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Anti-tumour effect of combinations of three acids isolated from *Taraxacum officinale*

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Abstract

Taraxacum officinale (TO) is a well-known medicinal plant used in folk medicine for its variety of biological activities. In this study a methanolic extract from roots was used to examine its anti-tumour effect by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay on two mouse tumour cell lines, fibrosarcoma and hepatoma cell lines. Normal hepatocyte and fibroblast cell lines were used as a control. Furthermore, three active compounds were isolated from the extract, caffeic acid, chlorogenic acid, and ursolic acid, in order to investigate their cytotoxicity and possible interactions between them in their combinations on the same tumour and non-tumour cell lines. The anti-tumour effect of the TO extract was confirmed on the fibrosarcoma cell line in a dose dependent manner. The anti-proliferative acting of each acid was described on both cancer cell lines and for the first time the combinations of these acids were investigated and their common effect in the mixtures reported. Further experiments to determine the mechanism of action and examine their action with conventional chemotherapeutics as a potential adjuvant therapy to enhance the chemotherapeutic effect and improve patient health with its hepatoprotective activity could be encouraged.

Chlorogenic acid, ursolic acid, caffeic acid, anticancer, dandelion

Taraxacum officinale (TO), commonly known as dandelion, is a plant traditionally used as a folk medicinal herb. The main investigated pharmacological properties include, in particular, its anti-inflammatory, antioxidative action or hepatic protection and many other effects (Seo et al. 2005; You et al. 2010; Park et al. 2011). Recently, scientific attention has been attracted to a *Taraxacum officinale* extract (TOE) and its anti-tumour effect (Han et al. 2018). The mechanism has not been fully elucidated yet, some specific bioactive compounds have been identified but their anticancer activity was not comparable with the whole TOE (Ovadje et al. 2016). Therefore, dandelion belongs among medicinal herbs with a considerable pharmacological, especially anti-tumour, potential.

In this study, we investigated the effect of TOE on viability of mouse fibrosarcoma and hepatoma cell lines compared to physiological fibroblasts and hepatocytes. Moreover, we isolated 3 substances from the TO extract, caffeic acid, chlorogenic acid, and ursolic acid, which, according to the current results, have an antitumor effect (Chang et al. 2010; Yamagata et al. 2018; Harmand et al. 2005) and we have shown that the isolated compounds and their combinations are able to decrease viability of the tumorous cell lines in a dose dependent manner.

Materials and Methods

Plant material

The underground parts of TO were collected from one area repeatedly from April to June 2016. A voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy of the Masaryk University. Fresh roots were washed and grated.

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Preparation of extract

Fresh grated roots were extracted using methanol at a 1:10 ratio for 48 h at room temperature. After filtration and collection of the methanolic extract, the sample was macerated again in chloroform (Penta Chemicals, CZ) at a 1:5 ratio for 24 h. Both extracts were combined and concentrated under vacuum, yielding a viscous material. This extract was dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA) and diluted with appropriate media to prepare a 10% stock solution. The stock solution was filtered by a syringe filter Filtropur S 0.2 (Sarstedt, Germany) and then diluted at selected final concentrations before use (20.0, 13.3, 10.0, 6.7, 4.0 mg/ml). The final concentration of DMSO was not higher than 0.5%, which had no effect on the cell viability.

Preparation of isolated acids

The extract was partitioned between chloroform and water. The aqueous part was extracted using ethyl acetate. This part was separated repeatedly on a silica gel column in the solvent system ethyl acetate:methanol = 9:1 with an increasing amount of methanol. Final purification was achieved by preparative thin-layer chromatography (TLC) of silica gel. Isolated compounds (mg amounts) were identified by spectral methods and comparing with standards. The necessary quantity of bioactive acids were obtained from Sigma-Aldrich, USA. Each acid was diluted with fresh culture medium to the tested concentration and then filtered by the syringe filter before use. We used isolated substances at the following concentrations: caffeic acid (3,000–100 μM), chlorogenic acid (2,000–100 μM), ursolic acid (100–10 μM), β-sitosterol (1,000–25 μM), and taraxacolide glucoside (500–50 μM).

