

In vitro antitumor efficacy of *Kochia indica* extract on human hepatocellular carcinoma cell line with or without 5-fluorouracil

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ABSTRACT

Aim: Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and the sixth most common cancer worldwide. The resistance to chemotherapy is a major obstacle in the treatment of HCC, necessitating the discovery of additional agents. There is a growing use of anticancer complementary and alternative medicine worldwide. Therefore, the aim of present study was focused on the confirmation of the suitability and validity of the new markers which would be achieved by demonstrating their significant change and reproducible expression during disease and disease management. **Methods:** HepG₂ cell line was used to provide a source for HCC cells. The cell cultures were divided into 4 groups: control untreated group, 5-fluorouracil (5-FU) treated group as a standard chemotherapy for HCC (positive control) with the following doses (15.625, 31.2, 62.5, 125, 250 µg/mL), *Kochia indica* extract treated group with the following concentration (12.5, 25, 50, 100, 200 µg/mL) and the group treated with a combination of 5-FU and *Kochia indica* in different ratios. **Results:** Treatment with *Kochia indica* extract, 5-FU and the combined treatment showed a significant cytotoxicity to HepG₂ cells, with different IC₅₀ values, when compared to the control. Regarding toxic effect, 5-FU showed IC₅₀ = 237.56 µg/mL which is lower cytotoxic in compared to *Kochia indica* with IC₅₀ = 120.5 µg/mL. The results also revealed that tumor cells were more resistant to 5-FU. Alternatively, the co-treatment with *Kochia indica* extract ameliorated the toxicity induced by 5-FU and enhanced its therapeutic potency, either by synergistic effect of both agents and/or due to its flavonoid components that may enhance the physiological properties of the cell membranes, facilitating 5-FU entrance into tumor cells. This decreased its therapeutic dose to less than 250 µg/mL by combination therapy. **Conclusion:** Present findings assume that *Kochia indica* extract co-therapy can ameliorate the side effects of 5-FU on HepG₂ by enhancing its cellular uptake.



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INTRODUCTION

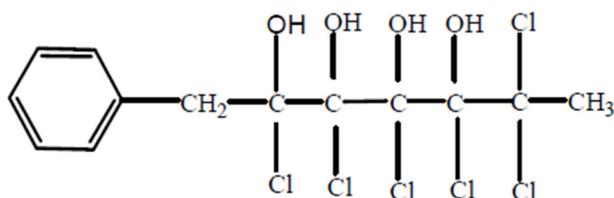
Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and the sixth most common cancer worldwide.^[1] Also, It is an aggressive malignancy with a poor prognosis and is currently the second most common cause of cancer-related mortality.^[2] It was found that about 80% of the estimated new cases of HCC occurred in less developed regions and its incidence is increasing worldwide in more developed countries^[3] including Egypt because of predominant diseases such as hepatitis C virus (HCV) and schistosomiasis.^[4]

It was recognized that the resistance to chemotherapy, 5-fluorouracil (5-FU), is a major obstacle in the treatment of HCC, necessitating the discovery of additional agents.^[5] Thus, the use of natural products in this respect is extensively under investigation.^[6] One of these products is *Kochia indica*, family chenopodiaceae [Figure 1]. It was originally introduced as an ornamental plant in some gardens. In the present time, it has spread and is now presented in both crop and no crop areas.^[7]

The elementary analysis of *Kochia indica* extract was previously carried out by Haroun^[8] using ultraviolet (UV) spectroscopy and infrared spectroscopy, gas chromatography and mass spectrophotometry. As shown in Figure 2, the chemical analysis of *Kochia indica* extract detects an active phenolic compound



Figure 1: A photograph showing *Kochia indica* whole plant



1-phenyl 2,3,4,5,6,6 hexa chloro 2,3,4,5 tetra hydroxy heptan

Figure 2: Phenolic active compound of *Kochia indica* plant extract^[8]

with proposed molecular formula $C_{13}H_{14}Cl_6O_4$, molecular weight 447 and chemical structure, 1-phenyl 2,3,4,5,6 hexachloro 2,3,4,5 tetra hydroxy heptane.

