Cynaropicrin Potentiates the Anti-Tumor Effects of Paclitaxel and 5-Fluorouracil on KYSE30 Human Esophageal Carcinoma

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Cynaropicrin potentiates the anti-tumor effects of paclitaxel and 5-fluorouracil on KYSE30 human esophageal carcinoma

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Abstract

Natural products or their use in combination therapy regimens may reduce side effects of chemotherapy and increase the effectiveness of treatments. The cytotoxic effects of cynaropicrin, a sesquiterpene lactone isolated from *Centaurea behen* were evaluated for the first time against esophageal squamous carcinoma cells (KYSE30). The synergistic effects of cynaropicrin with paclitaxel and 5-fluorouracil (5-Fu), conventional chemotherapeutic drugs used for esophageal cancer treatment, were also investigated. MTT results indicated that the 50% inhibitory concentration (IC_{50}) values of cynaropicrin on KYSE30 cells after 24, 48 and 72 h were 72, 43 and 28 μM, respectively, which were significantly different from those on normal HDF cells. It was also determined that cynaropicrin could induce the cell death via apoptosis. Our results indicated that cynaropicrin had synergism with both drugs. The best effects of cynaropicrin in combination with paclitaxel were observed at 48 h, in which dose reduction of paclitaxel reached 3 times in IC_{50}. Combination with 5-fluorouracil, resulted in 15 times dose reduction of 5-Fu in IC_{50} at 24 h. In conclusion cynaropicrin has selective cytotoxic effects on KYSE30 cells and we suggest its use in combination therapies with paclitaxel and 5-Fu, which would reduce the side effects of these conventional treatments.

Keywords: Esophageal cancer, Combination therapy, Cynaropicrin, Synergistic effects.

Introduction

The geographical distribution of esophageal cancer is variable in different parts of the world, reflecting the impact of environmental conditions, lifestyle and genetic factors on development of this disease (Mir *et al*., 2005; Wang *et al*., 2020). The incidence of this cancer is usually three to four times higher in men than women (Ferlay *et al*., 2015; Torre *et al*., 2015). In 2012, about 400,000 deaths from esophageal cancer and 456,000 new cases of this disease were documented (Ferlay *et al.,* 2013; Ferlay *et al.,* 2015., Liang *et al*., 2017). Nearly 80% of new cases occur in less developed regions of the world (Enzinger *et al*., 2003; Ferlay *et al.,* 2015; Wong *et al*., 2018). Eastern Asia, eastern and southern Africa have the highest incidence of esophageal cancer and western Africa has the lowest (Torre *et al.,* 2015; Liang *et al.,* 2017; Wong *et al*., 2018). This type of cancer is divided into two main forms with specific cause and distinct pathological features, including: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) (Jemal *et al*., 2011; Zhang *et al*., 2012; Malhotra *et al*., 2017). In 2012, nearly 90% of reported cases of esophageal cancer in the world were ESCC (Arnold *et al*., 2015; Abnet *et al*., 2018). In a region, often known as "esophageal cancer belt" extending from northern Iran through the Central Asian republics to the north central China, 91% cases of esophageal cancer are ESCC (Tran *et al*., 2005; Akbari *et al*., 2006; Siegel *et al*., 2015; Komal *et al*., 2019). ESCC is highly invasive and is usually diagnosed in advanced stages and thus it has a very poor prognosis (Quint *et al*., 1995; Jemal *et al*., 2005; Aghcheli *et al*., 2011; Abnet *et al*., 2018; Zhang *et al*., 2020). The survival rate of ESCC in developed countries is 10 to 15% (Moyana *et al*., 1996; Wang *et al*., 2017; Abnet *et al*., 2018). In advanced stages, that cancer has metastasized to the surrounding tissues such as lymph nodes, chemotherapy or combination of chemotherapy, radiotherapy and surgery are the preferred options for esophageal cancer treatment (Lagergren *et al*., 2017; Hou *et al*., 2019). However, despite the effectiveness of chemotherapy and radiotherapy, they often

cause very severe side effects (Qi *et al*., 2010; Shapiro, 2016; Turcotte *et al*., 2017). Studies suggest that radiations used for radiotherapy, increase the cancer invasion power, tumor migration and metastasis. In the case of chemotherapy, very high toxicity and low efficacy have caused pessimism to this treatment (Camphausen *et al*., 2001; Wild-Bode *et al*., 2001; Jung *et al*., 2007; Huang *et al*., 2017). Generally, the complexity of the formation and development of esophageal cancer prevents effective treatment (Qi *et al*., 2010; Yang *et al*., 2012; Hu *et al*., 2016).

During the past decades, use of targeted therapy has evolved greatly, however over the last few years, this strategy has been geared towards combination therapy (Devita *et al*., 1975; Chesney *et al*., 2000; Jukema and van der Hoorn, 2004; Weber and Noels, 2011; Gosh *et al*., 2018), because the traditional use of a single drug for treatment of complex diseases, such as cancer, is usually not effective. Using a combination of drugs, usually reduces their adverse effects (Nowak *et al*., 2016; Mokhtari *et al*., 2017). The combination of drugs can lead to additive, synergistic, antagonistic, or suppressive effects, however the best situation is a combination that leads to synergism as it reduces the dosage and drug resistance, increases the efficacy of treatment, and strengthens drug targeting. This type of treatment is considered as a standard method for cancer therapy (Devita *et al*., 1975; Jia *et al*., 2009; Zhang *et al*., 2012; Mokhtari *et al*., 2017). It can also be stated that the biological effects of compounds in a combined state are much greater than a single compound. The mixture of the compounds has multiple bioactive effects due to having different targets (Devita *et al*., 1975; Chesney *et al*., 2000; Jukema and van der Hoorn, 2004; Weber and Noels, 2011; Mokhtari *et al*., 2017).

