Glycosides	Benedict	Red ppt.	+
Flavonoids	Ethanol potassium hydroxide	Yellow solution	+
Phenols	Ferric chloride	Greenish-blue ppt.	+
Resins	Ethanol 95%→boiling→4%HCl	Turbid solution	+
Saponins	Convulse solution	Froth	+
Terpenoids	Chloroform anhydrous acetic acid and sulfuric acid	Brown solution	+
Alkaloids	Mayer 's reagent	White ppt.	-
Steriods	The same of Trepnoids reagent after one day	Blueish solution	-

(+) indicate the positive test.
(-) indicate the nagative tes.t

According to the results showed in Table (1) the aqueous extract *of origanum vulgare* plant contains Flavonoids, Phenols, Trepenoids and tannins ,etc.

The increased of inhibition rates when cancer cells treated with extract plant at different concentrations could be attributed to the Flavonoids such as Apigenin, Luteolin and Quercetin which have a cytotxic effects on growth cancer cell line *in vitro* and *in vivo* systems, therefore used as anticancer therapy [16].

The origanum extract exhibit antiproliferative effect and high antioxidant activity,due to a high content of Phenolic acids and Flavonoids [7,9].

Tannins compounds in aqueous extract which to led apoptosis and stopped one of cell cycle phases (G_1,S_1.G_2) on cancer cells [16]. Sadeghi etal [17] were studied the inhibition effect of Trepenoids in *Dophne mucronata* plant on human Myelogenous leukemia cell line K562 and has been found the cell cycle stopped in G_{1-} phase.

Study of Cytotoxic Effects

The inhibition rates of cancer cells which treated with two new copper (II)complexes 1,2 and aqueous *Origanum vulgare* extract were studied on cancer cell line (RD)with different concentrations comparaple with anticancer drug cis platin as a positive control after 72 hours exposure time.

The results showed significant differences ($p<0.05$) with concentrations increased for three treatments comparison with positive control, the inhibition rates were reached to (48.13% and 51.7%) when the cancer cells were treated with two new copper (II) complexes 1،2 respectively, While the inhibition rate of aqueous extract was reached to 51.63% at 250µg/ml was similar to effect of copper (II) complexes 1،2 with cis-platin drug. The result which showed no significant differences between aqueous extract and a new copper (II) complexes in different concentrations as shown in Table (2).

Table (2)
Inhibition effect on human cancer cell line (RD) with different concentrations of origanum vulgare extract , a new copper (II) complexes 1,2 and cis-pt after 72 hours exposure time.

Treatment / *Conc. µg/ml*	*Inhibition rates% (means ± standard deviation SD)*			
	Cis-pt	*CuL_1*	*CuL_2*	*Aqueous Extract*
31.25	**C,a** 10.50± 2.36	**D,a** 10.95± 3.89	**C,a** 15.92± 2.56	**C,a** 14.55± 4.03
62.5	**B.a** 23.72±3.10	**C,a** 23.50±4.18	**BC,a** 24.03±2.16	**C,a** 24.74±3.04
125	**B.a** 29.99±4.81	**B.a** 34.55±3.33	**B.a** 30.58±7.58	**B.a** 36.11±4.49
250	**A,a** 46.64±6.99	**A,a** 48.13±6.37	**A,a** 51.75±9.02	**A,a** 51.63±6.83

different letters(A,B,C)significant differences (p<0.05) as comparable between column.-different letter (a,b,c) significant differences(p<0.05) as comparable between row.

As shown in Fig.(1), the inhibition rate of cancer cells treated with copper(II) complex (1) was similar to copper complex (2) for four concentrations after72 hour exposure time comparable with cis –pt.

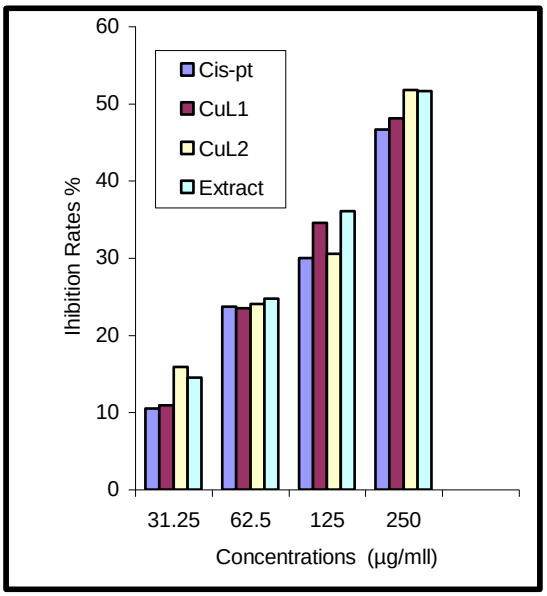

Fig.(1) The comparison of inhibition rates between three Treatments with cis-pt drug.