Previous investigators showed that *Kochia indica* has a great medicinal potency. It is used as a heart tonic agent^[9] and it has a strong tumoricidal properties.^[8] Additionally, resinous alkaloid, isolated from alcoholic extract of the plant showed nicotinic action on autonomic ganglia and neuromuscular junctions of voluntary muscles. Fruits and leaves of a related species, *Kochiascoparia* is used as cardiac tonic, anti-dermatitis and diuretic agent.^[10]

Also, ether extract of aerial parts of *Kochia* contains n-alkanes, free alcohols and a mixture of sterols, mainly sitosterol (70.9%). Ether plant extract exhibited antibacterial activity which is attributed to hydrocarbons and sterols present in its parts.

As known, one of the excellent chemotherapeutic drugs is 5-FU. Despite the excellent therapeutic effects of 5-FU, its cytotoxicity and genotoxicity in normal cells remain a major health problem.^[11] Therefore, thinking about herbal use is gradually growing worldwide to reduce the hepatotoxicity induced by 5-FU treatment. The present work was undertaken to test whether *Kochia indica* extract can exhibit antitumor effect by itself and can improve the antitumor efficacy of 5-FU treatment by reducing its probable side effects.

METHODS

Chemicals, cultures and reagents

The anti-cancer 5-FU obtained as an ampoule (10 mL) contains 250 mg of fluorouracil was stored below 30 °C, protected from light and preserved in sealed containers. It is manufactured by ACDIMA International (AiT, S.X. Haipu Pharmaceutical Co., Ltd.).

To prepare the standard anticancer treatment regimen of 5-FU, it was diluted with Dulbecco's modified Eagle medium (DMEM) to desired concentrations ranging from 10 to 250 μ g/mL. The final concentration of dimethyl sulfoxide (DMSO) in each cell culture did not exceed 1% v/v, to keep the cytotoxicity of DMSO at less than 10%. However, fluorouracil (25 mg/mL) stock solution was 100 fold diluted (10 mL + 990 mL medium) then it was mixed with the *Kochia indica* extract in various ratios, and each combination ratio was 2 fold serially diluted. The concentration of each agent in the combination was determined according to the following equation:

$N \cdot V$ [half maximal inhibitory concentration (IC_{50}) for agent separately] = $N \cdot V$ (in combination)
 $N = IC_{50}$ for the agent, V = volume of the agent separately = 1
 N = the concentration of the agent in the combination,
 V = the volume of the agent in the combination

Cell culture media, including DMEM and fetal bovine serum (FBS) were purchased from GIBCO® (Invitrogen). Penicillin and streptomycin, the culture antibiotics, were purchased from (Aldrich-Sigma Company, CA, USA). DMSO, fluorescence dye, Sodium bicarbonate ($NaHCO_3$), neutral red, 4-(4'-nitrobenzyl) pyridine (NBP) and 5-FU were also purchased from Sigma-Aldrich. All chemicals and reagents used in the experiments are of highly purified grades.

Hepatoma cell line

Hepatoma cell line (HepG₂) was obtained from Holding Company for Biological and Vaccine Production, Cairo, Egypt. HepG₂ was maintained in the cell culture laboratory, Medical Research Institute, Smouha, Alex, Egypt. HepG₂ is a perpetual cell line consisting of human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male patient who had a well-differentiated HCC.