Natural products constitute safe, endless, and rich sources of drugs for treatment of various diseases (Rates, 2001; Haefner, 2003; Butler, 2004; Raskin *et al*., 2004; Cragg *et al*., 2005; Mishra *et al*., 2011; Rey-Ladino *et al*., 2011; Alam *et al*., 2018). There has been a lot of interest in developing alternative chemotherapy regimens that tend to use natural compounds,

especially medicinal herbs (Fu *et al*., 2009; Kaur *et al*., 2011; Mansoori *et al*., 2019). From 155 anti-cancer drugs made since 1940, about 50% were either from natural products or their derivatives (Kinghorn *et al*., 2009; Ahmad *et al*., 2016; Newman *et al*., 2016; Elrayess *et al*., 2019). This tendency to natural products is mostly due to the mentioned disadvantages of conventional drugs, such as ineffectiveness, side effects and toxicity on normal cells (Fu *et al*., 2009; Kaur *et al*., 2011; Mansoori *et al*., 2019). Furthermore, natural compounds are safe and can lead to development of more effective therapeutic agents (Rates, 2001; Mansoori *et al*., 2019). Natural secondary metabolites with herbal origin play an important role in cancer chemotherapy (Mehhndiratta *et al*., 2011; Seca *et al*., 2018). Sesquiterpene lactones (SLs) are a large and diverse group of natural products found in more than 100 flowering plant families. The highest numbers of SLs have been extracted from the plants belonging to the family Asteraceae (Heinrich *et al*., 1998), which are rich in bioactive compounds (secondary metabolites) such as polyacetylenes, diterpenes and SLs (Herz, 1977). These compounds have many therapeutic activities including anti-inflammatory, anti-tumor, anti-microbial and anti-viral properties (Baruah *et al*., 1994; Zhang *et al*., 2005; Xavier-ravi *et al*., 2019). SLs have been reported as promising anti-cancer agents with potentials in both chemotherapy and chemoprevention (Akao *et al*., 2013; Quintana *et al*., 2018). They exert their properties by inhibiting inflammatory responses, preventing metastasis, and inducing apoptosis (Zhang *et al*., 2005; Quintana *et al*., 2018).

Centaurea L. with more than 700 species is one of the largest genera in the family Asteraceae (Bensouici*et al*., 2012). Members of this genus are spread all over the world (Forgo *et al*., 2012). The main and most important compounds identified in this group are SLs (Cardona *et a*l., 1994; Georgiadou *et al*., 2000). *Centaurea*is well known in folk medicine for its anti-microbial, anti-cancer, anti-diabetic, anti-rheumatic, anti-pyretic, anti-inflammatory, stimulant, tonic, and some other properties (Garbacki *et al*., 1999; Akkol *et al*., 2009; Koca *et*

al., 2009; Chougule *et al*., 2012). According to several experiments, SLs are responsible for the therapeutic properties of this genus (Chicca *et al*., 2011). *Centaurea behen* is an annual herb belonging to genus *Centaurea*. It is native to Iran, and is known as an Irano-Turanian plant. In Iran it is known as Bahman Sefid. *C. behen* also grows in countries such as Iraq, Pakistan, and India as well as North Africa and Europe (Khare, 2004; Mozaffarian, 2012; Mosaddegh *et al*., 2018). *C.behen* is used for traditional treatment of a number of diseases, including cystic fibrosis, central nervous system (CNS) disorders, and kidney stone, and is also used for itscardiotonic, sedative, anti-inflammatory,emmenagogue and aphrodisiac properties (Yadava *et al*., 2006, Mozaffarian, 2012; Mosaddegh *et al*., 2018). A number of SLs, including arguerin B, grosshemin and cynaropicrin, have been purified from this species (Oksuz *et al*., 1982). Researchers have proven that cynaropicrin has a wide variety of biological activities including: anti-tumor (Zong *et al*., 1994; Ha *et al*., 2003; Muhammad *et al*., 2003; Cho *et al*., 2004; Cho *et al*., 2004b; Choi *et al*., 2005; Kolli *et al*., 2012; Salvador *et al*., 2008; Yang *et al*., 2008; Butturini *et al*., 2013; liu *et al*., 2019), anti-inflammatory (Cho *et al*., 1998; Cho *et al*., 2000; Hayat *et al*., 2019), anti-parasitic (Schinor *et al*., 2004; Drab *et al*., 2005; Zimmermann *et al*., 2012; da Silva *et al*., 2013; Mokoka *et al*., 2013), anti-bacterial (Bachelier *et al*., 2006), anti-photoaging, anti-oxidant (Tanaka *et al*., 2013; Yamada *et al*., 2015) and anti-spasmodic, through inhibiting the production of cytokines such as TNFα and suppression of NF-κB. Anti-cancer effects of cynaropicrin on various cancers such as stomach, lung, breast and leukemia have been already reported (Cho *et al*., 2004b). According to the global burden of disease (GBD) statistics, esophageal cancer is among the six most common cancers that has imposed heavy burdens on Iranian society (Majidi *et al*., 2017), but despite these reports, little studies have been carried out on this cancer in Iran.In the present study we aimed to: 1) evaluate the cytotoxic and anti-cancer properties of cynaropicrin on ESCC cells for the first time, 2) determine the mechanism of cell death

induced by cynaropicrin, and 3) investigate its synergistic effects in combination with common drugs used in esophageal cancer chemotherapy, such as paclitaxel and 5-fluorouracil, in order to reduce the dose and subsequently, side effects of these medications.

Materials and Methods

Chemicals

Roswell Park Memorial Institute (RPMI)- 1640 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) was obtained from Sigma Aldrich. Anti-cancer drugs used in this study include: paclitaxel (pac) from Actavis and 5-fluorouracil (5-Fu) from Elewe PHARMA. FITC-Annexin V Apoptosis Detection Kit with propidium iodide (PI) was purchased from BioLegend. Cynaropicrin was extracted and purified from *C. behen* (voucher specimens: No. 12405) in our previous work (Shakeri *et al*., 2018).

Cell culture

Human esophageal cancer cell line (KYSE30) (Pasteur Institute, Iran) was cultured in RPMI-1640 with 10% FBS as a supplement, and human dermal fibroblast cell line (HDF) as normal cells (a generous gift from ACECR) were cultured in high glucose DMEM containing 15% FBS. Both cell lines were cultured at 37˚C in a humidified atmosphere containing 5% CO_2 (Lim *et al*., 2000; Pan *et al*., 2014).