-The correlation between concentrations and treatments

Table (3) shows the correlation between concentrations (31.25, 62.5, 125, 250)µg/ml and three treatments represented by by aqueous extract and 1,2 1,2 copper (II) complexes comparison with anticancer drug cis-pt ,the correlation of these treatments was approximate with cis-pt was reached to 0.982, 0.997 respectively and the correlation of *origanum vulgare* extracted was similar to copper complex (2) the results showed an evidence which to be found positive strong relation between two copper complexes and aqueous extract.

Table (3)
The correlation (p< 0.05) between all concentrations and each Group (new complexes CuL(1), CuL(2),aqueous extract and cis-platin) and between the same Groups.

Groups	*R*	*cis-platin*	*CuL 1*	*CuL 2*
cis-platin	0.982			
CuL_1	0.995	0.982		
CuL_2	0.96	0.997	0.96	
	5		8	
Extract	0.971	0.992	0.983	0.992

R=Correlation Factor.

The results in the Table (2) and Fig.(1) shows an evidence that new copper complexes have cytotoxic effect on cancer cell line by elevated of inhibition rates with concentration increased, this effect was similar to effect of anti-cancer drug cis-platin. In this study, we suggest the azo ligand in a new copper (II) complexes have inhibition effect, this effect was similar to Tsuda et al [18] studied on colon and liver cancer in miceand result in DNAdamge after shortly administration of relatively high dose, while carcinogenicity was detected after prolong treatment with low doses .Kenyon et al [19] showed the type of organic ligand (bis-8-hydroxyquinolin) coupled to tumor cellular copper forming potent protoseasome inhibitors and apoptosis inducers at copper concentration found in tumor tissues .

This study similar to our results which rised the inhibition rates with elevation of concentration. The new copper (II) complexes was similar effect to cis-platin that could be attributed to the cis-platin binding to and cross linking of DNA which ultimately triggers opoptosis (programmed cell death) [20].

Conclusions

The study showed the new copper (II) complexes and aqueous *origanum vulgare* extract have a cytotoxic effect on RD for four concentrations through exposure time 72 hour by increasing inhibition rates at high concentration 250 µg/ml, these effects were similar to effect of anticancer drug cis –platin.

References

[1] Pruefer**,** F. G.; Lizarraga F. Malonado , V.; and Melendez-Zajgla,J. "Participation of Omi Htra 2 Serine-portease Activity in The Apotosis Induced by Cisplatin on SW480 Colon Cancer Cells"; Chem.20, 348-54, **2008** .

[2] Kopf-Maier, P."Complexes of Metals Other Than Platinum as Antitumor Agents"; Euro.Clini. Pharm.47, 1-16, **1994**.

[3] ChuanDong, F.; Hua,S.; Jing;Z.; Baxiang, Z.;Shngi,Z.; Junying,M." a Novel Copper Complexes of Salicylaldhyde Pyrazole Hydrozone Induced Apoptosis Through Up-regultating Integrin β_4 in H_{322} Lung Carcinoma Cells"; Europ. Medic. Chem. 45, 1438-1446,**2010**.

[4] Xiao**,** Y.; Bi; C.;Fan, X.; Cui, C.; Zhang, X.; Dou, Op. "L-glutamine Schiff Base Copper.Fan et Complex As a Proteasome Inhibitor and an Apoptosis Inducer in Human Cancer Cells";Int. Onco. 33, 1073-9, **2008.**

[5] Eugen, B.; Jams, F.;David,W.;John,T. "Azo Compounds in Colon –specific Drug Delivery";Expe. Opin. Drug Deliv. 4, 547-560, **2007**.

[6] Giselle, C.; Ana Maria, F. "Oxindole and Copper Complexes with Oxidoles-Derivatives as Potential Pharmacological Agents";Braz. Chem. Soci. 17 ,261-268, **2006.**.

[7]Faleiro, L**.** "Antibacterial and Antioxidant Activities of Essential Oils Isolated From Thymbra Capitata L.(cav) and Origanum Vulgare L"; Agric. Food Chem. 53, 8162-8168 ,**2005**.

[8] Ahmed,H.; Amr, F.; Khalid, F. "Effect of Extraction Methods on The Chemical Activity of Egyptian"; Flav.. Frag.9, 54.61,**2002**.