Natural agents

Kochia indica leaf plant was collected nearby Tanta city, Al-Gharbiya Governorate, Egypt. The plant was authenticated visually in taxonomy laboratory at Botany Department, Faculty of Science, Tanta University, Egypt. The collected plants were washed under running tap water and blotted where they were cut into small pieces and kept for drying in oven at temperature 40 ± 2 °C for 5 days. The dried plant material was ground into powder and stored in air tight container as described by Prayong *et al.*^[12] The crude ethanolic extracts were dissolved in DMSO at 20 mg/mL as stock solutions which were then diluted with DMEM to desired working concentrations ranging from 10 to 250 µg/mL. The final concentration of DMSO in each sample did not exceed 1% v/v, to keep the cytotoxicity of DMSO at less than 10%.

Preparation of HepG₂ culture media

The HepG₂ culture media was prepared according to Van der Bliek *et al.*^[13] Briefly, the vial containing HepG₂ was placed in a 37 °C water bath until the contents were thawed and decontaminated immediately by dipping in or spraying with 70% ethanol. The vial contents were transferred to a centrifuge tube containing 9.0 mL of absolute DMEM. To prepare DMEM growth medium, FBS was added to reach 10% final concentration. Prepared cell culture media were spin at approximately

125 g for 5-7 min. The cell pellet was suspended again in the medium and dispense into a 25 cm² culture flask. The culture was incubated at 37 °C in a 5% CO₂ incubator and thereafter, was ready for sub culturing into working groups.

Experimental cell groups

HepG₂ cells were divided into 4 groups described as follow: (1) first group contained HepG₂ cells cultured in a media and left without any treatments to serve as a negative control; (2) second group contained HepG₂ cells cultured in a media treated with the following doses (15.625, 31.2, 62.5, 125, 250 µg/mL) of 5-FU as a standard chemotherapy for HCC and left to serve as a positive control; (3) third group contained HepG₂ cells cultured in a media and treated with the following doses (12.5, 25, 50, 100, 200 µg/mL) concentrations of *Kochia indica* ethanol extract alone and left as an experimental group; (4) fourth group contained HepG₂ cells cultured in a media treated with a combination of 5-FU and *Kochia indica* extract in different ratios (1:1, 2:1, 1:2, 1:9) and left as an experimental group.

Cell viability determination

The HepG₂ culture was sub-cultured at 37 °C under a humidified environment containing 5% CO₂ incubator. Cytotoxic effect of the tested agents on tumor cells was tested by the neutral red (NR) method described by Fotakis and Timbrell.^[14] In brief, tumor cells were seeded in 96-well plates (100 µL/well at a density of 3×10^5 cells/mL) and treated with various concentrations of the used agents for 24 h. Then, cells were washed twice with 1× phosphate buffer saline and the supernatant was discarded. A total of 100 µL of NR solutions (50 µg/mL) was added to each well and incubated at 37 °C for another hour. The NR dye was then dissolved in 100 µL of 0.33% HCl. Absorbance of NR dye was detected by a dual-wavelength UV spectrometer (Anthos 2010; Biochrom, UK) at 450 nm wavelength. The cytotoxicity was determined against untreated cells by the following equation of Machana *et al.*:^[15] cytotoxicity (%) = $[100 \times (\text{absorbance of untreated group} - \text{absorbance of treated group})] / \text{absorbance of untreated group}$. Therefore, the cell viability (%) = 100 - cytotoxicity.

To calculate the IC_{50} values of the agents under investigation, cytotoxicity (%) was plotted against agent's working concentrations^[14] to give linear equation from which IC_{50} was calculated. Also, the quantitative efficacy of the combination exposure between 5-FU and *Kochia indica* extract was determined as a combination index (CI) according to the following equation:^[16] $CI = (5-FU) \text{ in combination} / (5-FU) \text{ alone} +$