Cell viability assay

Cell viability was evaluated by MTT assay. To do so, cells were cultured (9000 cells/well for KYSE30 and 10000 cells/well for HDF) in 96 well plates (Orange Scientific), and treated with various concentrations of cynaropicrin (KYSE30 and HDF cells), pac, 5-Fu, cynaropicrin-pac and cynaropicrin-5-Fu (only KYSE30 cells) for 24, 48 and 72 h. Finally, cells were incubated with 20 µl MTT per well and after 4 h the absorbance of formazan crystals dissolved in dimethyl sulfoxide (DMSO) was measured at 570 nm by an ELISA plate reader (AWARENESS, USA).

Apoptosis detection

KYSE30 cells treated with 25 µg/ml cynaropicrin for 15 h were dually stained with FITC-annexin V and PI for detection of apoptotic cells. Following BioLegend's instructions, treated cells were first washed with staining buffer. After harvesting and centrifugation, annexin V binding buffer was added and cells were stained with FITC-annexin V and PI for 15 min (in the dark). Finally, cells were subjected to flow cytometry to analyze the mechanism of cell death induced by cynaropicrin.

Investigating the synergistic effects of cynaropicrin with chemotherapy drugs

KYSE30 cells were treated with cynaropicrin, pac and 5-Fu, separately. Inhibitory concentration (IC_{50}) for each agent was evaluated by MTT assay after 24, 48 and 72 h. According to median effect method of Chou-Talalay and using $IC_{50\ Cyn}/IC_{50\ drug}$ relationship or inverse, the combination ratio was evaluated for cynaropicrin-pac and cynaropicrin-5Fu in all three time periods. KYSE30 cells were then treated with a combination of cynaropicrin-pac and cynaropicrin-5-Fu for 72 h. Synergistic effects of cynaropicrin with any of the used drugs was determined through evaluating the combination index (CI) and dose reduction

index (DRI) using the Calcu-Syn software (Biosoft, Cambridge, UK). In this regard, CI<1 means synergistic effects, CI=1 means additive effects and CI>1 means antagonistic effects (Chou, 2010; Ashton, 2015).

Statistical analyses

Statistical analyses were performed using GraphPad Prism v. 6.07. Significant differences were ascertained by one-way ANOVA and multiple *t*-test. In the case of calculating the synergistic effects, Calcu-Syn software was used.

Results

Investigating the cytotoxic effects of cynaropicrin on KYSE30 cells

MTT assay was used to evaluate the cytotoxicity of cynaropicrin on esophageal cancer cells. To do so, cells were treated with different concentrations of the agent for 24, 48 and 72 h. IC_{50} values were calculated as 25.11, 15.39 and 9.94 µg/ml equal to 72, 43 and 28 µM for 24, 48 and 72 h, respectively. Dose-response curves and IC_{50} values are shown in Fig. 1, indicating that cynaropicrin cytotoxicity on KYSE30 cells is concentration- and time-dependent. (Supplementary Graph 1, Comparison: 5 and Supplementary Image: 1, 2 and 3).

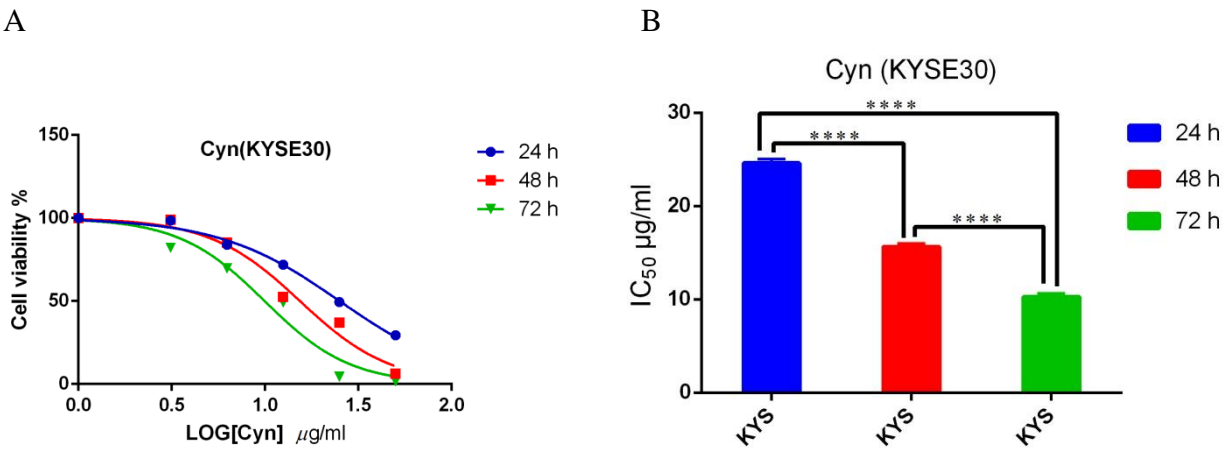


Fig. 1: A) Dose-response curves indicating the cytotoxic effects of cynaropicrin on KYSE30 cells. The graphs show the survival percentage of cells after treatment with different concentrations of cynaropicrin at 24, 48 and 72 h. The data are average of three repeats in each concentration with SEM. B) Comparing the IC_{50} values of cynaropicrin on KYSE30 cells at 24, 48 and 72 h. Columns are plotted with standard deviation. **** indicates the significance of the differences at the levels of $p<0.0001$.

To evaluate the anti-cancer effects of cynaropicrin, the same assay was performed on HDF, as normal cells. Values of 58.06, 46.31 and 33.40 µg/ml were calculated as IC_{50} of cynaropicrin at 24, 48 and 72 h, respectively. As indicated in Fig. 2, the results showed that cynaropicrin toxicity on this cell line is also concentration- and time- dependent. (Supplementary Graph 2, Comparison: 6 and Supplementary Image: 4, 5 and 6).

