

The Cytoprotective Effect of Crocin on Oxaliplatin Treated VERO cell line

In Vitro Study

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Abstract

Background

Cancer is a disease characterized by a shifted in the control mechanism that governs cell survival, proliferation and differentiation.

There are many types of treatment to the cancer like surgery, radiation, chemotherapy, immunotherapy and others. Each of these above treatments has its indication effect and has Side effect and / or adverse effect. The chemotherapy is one type of this treatment to the cancer that have the indication effect, side effect and adverse effect. There are many type of chemotherapeutic agent that used for this type of cancer one of them it is platinum compound (cisplatin, oxaliplatin and others).

There are two types of limitation in the use of these agents one of them is the side effect which is neurotoxicity, Ototoxicity, nephrotoxicity, and etc. and the second one is the resistance of cancer cells to these agents.

Aim of the study

To study the cytoprotection effect of Crocin on oxaliplatin treated VERO cell line

Material and methods:-

By using the tissue culture techniques which is a newly used techniques to check the cytotoxicity of many compound and the expected mechanism of action these techniques made a good jump in the research in many field

In this study we use VERO cell line and after exposure of this cell line firstly to different concentrations of oxaliplatin (1, 0.5, 0.25 and 0.125 mg/ml) for 48hr, secondly to different concentrations of Crocin (0.5, 0.25 and 0.125 mg/ ml) for 48hr. And thirdly to mix concentrations of oxaliplatin and Crocin in 48hr for the VERO cell line and measure the cytotoxicity of these exposures by crystal violet test (C.V. test) to show and compare the effect of these agents specially the Crocin.

The oxaliplatin has cytotoxic effect, its inhibitory concentration 50% (IC₅₀) in 48hr is (0.031mg/ml) which is the maximum cytotoxic effect in vitro. While the Crocin don't have cytotoxic effect at this tested concentration and for this cell line. After mixed different concentration of Crocin with different concentration of oxaliplatin the cytotoxicity of oxaliplatin reduce significantly.

Conclusion:-

From our study we can conclude the following:-

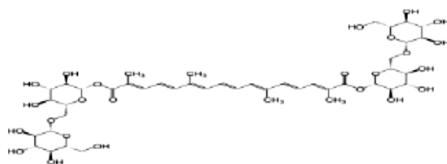
The Crocin has cytoprotective effect. The Crocin has no anti tumor and anti proliferative activity within these tested concentration and this cell line

Crocin

Chemical Name Crocin; Alpha-Crocin; Gardenia Yellow; Crocin 1; Crocin; Crocetin digentiobiose ester

Molecular formula; C₄₄H₆₄O₂₄

Molecular weight; 976.96456 g/mol

**1. Crocin occurrences in plants**

Genus species	family	Common name	part
<i>Crocus sativus</i>	Iridaceae	saffron	plant
<i>Cardenia jasminoides</i>	Rubiaceae	jasmin	fruit

Therapeutic Uses**Cytoprotective, anti inflammatory, free radical scavenger activity**

Crocin improve spatial cognitive abilities following chronic cerebral hypo perfusion and that these effects may be related to the antioxidant effects of these compounds (Hosseinzadeh et al., 2012)

the action of saffron extract (crocin.) as anti oxidant on oxidative stress following renal ischemia-reperfusion injury (IRI) that lead to the generation of reactive oxygen species and lipid per oxidation are associated with tissue injury after the ischemic reperfusion ,therefore, the use of antioxidants appears rational in the improvement of kidney diseases therapy. (Hosseinzadeh et al., 2005)

Many of these pharmacological action of saffron is due to its crude component like crocetin, Crocin and others that have potent antioxidant and free radical scavenger activity against a many of radical oxygen species and pro-inflammatory cytokines. (Mashmoul et al., 2013)

Anti tumor, anti proliferative action

Crocin is mainly extracted from *Crocus sativus* L., it is have anti tumor, antiproliferative effects on cancer cells, but the mechanisms of action are poorly understood it is showed that telomerase activity of HepG2 cells decreases after exposures with crocin, which is may caused by decreased the expression of the catalytic subunit of the enzyme.(Noureini and Wink, 2012) the results indicated that the crocin interact with DNA and induce some conformational changes in the duplex.(Bathaie et al., 2007)