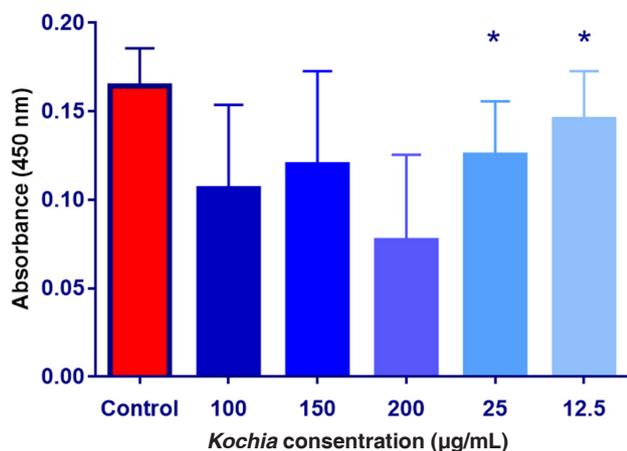


Figure 3: The absorbance values of HepG₂ cultured cells exposed to upgrading concentrations of *Kochia indica* extract. Data are expressed as means \pm SD ($n = 9$); * $P \leq 0.05$ vs. non-treated control group

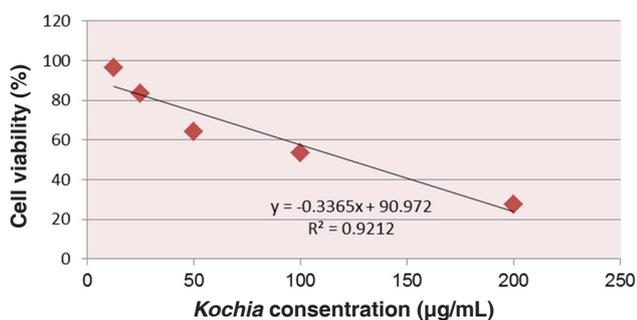


Figure 4: A linear relationship between calculated cell viability of HepG₂ cultured cells and concentrations of *Kochia indica* extract. This linear relationship resulted in $y = -0.3365x + 90.972$ and $R^2 = 0.9212$, from which value of IC₅₀ was obtained as 121.75 µg/mL

(*K. indica*) in combination/(*K. indica*) alone. If $CI < 1$, it means antagonistic effect; $CI = 1$, it means synergistic effect; and if $CI > 1$, it means additive effect.

Statistical analysis

Data were expressed as means \pm standard deviation ($n \geq 3$). Data of treated groups were statistically analyzed vs. control group by one way analysis of variance (ANOVA), followed by t -test using Graph Pad prism 6 Software, CA, USA. However, statistical difference was considered significant if $P \leq 0.05$ confidence interval.

RESULTS

Effect of *Kochia indica* extract on HepG₂

As shown on Figure 3, the mean values of absorbance were decreased after the exposure of HepG₂ cells to upgrading concentrations of *Kochia indica* extract compared to the control group. The obtained data were analyzed with ANOVA and showed that the difference among the absorbance values in the control group

Table 1: Effect of upgrading concentrations of *Kochia indica* extract on the HepG₂ cultured cells toxicity and viability ($n = 9$)

<i>Kochia indica</i> concentration	Cytotoxicity (%)	Cell viability (%)	t value	P value
Control	0	100		
12.5 µg/mL	3.80435	96.19565	3.761	0.0031
25 µg/mL	16.48551	83.51449	1.771	0.104
50 µg/mL	36.05072	63.94928	2.555	0.028
100 µg/mL	46.73913	53.26087	2.503	0.092
200 µg/mL	72.46377	27.53623	1.317	0.215
P value	0.0071			
F value	3.765			

and the other groups treated with *Kochia indica* was significant at $P \leq 0.05$. By application of t -test, the difference between means was significant after the exposure of HepG₂ cells to 12.5 and 25 µg/mL of the plant extract while the differences between means of other groups were not significant.

Data in Table 1 show the values of both cell toxicity and viability percentages after the exposure of HepG₂ cultured cells to all treatments. It was found that the cytotoxicity (%) was increased with increasing concentration of *Kochia indica* extract (12.5-200 µg/mL). Oppositely, the cell viability (%) of HepG₂ was gradually decreased with increasing concentration of the plant extract. Therefore, as the concentration of *Kochia indica* extract increases, the cytotoxicity (%) of HepG₂ increases, and vice versa for the % of the cell viability which decreases with the increase of the extract concentration [Figure 4].