Cell culture

We used two normal cell lines, mouse fibroblasts NIH/3T3 (ATCC® CRL1658™), mouse hepatocytes AML12 (ATCC® CRL2254™), and two tumour cell lines, mouse fibrosarcoma cells WEHI13VAR (ATCC® CRL2148™) and mouse hepatoma cells Hepa 1-6 (ATCC® CRL-1830™). The NIH/3T3 cells were incubated in minimal essential medium (MEM), supplemented with 10% foetal bovine serum (FBS) (Biosera, France) and 1% penicillin-streptomycin (P/S) (Sigma-Aldrich, USA). The AML12 were incubated in Dulbecco's modified Eagle's medium Ham's F12 w/ L-glutamine w/ 15 mM Hepes (DMEM F12) (Biosera, France) supplemented with 10% FBS, 1% P/S, 10 μg/ml insulin, 5.5 μg/ml transferrin, 10 ng/ml selenium and 10 ng/ml dexamethasone (Sigma-Aldrich, USA). The WEHI-13VAR were incubated in Roswell Park Memorial Institute Medium (RPMI) 1640 w/ L-glutamine (Biosera, France), supplemented with 10% FBS, 1% P/S. The Hepa 1-6 were incubated in DMEM high glucose w/ L-glutamine w/ sodium pyruvate (Biosera, France), supplemented with 10% FBS and 1% P/S. All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

LDH cytotoxicity assay

To screen a cytotoxic activity of isolated substances we used lactate-dehydrogenase (LDH) leakage assay by a commercial assay kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The cells were seeded in a 96-well plate at density of 2×10^4 cells/well in appropriate growth medium and cultured overnight in an incubator at 37 °C in an atmosphere of 5% CO_2. The next day we prepared and applied tested solutions and cells were further incubated under the same conditions for 24 h. The positive control cells were treated by using lysis buffer, the cells incubated with appropriate medium were used as a negative control. Then the reaction mixture was applied to each well and plates were stored protected from light at 37 °C for 30 min. After stopping the reaction by Stop Solution, the absorbance was read at 490 nm and 680 nm.

MTT Viability Assay

For detection of proliferative activity, we used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Cell Proliferation Assay (R&D Systems, Minneapolis, USA). On the first day, cells were seeded in a 96-well plate at a density of 1×10^4 cells per well in 200 μl of appropriate medium and incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2. The procedure on the second day was the same as in LDH assay. We used a Triton X-100 (SERVA Electrophoresis GmbH, Germany) as a positive control. Suitable controls with the equivalent concentration of DMSO were also included. On the third day, the medium was aspirated from the plates and 110 μl of diluted MTT reagent with fresh medium (1:10) was added into each well. Then the cells were incubated for 4 h in the incubator and 100 μl per well of the detergent were further added to the plates. The absorbance was measured on Infinite 200 PRO microplate reader (Tecan, Switzerland) at 570 nm after an overnight incubation in the dark at room temperature.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). For verification of normality of distribution Shapiro-Wilk test was used. Data were evaluated by Mann-Whitney or unpaired *t*-test. A P value < 0.05 indicated significance. All statistical analyses were performed using MedCalc (MedCalc Software Ltd, Belgium). All graphs were made in Microsoft Excel 2019 (Microsoft, USA).

Results

Effect of the whole extract

The cells were incubated with various concentration of TOE (4.0, 6.7, 10.0, 13.3 mg/ml) for 24 h. The TOE at a concentration of 4 mg/ml reduced the cell viability of WEHI 13-VAR by 20% against control, whereas at 13.3 mg/ml, the cell viability was reduced to almost 40% compared to the control. The cell viability of NIH/3T3 was significantly higher than the viability of WEHI 13-VAR in a dose dependent manner (Fig. 1).