A B

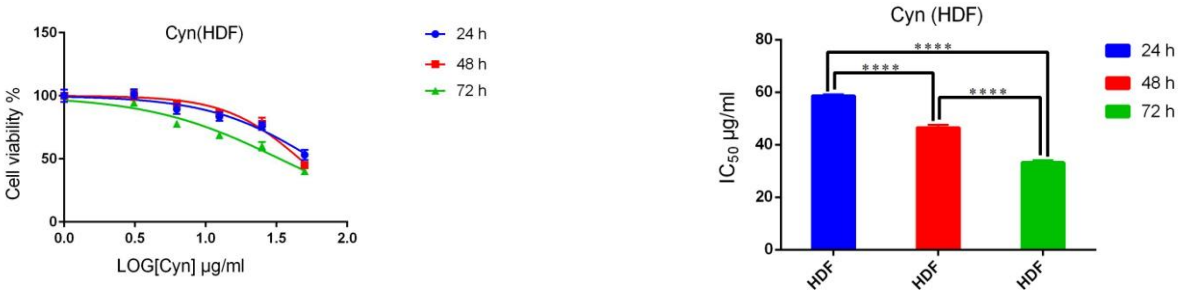


Fig. 2: A) Dose-response curves indicating the effects of cynaropicrin on HDF cells. The graphs show the survival percentage of cells after treatment with different concentrations of cynaropicrin at 24, 48 and 72 h. B) Comparing IC_{50} values of cynaropicrin on HDF cells at 24, 48 and 72 h. Columns are plotted with standard deviation. **** indicates the significance of the differences at the levels of $p<0.0001$.

Comparing the results between the two cell lines as shown in Fig. 3, indicated a selective cytotoxicity on the cancerous cells, which may confirm the anti-cancer effects of cynaropicrin. (Supplementary Comparison: 7).

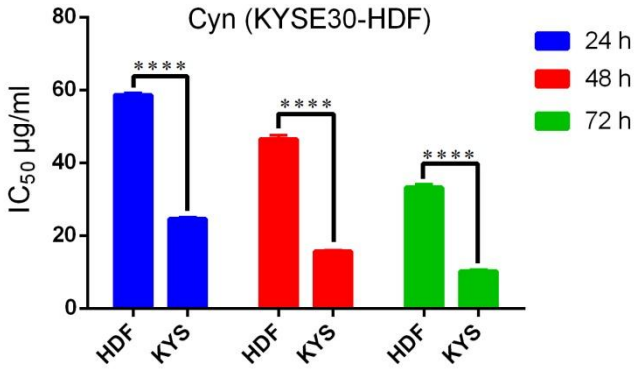
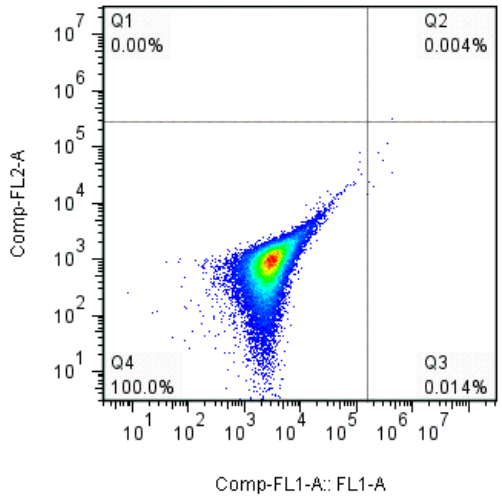


Fig. 3: Comparing the IC_{50} values of cynaropicrin on KYSE30 and HDF cells at 24, 48 and 72 h. **** indicates significance of differences at the level of $p<0.0001$.

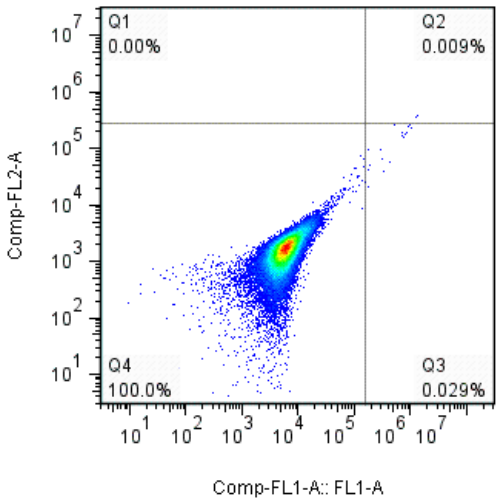
Cynaropicrin induces apoptotic cell death

To investigate the cell death mechanism induced by cynaropicrin, flow cytometric analysis was performed after staining with FITC-annexin V and PI. Cells were treated with the calculated IC_{50} value of cynaropicrin for 24 h, equal to 25 µg/ml, and stained after 15 h. A control group was also treated with DMSO to find out the effects of solvent on induction of apoptosis. Pac was used as a positive control and untreated cells were considered as negative control. Flow cytometry results are presented in Fig. 4. (Supplementary A, C and D).

A



B



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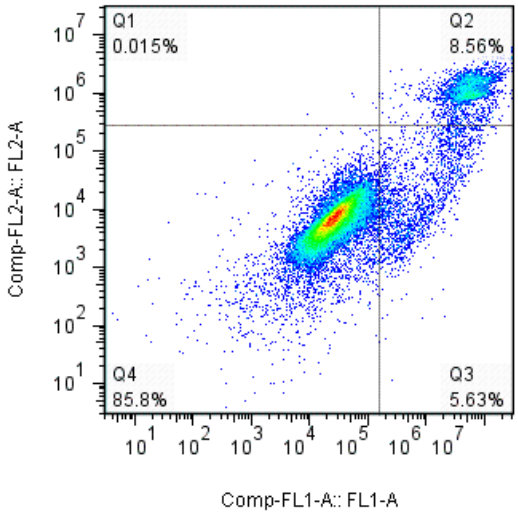