The crocin have a wide range of anti cancer action against a wide range of cancer murine tumors and human leukemia cell lines. The present report surveys the crocin serving as a chemo preventive agent. Dosage dependant cytotoxic effect of crocin to carcinoma, sarcoma and leukemia cells in vitro was noted. The crocin inhibite ascites tumor development and expanded the life expectancy of the treated mice contrasted with untreated controls by 45-120%. What's more, it decreased the onset of papilloma development, diminished rate of squamous cell carcinoma and delicate tissue sarcoma

in treated mice. It shows up now that saffron (dimethyl-crocin) disturbs DNA-protein cooperation's e.g. topoisomerases II, essential for cell DNA amalgamation

Anticancer activity of Crocin against a wide spectrum of murine tumors and human leukemia cell lines. The present report reviews the role of saffron in serving as a chemo preventive agent in modifying cancer risk. Dose-dependent cytotoxic effect to carcinoma, sarcoma and leukemia cells in vitro was noted. Saffron delayed ascites tumor growth and increased the life span of the treated mice compared to untreated controls by 45-120%. In addition, it delayed the onset of papilloma growth, decreased incidence of squamous cell carcinoma and soft tissue sarcoma in treated mice. Understanding the mechanisms of action of saffron has been solitarily based on their carotenoid-like action. Our results indicated significant inhibition in the synthesis of nucleic acids but not protein synthesis. It appears now that saffron (dimethyl-crocin) disrupts DNA-protein interactions e.g. topoisomerases II, important for cellular DNA synthesis.(Nair et al., 1995)

Crocin is the major constituent of *Crocus sativus* its highly inhibited the colorectal cancer and not affecting on the normal tissue. *Crocus sativus* extract mainly the crocin should be investigated for the treatment of colorectal cancer on three colorectal cancer cell lines (HCT-116, SW-480, and HT-29).(Aung et al., 2007)

4-5. Pharmacokinetic properties of crocin

If the crocin is administered orally is not absorbed either after a single or repeated dosage. This crocin is discharged to a great extent through the intestinal tract taking after oral administration, plasma crocin level

don't have a tendency to collect with repeated oral dosage of crocin , the intestinal tract serves as an essential site for crocin hydrolysis.(Xi et al., 2007)

Materials and Methods

2. Methods

2.1 Cell lines used: we can know the types of the cell lines that used in this study and its properties

VERO cell line

Organism *Cercopithecus aethiops*

Tissue kidney

Product Format frozen

Morphology epithelial

Culture Properties adherent

Disease normal

Age adult

Applications

This cell line can be used for the detection of verotoxin.

This cell line can be used for efficacy testing.

This cell line can be used to study malaria biology.

This cell line can be used for media testing.

This cell line can be used for mycoplasma testing.

This cell line is a suitable transfection host.

This cell line can be used for the detection of virus in ground beef.

Complete Growth Medium The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium and RPMI , to make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.(ATCC)

3. Preparation of reagents and solution (Kenkel, 2010) (Freshney and Freshney, 2004)

a. Phosphate buffered saline (PBS).

The substance of one vial of PBS was disintegrated in 1L. Of de ionize distilled water (D.D.W) and blended with mixing. Disinfection was finished

via autoclaving at 120 C° for 20 minutes and store in sterile beaker until use

b. Antibiotics stock solution

Garamycin stock solution

Garamycin vial of 80 mg/ 2ml solution was considered as stock solution stored at 4C° for uses. 6

d. Sodium hydroxide (1N) solution

1N of NaOH was set up by expansion of 40 grams of NaOH to roughly of 900 ml de ionized water(DDW) with cooling and mixing until it broke down totally, then it was finished to 1L. Disinfection was finished by filtration through 0.2 µm Nalgene filter.