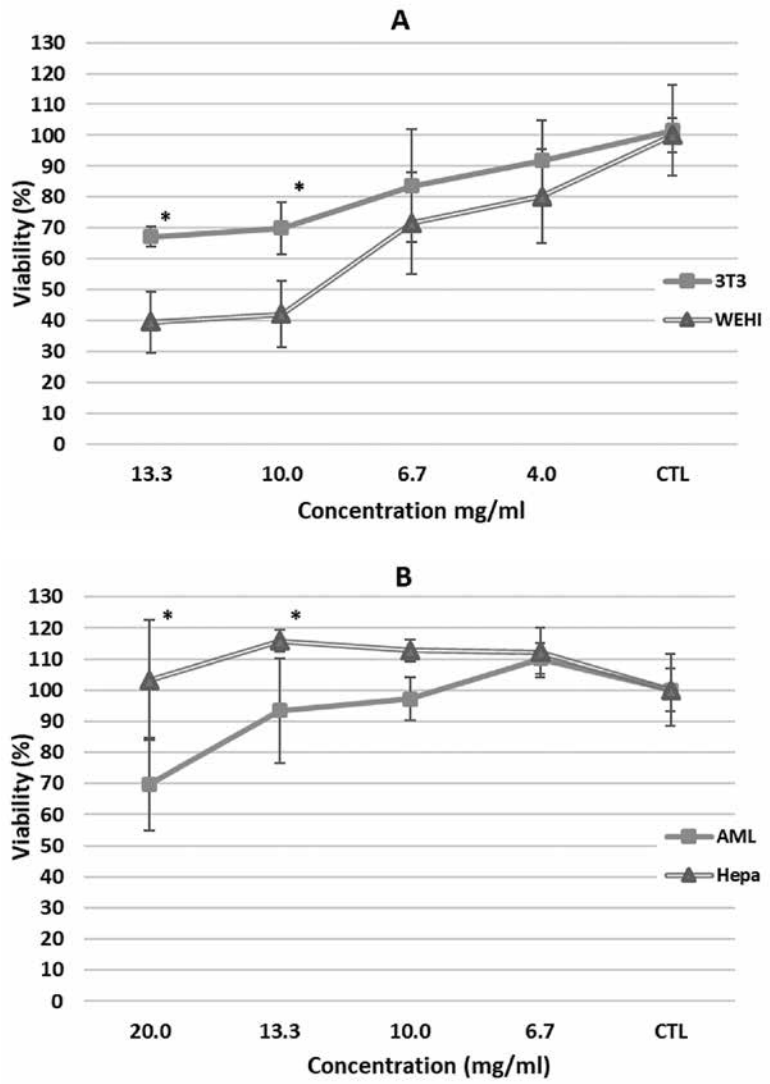

Fig. 1. (A) The cytotoxic effect expressed by reduced viability of the methanolic extract of Taraxacum officinale was significantly ($P < 0.05$) higher on the fibrosarcoma cell line (WEHI) than on the normal fibroblast cell line (3T3) at concentrations of 13.3 mg/ml and 10.0 mg/ml. (B) Viability of normal hepatocytes (AML) was significantly ($P < 0.05$) lower than viability of cells of hepatoma (Hepa). CTL - control cells without treatment; * - $P < 0.05$

The viability of normal hepatocytes was significantly lower than the viability of the hepatoma cell line. Viabilities of treated tumour and normal cells were similar as untreated cells. The dose of 6.7 mg/ml TOE significantly improved the proliferation of both treated cell lines compared to untreated cells.

LDH cytotoxicity of bioactive compounds

LDH cytotoxicity was measured to determine any bioactivity of isolated substances. Three compounds showed significantly different effect on physiological and tumour liver cell lines during the 24-h incubation (Fig. 2). One of them was caffeic acid (CA). LDH cytotoxicity assay showed decreased viability of the Hepa 1-6 cell line without any effect on viability of the normal cell line after incubation with 1000 μM CA (Fig. 2). The second compound was chlorogenic acid (CGA). Our results showed that CGA significantly decreased viability of tumour cells and had no effect on the normal cell line at a 1000 μM concentration (Fig. 2). The third substance was determined as ursolic acid (UA). The results showed that UA had a cytotoxic effect on the hepatoma cell line without any influence on the liver cell line at a concentration of 50 μM (Fig. 2). We tested two other substances in our study (β-sitosterol, taraxacolide glucoside), but no effect on cell lines' viability was found (data not shown).