Effect of 5-FU concentrations on HepG₂

As shown on Figure 5, the absorbance values were decreased after the exposure of HepG₂ cultured cells exposed to various concentrations of 5-FU compared to the control group. ANOVA showed that the difference among the absorbance values in the control group and the other groups treated with 5-FU was significant at $P \leq 0.05$. To determine the significance, t -test was applied on the obtained data and showed that the difference between means was significant after the exposure of HepG₂ cells to 125 and 250 µg/mL of 5-FU, but not significant with other groups.

Results in Table 2 demonstrates that the percentage of cytotoxicity increased gradually by increasing the concentration of 5-FU, while the viability (%) of HepG₂ cultured cells was decreased. The absorbance means of two groups exposed to both concentrations 125 and 250 µg/mL of 5-FU were significantly different ($P \leq 0.05$) compared to the control.

Data depicted in Figure 6 show the relationship

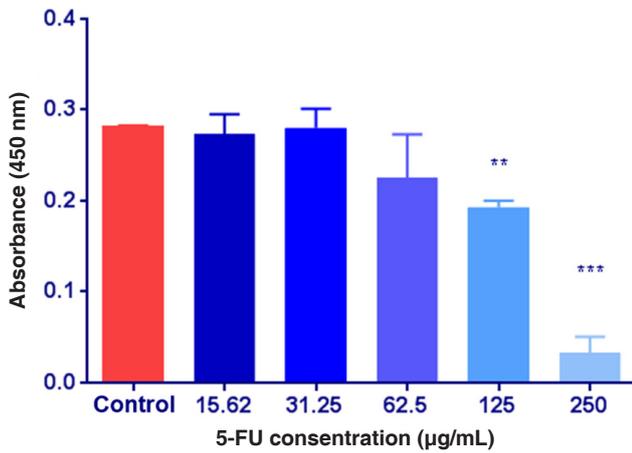


Figure 5: The absorbance values of HepG₂ cultured cells exposed to 5-fluorouracil (5-FU) various concentrations. Data are expressed as means ± SD (n = 9, **P ≤ 0.01, ***P ≤ 0.001, vs. non-treated control group)

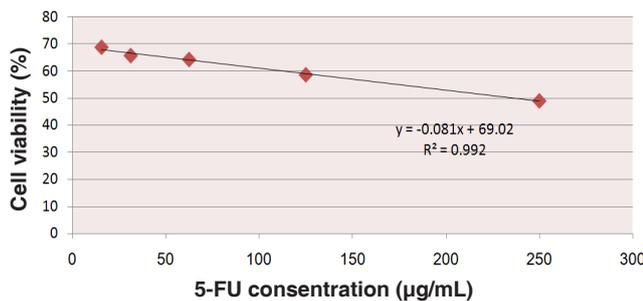


Figure 6: The linear indirect relationship between cell viability percent of HepG₂ cultured cells against upgrading concentrations of 5-fluorouracil (5-FU), value of IC₅₀ that gives 50% viability was obtained as 234.342 µg/mL