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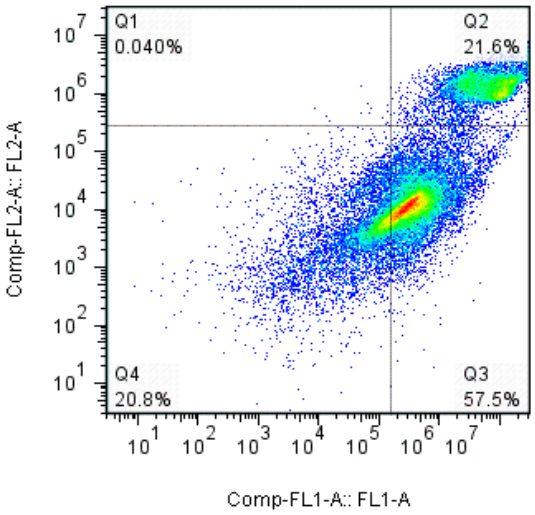


Fig. 4: Cynaropicrin induces apoptosis in KYSE30 cells as shown with PI and FITC-annexin V double staining and flow cytometry. A) Untreated cells, B) cells treated with DMSO, C) cells treated with paclitaxel, and D) cells treated with cynaropicrin.

The diagram of treated cells with cynaropicrin shows the presence of the majority of cells in quarter 3, which indicates the occurrence of apoptosis in cells caused by cynaropicrin. The cells in this quarter are annexin V^+ and PI^-.

Cynaropicrin had synergistic effects with paclitaxel and 5-Fu

The median effect method of Chou-Talalay was used for investigating the synergistic effects of cynaropicrin with different drugs. To do so, acquiring data such as the IC_{50} and slope of dose-response diagrams for cynaropicrin, pac and 5-Fu at three time periods was necessary. As shown in Fig. 5, IC_{50} values estimated for pac, were equal to 7.93, 5.92 and 3.90 µg/ml and for 5-Fu, 79.98, 25.22 and 15.16 µg/ml at 24, 48 and 72 h, respectively. (Supplementary Gragh3,4 and Supplementary Image: 7, 8, 9, 10, 11 and 12).

A B

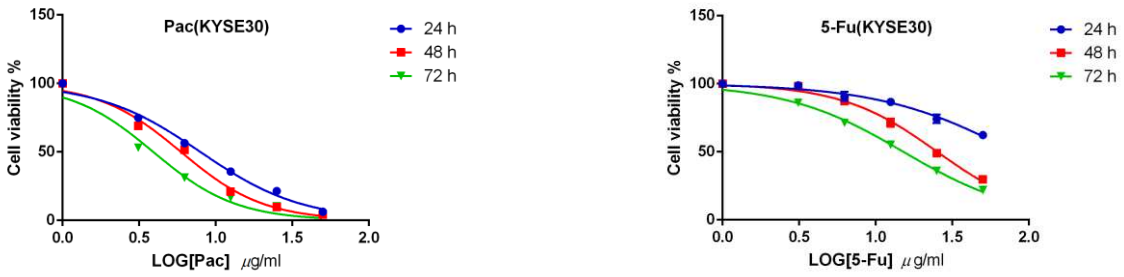


Fig. 5: Dose-response curves of A) pac and B) 5-Fu on KYSE30 cells. The graphs show the survival percentages of cells after treatment with different concentrations of drugs at 24, 48 and 72 h.

The results indicated that pac had cytotoxic effects on KYSE30 cells in a time and concentration-dependent manner. Moreover, 5-Fu showed similar but weaker effects, especially on the first day of treatment. The combination experiments for cynaropicrin and the two drugs were designed and cells were treated for 24, 48 and 72 h, based on the principles of the median effect method. The results of both combinations, in all three time periods, are presented in Figs. 6 and 7 for pac and 5-Fu, respectively. Diagrams were drawn in the form of combination and dose reduction indices. With regard to pac-cyn combination, the graphs represented an additive effect at low concentrations and an antagonistic effect at high concentrations of combination within 24 h. A synergistic effect at 48 h and a moderate synergism at 72 h were observed. In both time periods, the CI value had been reduced at higher concentrations and the synergism was strengthened. The results of the cynaropicrin-pac combination in three periods are listed in table 1in three sections A, B and C. (Supplementary S1, S2 and S3).