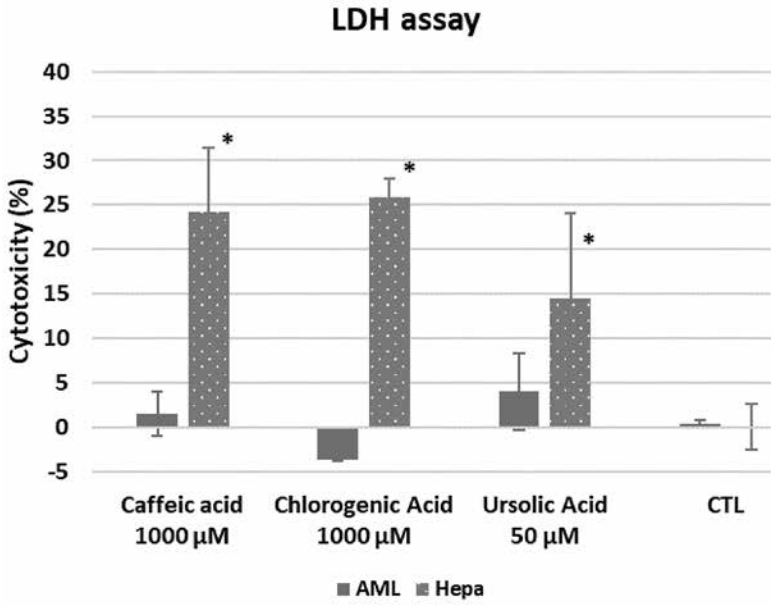

Fig. 2. The cytotoxic effect of isolated substances - caffeic acid, chlorogenic acid and ursolic acid measured by lactate-dehydrogenase (LDH) assay was significantly ($P < 0.05$) higher on tumour cells (Hepa) than on normal cells (AML) at concentrations of 1000 μM for caffeic acid and chlorogenic acid and 50 μM for ursolic acid. CTL - control cells without treatment; * - $P < 0.05$

Effect of combinations of bioactive compounds

We used two normal cell lines and two tumour cell lines and tested the acids at the most effective concentrations. The combinations used are shown in Table 1. They were evaluated for their effect on cell proliferation. We used MTT viability assay, which is focused on live cells, therefore it is affected by any alteration of cell proliferation (cytotoxic effect, slowing or improving of proliferation).

Table 1. The combination of each acid at two concentrations.

Extracted acids	UA 25 μM	UA 10 μM	CGA 2000 μM	CGA 1000 μM
CGA 2000 μM	CGUH			
CGA 1000 μM		CGUL		
CA 500 μM	CUH		CGCH	
CA 250 μM		CUL		CGCL

UA - ursolic acid; CGA - chlorogenic acid; CA - caffeic acid; CGUH - combination of 25 μM ursolic acid with 2000 μM chlorogenic acid; CGUL - combination of 10 μM ursolic acid with 1000 μM chlorogenic acid; CUH - combination of 25 μM ursolic acid with 500 μM caffeic acid; CUL - combination of 10 μM with 250 μM caffeic acid; CGCH - combination of 2000 μM chlorogenic acid with 500 μM; CGCL - combination of 1000 μM chlorogenic acid with 250 μM caffeic acid