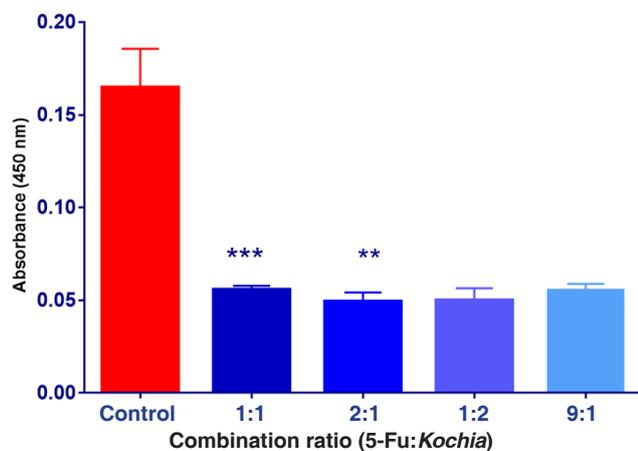


Figure 7: The absorbance values of HepG₂ cultured cells exposed to combination ratios of 5-fluorouracil (5-FU) and *Kochia indica* extract various concentrations. Data are expressed as means ± SD (n = 9), **P ≤ 0.01, ***P ≤ 0.001, vs. non-treated control group

between the percentages of the HepG₂ cell viability and upgrading concentrations of 5-FU. This relationship gives rise to a linear equation: $y = (-0.081x + 69.02)$ ($R^2 = 0.992$) from which IC₅₀ be calculated as

Table 2: Effect of upgrading concentrations of 5-FU on the HepG₂ cultured cell toxicity and viability (n = 9)

5-FU concentrations	Cytotoxicity (%)	Cell viability (%)	P value (t-test)	t value (t-test)
Control	0	100		
15.625 µg/mL	31.34058	68.65942	< 0.626	0.569
31.25 µg/mL	34.42029	65.57971	< 0.891	0.1549
62.5 µg/mL	35.86957	64.13043	< 0.2339	0.1686
125 µg/mL	41.48551	58.51449	< 0.0031	18.72
250 µg/mL	51.08696	48.91304	< 0.0001	17.8
P value (ANOVA)	< 0.0001			
F value (ANOVA)	78.11			

5-FU: 5-fluorouracil; ANOVA: analysis of variance

Table 3: Effect of combination therapy of 5-FU and *Kochia indica* extract on the HepG₂ cultured cells toxicity and viability (n = 9)

Combination ratio 5-FU:Kochia (v:v)	Cytotoxicity (%)	Viability (%)	P value (t-test)	t value (t-test)
Control	0	100		
A ratio of 1:1	42	58	< 0.0005	1.587
A ratio of 2:1	53.2	46.8	< 0.0018	2.321
A ratio of 1:2	52	48	< 0.9319	18.647
A ratio of 9:1	57	43	< 0.736	15.265
P value (ANOVA)	< 0.0001			
F value (ANOVA)	67.08			

5-FU: 5-fluorouracil; ANOVA: analysis of variance

234.342 µg/mL of 5-FU.

Combination effect of 5-FU and *Kochia indica* extract on HepG₂ cells

Data shown on Figure 7 shows means of the absorbance values of HepG₂ cells exposed to various combination ratios of 5-FU and *Kochia indica* extract. It was observed that the obtained absorbance in the experimental groups was reduced compared to the control group. Statistical analysis revealed that means of treatments in ratio of 2:1 was more significant than 1:1, compared to the control.

Results in Table 3 show the combined effect of both 5-FU (250 µg/mL) and *Kochia indica* extract (100 µg/mL) on the cell toxicity and viability of HepG₂ cultured cells. After the exposure of HepG₂ to the combination therapy, data obtained were determined as mean values of the absorbance. Based on the absorbance, the percentage of cytotoxicity was increased while the percentage of the cell viability was decreased by increasing the combination ratios. The most effective ratio was found to be (1:2, 5-FU/*Kochia*) of both agents and this ratio led to cytotoxicity of 52% and cell viability of 48% on HepG₂ cultured cells.

By the application of CI equation on the ratio of 1:2, CI can be calculated as: $CI = 83.33/250 + 66.66/100 = 0.33$

+ 0.6666 = 0.9966 = 1 in approximately (synergistic effect). Accordingly, when 2 v of *Kochia indica* extract was added to 1 v of 5-FU, there is a synergistic effect on the cell viability of HepG₂.