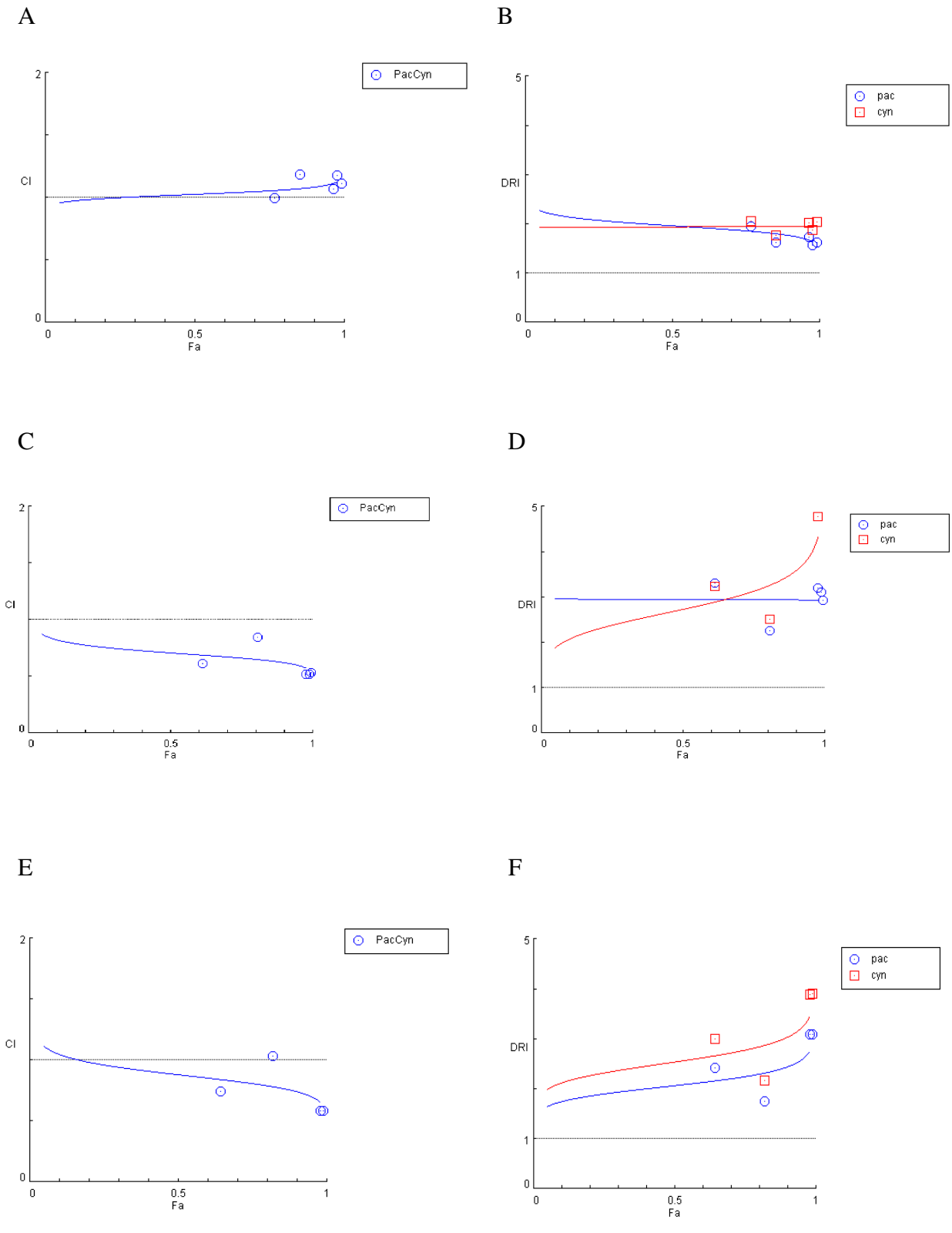


Fig. 6: A, C and E): Combination index diagrams show the simultaneous effects of pac and cynaropicrin on KYSE30 cells at 24, 48 and 72 h, respectively. B, D and F): Dose reduction diagrams express a multiplier reduction in the dose of pac and cyn in a combination treatment over 24, 48 and 72 h. CI: Combination Index, Fa: Fraction affected, CI doesnot have unit.

411 Table 1: CI and the DRI related to the combination of cyn-pac at A) 24, B) 48, C) 72 h.

412

413 A

24 h	**CI value**	**DRI Cyn**	**DRI Pac**
IC_{50}	1.02	1.93	1.96
IC_{75}	1.05	1.94	1.85
IC_{90}	1.08	1.94	1.75
IC_{95}	1.10	1.94	1.69

414

415 B

48 h	**CI values**	**DRI Cyn**	**DRI Pac**
IC_{50}	0.70	2.72	2.72
IC_{75}	0.65	3.12	2.93
IC_{90}	0.61	3.58	2.93
IC_{95}	0.59	3.93	2.93

416

417 C

72 h	**CI value**	**DRI Cyn**	**DRI Pac**
IC_{50}	0.87	2.53	2.06
IC_{75}	0.80	2.77	2.24
IC_{90}	0.73	3.02	2.43
IC_{95}	0.69	3.22	2.58

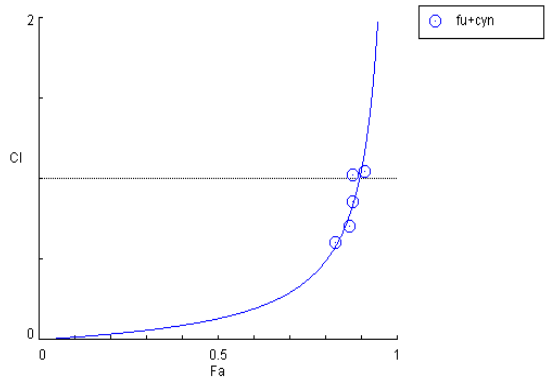
418

419

420 The combination of cynaropicrin and 5-Fu demonstrated a very strong synergism, so that CI=
421 0.13 and DRI for both substances in the IC_{50} was about 15. However, at higher
422 concentrations, the observed synergism was very weak, which ultimately ends to antagonism.
423 The results for this combination at 48 and 72 h indicated a synergism and a moderate
424 synergism, respectively.

In Fig. 7, the CI and the DRI charts referring to cyn-5-Fu combinations at 24, 48 and 72 h are presented. The results of the cynaropicrin-5-Fu combination in three periods are listed in table 2 in three sections A, B and C. (Supplementary S4, S5 and S6).

A



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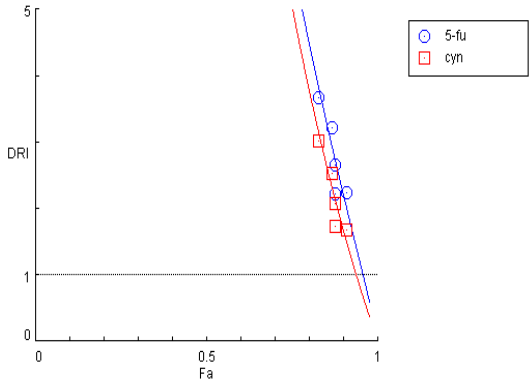

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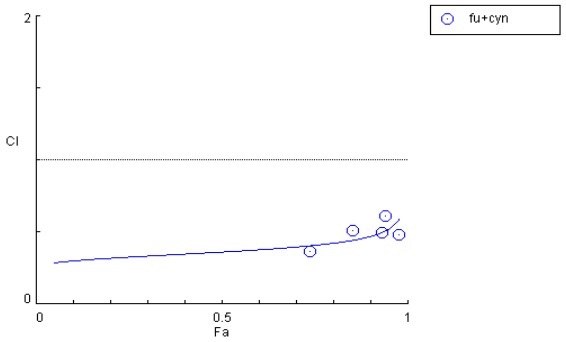