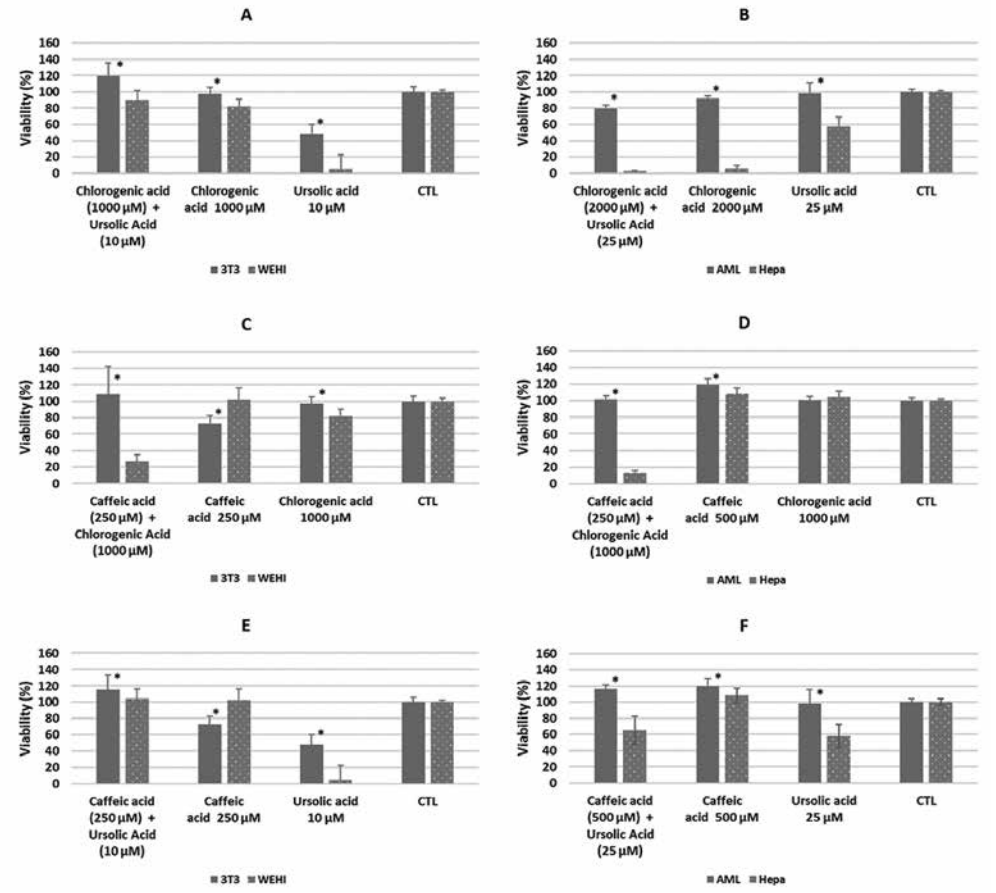

Fig. 3. The combined effects of (A, B) chlorogenic acid and ursolic acid; (C, D) caffeic acid with chlorogenic acid; (E, F) caffeic acid and ursolic acid on viability of NIH/3T3 (3T3), WEHI-13VAR (WEHI), AML 12 (AML) and Hepa 1-6 (Hepa) cell lines. CTL - control cells without treatment; * - $P < 0.05$

The most promising mixtures were those that contained CGA. Viability of the 3T3 line was significantly higher after incubation with a combination of a lower concentration of chlorogenic acid and ursolic acid (CGUL) compared to incubation with both acids used individually at the same concentration as in the combination. There was no significant difference between viability of the WEHI-13VAR line after incubation with CGUL and after incubation with chlorogenic acid (1000 μM). The combination of a higher concentration of chlorogenic acid and ursolic acid (CGUH) used on the liver cell lines significantly decreased proliferation of non-tumour cells. The proliferation of tumour cells with CGUH was not significantly different from the proliferation of them with CGA but in both cases the means of viability were under 4%. Viability of both tumour cell lines was significantly lower after being exposed to a lower concentration of chlorogenic acid and caffeic acid (CGCL) than after being exposed to each acid individually, without any effect on viability of normal cell lines (Fig. 3).

Discussion

Proliferation of four cell lines exposed to TOE was assessed by MTT assay. The results for fibroblast and fibrosarcoma cell lines demonstrated that the crude TOE restricted tumour cell proliferation, whereas its effect on the non-tumour cell line was low, confirming the anti-tumour activity of TOE. Similarly to our results, a previous study reported anti-proliferative activity against various types of tumour cell lines (Ovadje et al. 2012; Zhu et al. 2017). However, the difference between viability of liver tumour and no-tumour cell lines was minor and there was no significant decrease of their proliferation. This effect could be explained by hepatoprotectivity of TOE (Tabassum et al. 2011). These results suggest that each cell line can be affected by the natural extract in a different way. Chatterjee et al. (2011) described a similar effect of TOE on human melanoma cells. Their viability assay showed that the melanoma cell line G361 was suppressed by a 4 × higher concentration of TOE than the melanoma cell line A375s due to an up-regulated expression of anti-apoptotic genes and down-regulated expression of pro-apoptotic genes. The mechanism of action is still not clear and recent studies have suggested that TOE affects tumour cells through multiple death signalling pathways (Koo et al. 2004; Ovadje et al. 2016; Rehman et al. 2017).