Hydrochloric acid (1N) solution

1N of HCL was set up by expansion of 58.8 ml of the 36% HCl drop by drop to 900 ml of de ionized water (DDW) in volumetric beaker with persistent blending. At that point it was finished by de ionized water into 1L. Disinfection done by 0.22 µm Millipore filter unit, and kept sterile until need

f. Trypsin-EDTA solution

A weight of 12.4 gm of Trypsin-EDTA powder (0.05% w/v Trypsin + 0.53mM EDTA) was dissolved in 900ml of D.D.W., with continuous mixed via stirring. And then the acidity of the solution (pH) adjusted to 7.2. And then the volume of solution completed to 1 L by DDW. The solution was sterilized by filtration using 0.45 and 0.22 µm Nalgene filters. The solution stored at - 20C° and dissolve to 37 C° when used.

g. Trypan blue dye

A stock solution of trypan blue dye (4mg/ml) was prepared by mixing PBS to 0.4 g of trypan blue into a graduated chamber with blending until it broke down. The volume was finished with PBS to 100 ml and put away at room temperature.

4. Preparation of the media

4.1. Tissue culture media

Liquid medium was prepared from powdered medium according to the US

biological product manual as following:

A 16.650g of RPMI powder with HEPES support, L-glutamine and phenol red was broken down in roughly 900 ml of de ionized water in volumetric beaker. Alternate segments include: 2g sodium bicarbonate powder or as indicated by need, 2.5 mg amphotercin B dry powder, 1.25 ml gentamycin arrangement and 0.5ml streptomycin arrangement had been included with constant mixing. The volume approximated to around one litter and the pH of the media changed in accordance with 7.4 by utilizing 1N NaOH and a pH meter. The volume was finished to one litter by including D.D.W. sterilization was finished by 0.4 and 0.2 µm Nalgin filter in this manner.⁷

5 ml of the media was hatched in sterile flask for 4 days with day by day examination for indications of bacterial and contagious tainting. It was viewed as clean just if there should arise an occurrence of no indications of tainting amid four days of incubation. At that point the media was put away at 4°C in until utilize

4.2. Maintenance media

Its preparation was similar to those of tissue culture media listed above except it was free of fatal bovine serum (Freshney and Freshney, 2004)

4.3. Freezing media

10 ml stock solution was prepared from different constituents including: 6 ml serum free media, 3 ml fatal, bovine serum, 1ml dimethyl sulfoxide (DMSO) was added drop by drop with mixing. The stock was stored at - 20C° in between uses.(Riddle et al., 1993)

7. Crystal violet assay

Crystal violet (CV) assay was utilized to decide the optical thickness of the cell growth in every well of the micro titer plate, by utilizing plate spectrometer. After the end purpose of cytotoxicity examine, the maintenance

medium with the test material was disposed of out and the wells washed with 100 μ l of icy PBS via programmed pipette. At that point the cell cultures were fixed with 10 % buffered formalin for 20 min at room temperature. Fixative arrangement was disposed of and 100 μ l of 0.1 % CV solution was added to every well. The specimens were brooded at room temperature for 20 min with shaking gently . After that the plates were washed by submersion in streaming tab water for 15 min. Micro plates were permitted to air dry and 0.2 % Triton X-100 in water was added to every well and brooded for 30 min at room temperature with delicate shaking to break up the color. At that point, 100 ml from every well was moved into another 96-well micro plate and the absorbance was examined at 570nm by a micro plate spectrometer (Castro-Garza *et al.*, 2007)

The percentage of viability was calculated according to the following equation:

Viability% = optical density of test well/ optical density of control X 100.

The percentage of viability was calculated according to the following equation: (Chiang *et al.*, 2003)

Inhibition % = (optical density of test wells/optical density of Control wells) x 100

8. Cytotoxicity assays

Data in table (3-1) revealed that the viability percent of VERO cell line in the

As indicated by Freshney (2004), the cytotoxicity measures were connected for examined the effect of oxaliplatin and Crocin on SW-480 cell line culture. Different groupings of oxaliplatin and Crocin were tried for a specific time terms. At the point when the growth in the flask got to be as monolayer before it achieved the exponential stage, the cell monolayer were reaped and re-suspended with a growth medium in a centralization of 5×10^5 cell/ml and seeded in a 96 well microtiter plate. At the point when the cell developments achieve 80%, the wells were presented to serial exposure of the test chemicals as in the following analyses.