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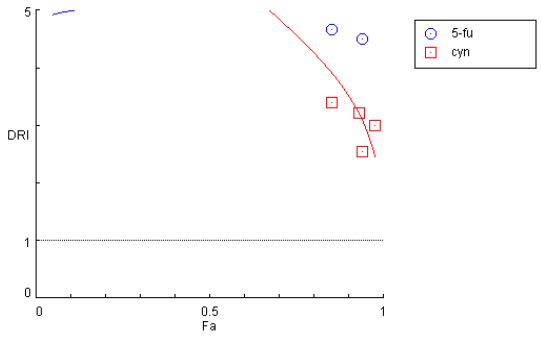

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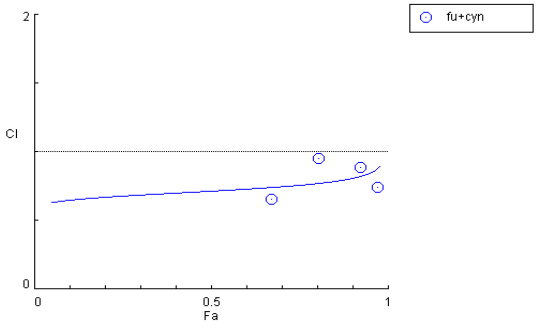

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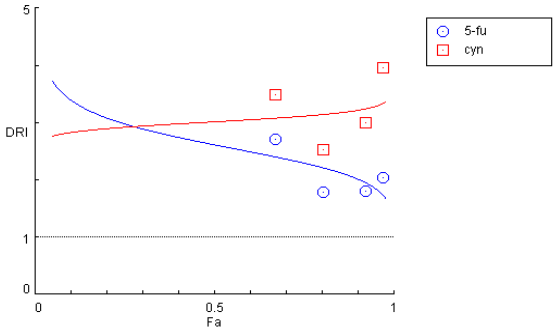

Fig. 7: A, C and E): Combination index diagrams showing the effects of 5-Fu and cynaropicrin on each other at 24, 48 and 72 h. B, D and F): Dose reduction diagrams, expressing a multiplier reduction in the dose of 5-Fu and Cyn in a combination treatment over 24, 48 and 72 h. CI: Combination Index, Fa: Fraction affected, CI does not have unit.

Table 2: CI and the DRI related to the combination of cyn-5-Fu at A) 24, B) 48, C) 72 h.

A

24 h	**CI value**	**DRI Cyn**	**DRI 5-Fu**
IC_{50}	0.13	15.26	15.07
IC_{75}	0.37	5.03	5.75
IC_{90}	1.07	1.66	2.19
IC_{95}	2.15	0.78	1.14

B

48 h	**CI value**	**DRI Cyn**	**DRI 5-Fu**
IC_{50}	0.36	5.92	5.18
IC_{75}	0.40	4.56	5.27
IC_{90}	0.47	3.51	5.37
IC_{95}	0.52	2.93	5.44

C

72 h	**CI value**	**DRI Cyn**	**DRI 5-Fu**
IC_{50}	0.71	3.01	2.61
IC_{75}	0.75	3.11	2.29
IC_{90}	0.80	3.21	2.01
IC_{95}	0.84	3.28	1.83

Table 2: CI and the DRI related to the combination of cyn-5-Fu at A) 24, B) 48, and C) 72 h.

Discussion

The high incidence and mortality rate of esophageal cancer, which is one of the common cancers of the digestive system in Asia (Ferlay *et al*., 2010), indicate the serious threats of this malignancy (Napier *et al*., 2014). The absence of a comprehensive diagnostic system leads to late detection, poor prognosis and high mortality rates (Polednak, 2003; Qureshi *et al*., 2009; Pourhoseingholi *et al*., 2015). Some predictions suggest a decrease in the incidence of cancer in the coming decades, but it does not include esophageal cancer. The incidence of esophageal cancer is expected to increase by 140% up to 2025 (Lambert *et al*., 2007).

In the present study, the toxicity and anti-cancer effects of cynaropicrin were investigated on KYSE30 cells and the mechanism of cell death induced by this compound was examined. The synergistic effects of cynaropicrin with common chemotherapeutic drugs used for esophageal cancer, pac and 5-Fu, were also investigated and calculated by Chou-Talalay median effect method. The results indicated significant differences (p<0.0001) in cytotoxic effects of cynaropicrin on normal HDF cells compared to cancerous cell line, which proves selective toxicity and anti-cancer effects of this compound on KYSE30 cells.

As mentioned earlier, many studies have been carried out on cynaropicrin in which several investigations are related to cytotoxic and anti-cancer effects of this compound. Some researchers have focused only on cytotoxic effects of cynaropicrin (Choi *et al*., 2005; Yang *et al*., 2008; Bruno *et al*., 2005; Ha *et al*., 2003; Kang *et al*., 2007 and Butturini *et al*., 2013). In these studies, cytotoxic effects of cynaropicrin were proven on different cell lines including prostate, breast, stomach, colon, lung, ovary and cervix cancerous cells and also myeloma cells. The lowest and highest reported IC_{50} values were 0.83 µM for HCT15 (colon cancer) and 24.51 µM for A549 (lung cancer), respectively. In none of these studies, the selective anti-cancer effects of cynaropicrin were investigated.

In a study by Cho and colleagues (2004b), several leukocyte cancer cell lines including U937, 1-EO1 and Jurkat T cells were treated with cynaropicrin and its toxic effects were confirmed. The treatment was also performed on HDF-1 and Chang liver cells (human liver cells) as normal cell lines. The significant difference observed between the IC_{50} values of cancerous and normal cells confirms the anti-cancer effects of this compound (Cho *et al*., 2004b). In a similar study, various cancer cell lines were treated with cynaropicrin. The lowest and highest IC_{50} values were related to SK-MEL (melanoma) equal to 5.77µM and SK-OV-3 (ovary cancer) equal to 18.18 µM, respectively. VERO cell line (kidney epithelial cells from African green monkey) as normal cells, was used to confirm the anti-cancer effects of cynaropicrin. No significant difference was observed between its IC_{50} values on normal and cancerous cells (Muhammad *et al*., 2003). It can be concluded that VERO cells were not an appropriate control in this study. The findings of these reports are in agreement with the results obtained in present study.