[9] Abdel-Massih, RA.; Fares,R.; Bazzi ,S.;El-Chami ,N.;Baydoun,E. "The Opoptotic and Anti-Proliferative Activity of Origanum Majorana Extracts on human Luekemic Cell Line"; Leuk. Rese. 22, 41-48, **2009**.

[10] Sanaa**,** A. "Preparation and Identification of Some a New Derivative for Trimethoprim Drug"; Univ. Anba. Pure Scie. 3, 48-54, **2009**.

[11] Salmman,I.;"Effect of Crude Extract Silybum Marianum L.Seeds on Cancer and Normal Cell Lines"; Thesis,Education College for Women, Baghdad,48-50,**2008**.

[12] Ayoola, G.; Coker, H.;. Adesegun S.;Adepoju- bello, A.; Ezennia, E.; Alangbayila, T. "Phetochemical Screeng and Antioxidant Activities of Some Selected Medicinal Plant Used for Malaria Therapy in South Western Nigeria";Topi. Pharm. Rese. 7**,** 1019-1024, **2008**.

[13] Freshney, R.I.;"Cultured of Animal Cells: Amanual for Basic Techniqus ";Inc. Publication ,New York;pp64-69**; 2000**.

[14] Gao, X.; Xu, X., Janakiraman, N. ; Gautam, S.C. "Disparate in Vivo Antileukemic Effect of Reseveratrol a Natural Polyphenolic Compound Found in Grapes ";Nutr. 132, 2076-2081,**2002**.

[15] Al-Mohammed**,** N.T.; Al-Rawi, K.M. ; Younis, M.R.; AL-Morani,W.K." Prinsiples of Statistics";.AL-Mous. Univ. 7, 50-52 , **1986.**

[16] Birt, F.D.; .Hendrich,S.; Wang, W. ;" Dietary Agents in Cancer Prevention Flavonids and Iso Flavonoids Pharmacology and Therapeutics"; USA; pp157-177;**2001**.

[17] Sadehi, H.; Yazdanparast, R. "Anti-tumor Activity and Cell Cycle Arrest of a new Diterperne Ester Form Daphane Mucronatab Using K562 Cell";Iran Biom. 7, 127-131, **2003**.

[18] Tsuda, S.; Matsusaka, N.; Madaram H.; Ueno, S.;. Susa, N.; Ishida, K.; Kawamura, N.; Sekihashi, k.; Sasak,Y. "The Comet Assay in Eight Mouse Organs Results with 2 Aso Compounds"; Mutat.Res. 465, 1139-1151, **2000**.

[19] Kenyon, G.; Puja,G.; Harbach,R .; Wayne, G.; Ping Dou, Q."Organic Copper Complexes as New Class of Proteasome Inhibitors and Apoptosis Inducers in Human Cancer Cells"; Bioc. Pharm. 67, 1139-1151, **2004**.

[20] Stordal B.; Davey, M. "Vander Standing Cisplatin Resistance Using Cellular Models"; IUBMB Life . 59, 696-9, **2007**.

الخلاصة

تم دراسة تأثير معقدات النحاس (II) 1و2 الجديدة حيث يكون الليكاندL_1 بالمعقد (1) هو 2-امينو- 5-[2-امينو-5-(3و4و5- تراي ميثوكسي-بنزيل)-بيرميدنايل-4-ازو]-فينول بينما L_2 في المعقد (2) هو 2-[2-امينو-5-(3و4و5- تراي ميثوكسي -بنزيل)-بيرميدنايل- 4-ازو]-4-بروموفينول والمستخلص المائي لنبات

المردقوش *Origanum Vulgare L.* (بعد أجراء الكشف الكيماوي له) على نمو الخط الخلوي السرطاني للعضلة البشرية (RD) للإنسان باستخدام نظام خارج جسم الكائن الحي *in vitro* مقارنة بالعقار المضاد للسرطان السزبلاتين(cis-pt) كسيطرة موجبة. تم معاملة الخلايا السرطانية بتراكيز مختلفة هي (250, 125, 62.5,31.25) مايكرو غرام/مل لكل من المعاملات الثلاث وعقارالسزبلاتين بعد مدة تعريض 72 ساعة. تم اختبار الفعالية السميةالخلويه من خلال مقياس معدل التثبيط.أظهرت النتائج فروقات معنويه ($P< 0.05$) بين معدلات التثبيط لكل من المعاملات الثلاث مع ازدياد التركيز.وقد بلغت مستويات التثبيط 51.63%, 51.75%, 48.13% على التوالي عند التركيز250 مايكروغرام مل.تم ايجاد علاقة قوية بين التراكيز المختلفة والمعاملات الثلاث مقارنة مع السزبلاتين.