DISCUSSION

The *in vitro* cytotoxicity test was mainly performed to screen potentially toxic compounds that affect basic cellular functions. This toxicity is measured with cellular damage using NR which is a weak cationic dye that penetrates and accumulates in the lysosomes of living cells. Therefore, NR assay was used to determine the cell viability or, in other words, the toxicity of the test compounds.^[14]

Data obtained from *in vitro* study on *Kochia indica* extract, 5-FU and combination treatment showed a significant cytotoxic effect to HepG₂ cultured cells, with different IC₅₀ values, when compared to the control. For example, the value of IC₅₀ of *Kochia indica* extract was found to be 120.5 µg/mL. Also, a previous study showed that *Kochia indica* seeds exerted lethal effect on tumor cells IC₅₀ = 0.147 mg/mL which may indicate that the seeds of this plant have serious toxic side effects than other parts.^[8]

Other studies showed different results between aqueous and alcoholic extracts of *Kochia indica* where the anticancer activity of the aqueous extract was 2.88 and anticancer activity of the ethanolic extract was 1.6. It was observed that the aqueous extract is more potential than the ethanolic one.^[15]

Many studies showed that *Kochia indica* extract is rich in flavonoids^[16] and alkaloids.^[9] Therefore, it has many biological properties about its mode of action such as antioxidant,^[17] heart tonic^[9] and anticancer effect.^[5,9,17] Many cancers develop resistance to chemotherapy, thus lowering its anticancer efficacy. As current treatments become inadequate, higher doses of anticancer therapies such as 5-FU and other novel therapies are used in treating cancers. Such higher dose therapies can lead to cytotoxic and genotoxic side effects in normal cells.^[18]

According to the above information, 5-FU is a widely used anticancer drug, but the response to it shows low efficacy, approximately 20%.^[19] Thus, many studies have investigated 5-FU with other anticancer drugs such as antioxidants.^[20] However, cytotoxicity is nearly inevitable when a combination of 5-FU and antioxidants is used, which remains a major problem in chemotherapy.^[21]

In the present study, 5-FU showed IC₅₀ = 237.56 µg/mL

which is a lower cytotoxic in comparing with *Kochia indica* with IC₅₀ = 120.5 µg/mL which emphasize the resistance of HepG₂ cells to 5-FU as a chemotherapeutic agent. The current work showed also additive synergistic effect when 5-FU combines with *Kochia indica* extract by ratio of 2:1 (v/v) in the treatment of HepG₂ cultured cells. A previous study showed two dual effects when 5-FU enhanced with antioxidant such as, Bupleuri Radix that enhanced 5-FU induced cytotoxicity in HepG₂ hepatoma cells, and protected normal lymphocytes from 5-FU induced cytotoxicity.^[22]

Results of IC₅₀ obtained above showed that the treatment of HCC with either 5-FU or *Kochia indica* extract alone can lead to a cytotoxic effect to the target tumor cells. Instead, the combination between a well-known chemotherapy such as 5-FU and antioxidant such as *Kochia indica* extract can ameliorate the toxicity induced by 5-FU as well as enhance the therapeutic potency of 5-FU toward cancer cells by what is known synergistic effect of both agents. The present results can also suggest that flavonoids found in *Kochia indica* extract enhance the physiological properties of the cell membranes facilitating 5-FU entrance into tumor cells which were resistant to 5-FU, and hence decrease the therapeutic dose to be less than 250 µg/mL as shown in combination therapy. These results are considered as a first line in studying the possible efficacy of *Kochia indica* extract as an anti cancer agent or as adjuvant with 5-FU, however, further *in vivo* studies are suggested at experimental animal sessions.

Authors' contributions

Study design: N.M. Abdel-Hamid
Experimental work: W.O. Zeina, G.A. Tabl
Manuscript drafting: Y.E. El-Bolkainy
Manuscript revision: N.M. Abdel-Hamid, W.O. Zeina

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

University of Tanta approved the whole work prior to any work.

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