The varying effect on cancer cell lines, unclear amount of bioactive substances and undefined mechanism of action are the disadvantages of crude extracts. Nevertheless, many anti-cancer drugs derived from natural sources have been introduced over the last decades (Lee 1999). An isolation of the bioactive compounds and the definition of their effect could solve this problem.

Therefore, we isolated three substances from TOE and tested their cytotoxic activity on normal liver cells as an example of important detoxifying organ and on tumour liver cells. One of the substances was CA. It is a simple phenolic compound which has been reported to be a powerful antioxidant (Gülçin 2006). Moreover, its anti-tumour effect has been described in some studies (Chang et al. 2010; Prasad et al. 2011). The second compound was CGA, an ester of CA and quinic acid. It belongs to a group of plant polyphenols abundantly occurring in human diet, especially in coffee (Farah and de Paula Lima 2019). It is known mostly for its antioxidant and anti-inflammatory effects (Liang and Kitts 2015). Moreover, its anti-tumour effect has been evaluated (Yamagata et al. 2018). The third bioactive substance was determined as ursolic acid, a pentacyclic terpenoid exhibiting a wide range of pharmaceutical properties, e.g. anti-inflammatory and antioxidant effects (Yin and Chan 2007; Chun et al. 2014), improvement of the condition and functioning of the liver, heart, and brain (Saravan et al. 2006; Radhiga et al. 2012; Li et al. 2013). Moreover, strong anti-microbial activity against a wide spectrum of bacterial strains has

been reported (Fontanay et al. 2008; do Nascimento et al. 2014). Many studies have shown that UA is a strong multi-tasking anticancer agent, which has been tested on various cell lines (Harmand et al. 2005; de Angel et al. 2010). We evaluated the anti-tumour effect of the three mentioned acids on the mouse hepatoma cell line. Previous studies have reported that natural active substances might have different effects in the extract than when they act alone due to their possible interactions with other compounds of the extract and due to the changes in their amounts during the plant ontogenesis (Németh 2005; Cirak et al. 2013; Casanova and Costa 2017). Therefore, we also used our isolated substances in combinations to investigate their common effect.

We used isolated acids at their most active concentration and made their combinations to detect their possible interactions. The most promising mixtures contained CGA (Fig. 3). In our study, CGA with UA improved viability of fibroblasts and the strong cytotoxic activity of UA was not manifested in the presence of CGA. The combined effects of CGA and UA have not been investigated yet. Our results suggest that the effect of this combination is determined by cytotoxicity of CGA. The key role of CGA was seen with both tumour cell lines, where the cytotoxic effect of this combination was similar to the cytotoxic effect of CGA, despite a possible effect of UA. This could be useful for studies of other pharmacological properties of UA such as e.g. anti-microbial activity, where the cytotoxicity of UA is a limiting factor for an *in vivo* use (Fontanay et al. 2008). A more effective mixture was CGA with CA. We evaluated the synergic effect between them. Similarly to our results, previous studies have reported that both acids (CGA and CA) alone exhibited strong anti-proliferative activity on tumour cells (Chang et al. 2010; Yamagata et al. 2018). However, their combination was investigated only as an equimolar mixture with other 2 substances, 3-phenylpropionic acid and benzoic acid where the mix of them had a markedly lower EC50 than the EC50 of the two compounds individually (Sadeghi Ekbatan et al. 2018). A study of the action of a mixture of CGA and CA has not been published yet, but our findings suggest that the other two substances are not necessary for the effect of the combination and the acid ratio can significantly affect the final effect. We also tested a combination of CA with UA. The results showed a similar effect to the combination of CGA with UA, which could be explained by partly the same structure of CGA with CA.

Our current study extends previous findings about these three acids by demonstrating the effects of their combinations. The present results proved a strong anti-proliferative effect especially of the mixture of CGA with CA. However, the mechanism of action of these combinations is not clear and further experiments are required to clarify this. Moreover, the natural health products are considered as an adjuvant therapy to chemotherapy and radiotherapy to enhance their effect and patient health condition. The TOE was investigated as an adjuvant therapy, which enhances the ability of two chemotherapeutic drugs, taxol and mitoxantrone, to induce apoptosis (Fantini et al. 2015; Nguyen et al. 2019). Our findings also provide support for a further study of the combinations of isolated substances from *Taraxacum officinale* as potential enhancers to conventional cancer therapy.

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