Experiment

Measuring the cytotoxic effect of different concentration Of oxaliplatin on the cell line after 48 hr and calculate the ID50 of oxaliplatin in Vero cell line. Measuring the cytotoxic effect of different concentrations Of Crocin on the cell line after 48 hr and calculate the ID50 of Crocin if it found in Vero cell line. Measuring the cytotoxic effect of mixing different concentrations of Crocin with different concentration Of oxaliplatin on the cell line after 48 hr and calculate the net effect for them in Vero cell line.

3. The results

3.1. in vitro cytotoxicity study:

3.1.1. Effects of oxaliplatin cytotoxicity on VERO cell line (crystal violet assay) (C.V.):

Oxaliplatin concentration	The percent of viability of oxaliplatin by C.V. test (mean $\pm$ SD) in 48hr.
Ox 00 mg/ml (control)	100 $\pm$ 0.446
Ox 1mg mg/ml	17.2 $\pm$ 0.978*
ox 0.5 mg/ml	21.21 $\pm$ 1.093*
ox 0.25 mg/ml	29.94 1 $\pm$.006*
ox 0.125 mg/ml	37.27 $\pm$ 1.103*

* = the difference is significant (P<0.05) as compared with control.

Table (3-1) cytotoxicity of oxaliplatin on VERO cell line (OX= oxaliplatin)

presence of different concentrations of oxaliplatin (1, 0.5, 0.25 and 0.125 mg/ml) was ranged between (17.2 ± 0.978 - 37.27 ± 1.103) after 48h

There were significant differences ($P < 0.05$) in the percent of viability 48h groups of treatment throughout this period of incubation used in the study as compared to control group (without oxaliplatin) the viability of VERO cell line decreased through increasing the concentration of oxaliplatin. While there were no- significant differences ($P < 0.05$) in the percent of viability between 9 48hr and 72hr groups of treatment because the oxaliplatin under goes decomposition after reconstitution after 72hr.in vitro.

We can calculate the ID50 of oxaliplatin for 48hr (0.031mg/ml) and 72hr of incubation. So the maximum effect of oxaliplatin is after 48hr in vitro

ID50 is the concentration of oxaliplatin that needs to produce 50% viability after specific time of exposure

3.1.2. Effects of Crocin on VERO cell line:

Data in table (3-2) revealed that the viability percent of VERO cell line in the presence of different concentrations of Crocin (0.5, 0.25 and 0.125 mg/ml) was ranged between (100.259 ± 0.409 – 100.72 ± 0.452) after 48h incubation.

Conc.Of Crocin	The percent of viability of Crocin by C.V. test (mean $\pm$ SD) in 48hr.
00mg/ml control	100 $\pm$ 0.446
0.5mg/ml	100.259 $\pm$ 0.409
0.25mg/ml	100.367 $\pm$ 0.345
0.125mg/ml	100.72 $\pm$ 0.452

Table (3-2) cytotoxicity of Crocin for cancer cell in different concentrations

There was non-significant differences ($P < 0.05$) in the percent of viability for all groups of treatment throughout the period of incubation used in the study as compared to control group (without Crocin).

. Effect of concomitant use of different concentration of oxaliplatin with different concentration of Crocin on VERO cell line:

The viability percent of VERO cell line for different concentrations of oxaliplatin (1, 0.5, 0.25, 0.125mg/ml) used in concomitant with different concentration of Crocin (0.5, 0.25, 0.125 mg/ml) for 48h show in the table (3-3)

Cro Conc.	conc. ox.	0.125mg/ml	0.25mg/ml	0.5mg/ml	1 mg/ml	00mg/ml control
00 μ g/ml control		37.27 $\pm$ 1.103*	29.94 $\pm$ 1.006*	21.21 $\pm$ 1.093*	17.2 $\pm$ 0.978*	100 $\pm$ 0.446
500 μ g/ml		65.50 $\pm$ 1.09*	60.46 $\pm$ 1.13*	49.66 $\pm$ 6.56*	38.80 $\pm$ 3.40*	100.259 $\pm$ 0.409
250 μ g/ml		63.27 $\pm$ 0.30*	58.81 $\pm$ 1.35*	48.06 $\pm$ 1.1*	38.75 $\pm$ 1.84*	100.367 $\pm$ 0.345
125 μ g/ml		62.11 $\pm$ 0.22*	49.95 $\pm$ 1.43*	42.97 $\pm$ 1.21*	31.87 $\pm$ 2.15*	100.72 $\pm$ 0.452