Chen and colleagues (2007) proved that the activation of EGFR-encoding gene contributes to the creation and progression of ESCC. The role of inhibiting this pathway in the treatment of esophageal cancer was discovered using erlotinib and afatinib inhibitors. For this purpose, seven different ESCC cell lines were examined. Observations indicated that these drugs inhibited the pathway and led to a decrease in the growth of cancerous cells. However, KYSE30, TE8 and KYSE250 were introduced as cell lines which significantly had less growth inhibition, and were more resistant than other ESCC cell lines used in their study (Chen *et al*., 2017). This result can be a reason for the numerical difference between IC_{50} values of cynaropicrin on KYSE30 cells compared with other cancerous cell lines.

The occurrence of apoptosis after treatment with cynaropicrin was confirmed by flow cytometry. Several other studies have also shown the apoptosis inducing effects of cynaropicrin in different cell lines. For example, according to Cho *et al*. the production of

reactive oxygen species by cynaropicrin led to induction of apoptosis. To prove this, cells were treated simultaneously with cynaropicrin and two scavengers of reactive oxygen species including L-cysteine and N-acetyl-L-cysteine. The two scavengers inhibited the morphological changes and DNA fragmentation induced by cynaropicrin (Cho *et al.*, 2004b). Moreover, in a study by Butturini *et al*. cynaropicrin was considered as an inhibitor of STAT3 activation and IL-6 production stimulus. They showed that cynaropicrin as the receptor of the Michaelis-Menten reaction, induces the addition of glutathione to STAT3 and inhibits this transcription factor. The inhibition of STAT3 leads to suppressing the expression of two anti-apoptotic genes, including BCL2 and Survivin in DU 145 cells. STAT3 is active in a variety of cancers and plays an important role in inhibition of apoptosis and inducing drug resistance (Butturini *et al*., 2013).

As noted above, Chou-Talalay method was used to investigate the combination effects of cynaropicrin with pac and 5-Fu drugs. The reason for this selection is the advantages of this method including: detection of various types of behaviors in combination (synergism, additive and antagonism), significant reduction in drug consumption, time saving, estimation of the behavior of materials in combination with the lowest number of data, presenting results numerically and independent of unit, and in particular, being functional at both *in vitro* and *in vivo* levels (Chou, 2006; Chou, 2008). Additive effects were observed in the combination of cynaropicrin with pac using this method at 24 h. The strongest synergistic effects were revealed at 48 h. DRI for pac was about three times, and for cynaropicrin was about four times. These synergistic effects were also observed at 72 h at which DRI for pac was greater than two times and for cynaropicrin it was higher than three times. It must be noted that having a dose reduction in combination does not merely mean synergistic effects. So that, even in the case of minor antagonism, there is a possibility of dose reduction. Therefore, CI is the primary reference for the diagnosis of synergistic effects. All in all, cynaropicrin had a

synergistic effect in combination with pac in a time-dependent manner. In the combination of cynaropicrin with 5-fluorouracil in IC_{50}, the CI was equal to 0.13 with a DRI= of about 15, that indicate a very strong synergistic effect. However, our data showed that with an increase in IC, the CI increases. So that, in IC_{95} the CI reached over two, which indicates the antagonistic effect. Hence, cynaropicrin and 5-Fu have the most synergistic effects at lower concentrations. At 48 h, this combination also showed a good synergistic effect in IC_{50} in which the CI was equal to 0.36. Regarding the remaining synergistic effects, even at IC_{95} at 48 h, it can be concluded that the synergistic effects of cynaropicrin and 5-Fu at high concentrations are time-dependent. At 72 h, synergistic effects were observed for all concentrations of the combination. To summarize, combination of both drugs with cynaropicrin had the most logical synergistic effects within 48 h. However, the strongest synergistic effects on the combination of cynaropicrin and 5-Fu was observed at 24 h.

Study of Butturini *et al.* (2013) is the only trial in which the combination of cynaropicrin with chemotherapy drugs was examined. In this study, the effects of cynaropicrin in combination with cisplatin and docetaxel were investigated on a prostate cancer cell line (DU 145). In both cases, the synergistic effects of cynaropicrin were observed (Butturini *et al.*, 2013). In another study, the effects of DMSO on platinum compounds such as cisplatin and carboplatin were investigated (Hall *et al.*, 2014). As a result, it was reported that DMSO reacts with platinum and prevents the toxicity of these compounds (Hall *et al.*, 2014). According to this result, the cisplatin combination with cynaropicrin in the study of Butturini and colleagues (2013), requires further approval with another suitable solvent for cynaropicrin. Based on the Chou-Talalay method, the smaller CI will have a greater DRI, but in the case of combination of docetaxel with cynaropicrin in the latest study, the result is quite the opposite; when the strongest synergistic effect was observed, there was the lowest dose reduction and in the weakest synergistic effect, the highest reduction in the dosage were recorded, which does not

seem to be logical (Butturini *et al.,* 2013). Therefore, the results of the combination of docetaxel and cynaropicrin also need to be reviewed.

Conclusion

To conclude, the present study reported cynaropicrin as a potent candidate with anti-cancer properties for treatment of ESCC. It has a very high potential for combinational therapy which can reduce the dose of chemotherapy drugs and subsequently reduce their side effects. Nevertheless, further studies are needed to investigate the effects of cynaropicrin on other cancer cell lines, as well as its synergistic effects with other chemotherapy drugs in both *in* vitro and in vivo models.

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Authors Contributions

M.M.M.and A.R.B. conceived and designed the experiments, and supervised the project; S.N. conducted the experiments and wrote the manuscript; S.N. and N.G. analyzed the data; M.I. and A.S.extracted and purified the tested plant composition; M.M.M. and A.S. proofread the manuscript. All authors discussed the results and commented on the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Declarations

Consent to participate:

The authors declare consent to participate in this study.

Consent for publication:

The authors declare consent to publish this study.

Conflict of interest:

The authors declare that they have no conflict of interest.

Ethics approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

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