* = the difference is significant ($P < 0.05$) as compared with control

Ox= oxaliplatin , cro= Crocin

Table (3-3) viability percent after 48 hour (mix oxaliplatin + Crocin) in different concentrations

4. Discussion

4.1. Cytotoxicity test c.v.:

We can notice that the oxalo- platin has cytotoxicity effect on VERO cells in

response to the concentration of drug and the time of exposure. So when the concentration of drug increased the viability of the cells decreased or the

killing effect of the drug increased (Suzuki et al., 2008).

The IC₅₀ of the oxaliplatin after 48hr. is (0.031mg/ml). So there is a significant reduce in the viability of the VERO cells in 48hr. and there is a non significant difference in the viability of the VERO cells between the 48hr. and 72hr. the reasons of this that the oxaliplatin after the reconstitution remained stable for 48hr in vitro and after this period the drug is decomposed . And for these reasons it does not give more effect (Ibrahim and Mauvernay, 1998) (Hospira Company).

So in vitro the maximum effect of this drug, it is reached after 48hr. and for these reasons all our cytotoxicity effect will be measured for 48hr. So the maximum effect of oxaliplatin is after 48hr. and the IC₅₀ is (0.031mg/ml)

For the Crocin we can notice that this crude herbal material does not have any cytotoxic effect on VERO cell line. Because after the exposure of VERO cells to the Crocin in different concentration (0.5, 0.25, 0.125mg/ml) for 48hr. the Crocin does not exhibit any cytotoxic effect on the VERO cells and this conclusion is fitted with the following researches.

Argyraki et al they said that the Crocin have cytogenic and/ or cytoprotective effect at tested dose (Argyraki et al.).

Lari et al said that the Crocin reduces the hepatotoxicity and protective effect agonist diazion throughout the reduction in inflammation mediator and decrease the caspases activity (Lari et al., 2015)

While there are other researches aren't fitted with these data examples of these researches are as following:

Kim et al said the Crocin and crocetin which is the carotinoid of saffron induce cytotoxic effect (Kim et al., 2014)

Aung et al also proved that Crocin from *Crocus sativus* possesses significant anti-proliferation effects on human colorectal cancer cells through the p-53 protein (Aung et al., 2007)

The above researchers said that there is a anti tumor and/ or anti proliferative effect for the Crocin. And these different in the result as we expected is due to either once or more from the following causes;

1. The data or this effect (cytotoxic) effect is measured on the other cell line (ex. HeLa cell line and HepG2). (Tavakkol-Afshari et al., 2008) Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines HeLa HepG2 via the p53 activity. 11

2. As mentioned in some of these researches they dissolve the Crocin powder in DDW and then applied on the wells that contained the cells as done by (Li et al., 2012) they dissolve the Crocin in DDW while we think is better to dissolve the Crocin in SFM

And this may affect the constituent of the growth media. This may lead to change the concentration of nutrients, minerals, growth factors and co factors and / or PH or the buffer system of the growth media, this disturbance in the media may lead to killing effect to the cells and as we know that a tissue culture experiment is highly sensitive experiment

3. There is another type of crud herbal material that obtained from saffron (ex. Safranal, crocetin) this crud material is examined to have cytotoxic effect on the different cell lines and there is a confusion between the crocetin (have cytotoxic effect) and Crocin (don't have cytotoxic effect) as said by (Li et al., 2012) some time mention Crocin and other mention crocetin.

In the concomitant use of oxaliplatin and Crocin or mixed the oxaliplatin

and Crocin in different concentration for 48hr (maximum effect of oxaliplatin), we can notice that the cytotoxic effect of oxaliplatin decreased when mixed with Crocin

The data that obtained we can concluded that the Crocin don't have cytotoxic effect and in reverse, it has cytoprotective effect within this range of concentration and this cell line

Conclusion

From our study we can conclude the following:

1. The Crocin has cytoprotective

2. The Crocin has no anti tumor and anti proliferative activity within these tasted concentration and this cell line

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