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Original Research Article

Cytotoxic Effects of *Anacyclus Pyrethrum*, *Terminalia Chebula*, and *Brassica Nigra* Ethanol Extracts on KB Cancer Cell Line

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ABSTRACT

The prevalence of oral squamous cell carcinoma (OSCC) has increased in the last 2 decades. For the treatment of this disease, various herbal anticancer agents have been introduced. In this study, for the first time, we analyzed the apoptotic and cytotoxic effects of herbal plants, including *Anacyclus pyrethrum*, *Terminalia Chebula*, and *Brassica nigra*, in KB cancer cell lines. For analyzing the cytotoxic effects of ethanol extracts, MTT reduction assay was performed in the KB cells. Moreover, the apoptosis-inducing effect of plants on KB cells was assessed using TUNEL and DNA fragmentation assays. The results showed that *Anacyclus pyrethrum* and *Terminalia Chebula* extracts significantly inhibited cell viability. TUNEL and DNA fragmentation assays showed that the main mechanism of cell death was apoptosis induction by *Anacyclus pyrethrum* and *Terminalia Chebula* extracts. Our results suggest that these ethanol extracts may contain bioactive constituents, which can be helpful in OSCC treatment.

Keywords: *Anacyclus Pyrethrum/Pellitory*, *Terminalia Chebula*, *Brassica Nigra*, Oral Squamous Cell Carcinoma, Apoptosis.

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Introduction

Nearly 1.5% of all malignant neoplasms in the human body result in oral squamous cell carcinoma (OSCC). The rate of increase has been immense in the last 2 decades (10% to 15% every year) [1]. More than 30 000 cases of oral and pharyngeal cancers are detected every year, and more than 8000 people do not survive the disease. The incidence of OSCC has increased over the past 3 decades in the United States (0.3 per 100 000 people). In SEER registries, the 5-year survival rate was estimated at 60.2% for oral tongue cancer patients in 2007, showing only a slight improvement in the past 2 decades [2]. In search of effective medicinal treatments, traditional medicine has been taken into consideration. Despite the introduction of novel screening methods and high-throughput screening, traditional knowledge systems can still help find agents with higher efficiencies [16-17]. Generally, customary medicinal herbs are low-cost, easily usable, and regionally available both in raw form and simple medicinal preparations.

Anacyclus pyrethrum is an herbal medicine from the *Asteraceae* family (genus, *Anacyclus*), which is endemic to Iran, India, and Arab countries [3]. Pyrethrine is identified as the active component of *Anacyclus pyrethrum*. Other compounds of this plant are resinous and include tannin, pelletonin, gum, potassium sulfate, carbonate, calcium phosphate, and potassium chloride [4].

Terminalia chebula Retz. is grown widely as an ornamental tree in tropical regions [5] and is used in traditional Mongolian medicine. Dried *T. chebula* galls are available in the markets of China and Southeast Asia [6-7]. *T. chebula* extract (TE) has shown different biological characteristics, such as antidiabetic, anticancer, antibacterial, antimutagenic, and anticaries effects [8-12]. *T. chebula* fruits contain 14 hydrolysable tannin components, such as gallic acid, chebulic acid, chebulanin, ellagic acid, punicalagin, neochebulinic acid, corilagin, terchebulin, and casuarinin [6].

Brassica nigra (black mustard), which belongs to the *Brassicaceae* family, has been used in different regions for its medicinal and flavoring properties. In traditional medicine, the seeds of *B. nigra* are used to manage conditions, such as cardiovascular diseases, diabetes, and associated complications [18]. Despite the introduction of antioxidant constituents in the seeds of other *Brassicaceae* members, limited research has evaluated the antioxidant properties of *B. nigra* and quantified its antioxidant constituents [19].

Cell apoptosis refers to programmed cell death and is recognized as an important process in the prevention of cancer [13]. It is activated through intrinsic and extrinsic pathways. The latter pathway involves caspase-8 activation, whereas the former pathway involves caspase-9 cleavage and

cytochrome C secretion [14]. Mitochondria contribute to cell apoptosis. The intrinsic pathway of mitochondria-dependent apoptosis involves Bcl-2 family, consisting of pro- and antiapoptotic proteins, which sustain a dynamic balance of cell death and survival via interactions with each other or other proteins [15].

In this study, KB cell line of OSCC was used to investigate the effects of ethanol extracts of 3 herbal plants, including *A. pyrethrum*, *T. chebula*, and *B. nigra* on cytotoxicity and apoptosis induction.

Materials and Methods

Preparation of plant extracts

A. pyrethrum, *T. chebula*, and *B. nigra* were supplied by Pajuhan Sabz Company (Iran). The plants were confirmed at the herbarium of School of Pharmacy. The plant samples were crushed in a mechanical grinder to optimize the particle size. Then, the plants were transferred to a 4-L Erlen flask. For each 250 g of *A. pyrethrum*, *T. chebula*, and *B. nigra* powder, 2 L of ethanol was incubated for 3 days and mixed occasionally. A Whatman paper was used to filter all the extracts. The filtrate was evaporated to collect a gummy mass under reduced pressure, and it was stored at 4°C for further analysis.

Cell line and culture

The KB cell line was purchased from Pasteur Institute, Tehran, Iran. It was grown in RPMI-1640 medium (Sigma-Aldrich, MO, USA), which contained 10% fetal bovine serum (Sigma-Aldrich, MO, USA) and 1% antibiotics (100 IU/mL of penicillin and 100 µg/mL of streptomycin; Sigma-Aldrich, MO, USA) in a 5% CO₂ atmosphere at a temperature of 37°C. For cytotoxicity test, after trypsinizing the cells, they were added to 96-well round-bottomed plates of tissue culture. For cell stabilization, KB cell lines were stored for 1 day.

Cell proliferation assay

MTT assay for cytotoxicity

MTT assay, as a colorimetric test, evaluates the potential of mitochondrial reductase enzymes inside living cells in the reduction of yellow MTT to purple formazan dye [20]. By adding an organic solvent (dimethyl sulfoxide or DMSO), the insoluble purple product is solubilized, producing a purple solution. In order to examine cytotoxicity caused by the herbal extracts, MTT assay was carried out. A total of 15 000 cells were added to plates and incubated overnight. Then, the cells were treated with

A. pyrethrum, *T. chebula*, and *B. nigra* extracts, while untreated cells were considered as the controls. Incubation of cells was performed at 37°C for 48 hours, and then, MTT (5 mg/mL) was added. After 4 hours, the content of each well was removed and DMSO (200 µL) was added. Absorbance was read at 570 nm after 30 minutes. GraphPad Prism version 6.01 (San Diego, CA, USA) was applied to determine the concentration causing 50% cytotoxicity (IC₅₀).

TUNEL assay

DNA damage was detected using TUNEL assay (Roche, Germany). This assay examines the activity of terminal deoxynucleotidyl transferase (TdT; Roche, Germany), which adds fluorescent conjugated nucleotides to broken DNA strand ends. These nucleotides attach to free 3'-hydroxyl ends of single- or double-stranded DNA. In this study, after seeding 15 000 cells in 96-well plates, they were incubated overnight. Then, they were treated with the ethanol extracts of *A. pyrethrum*, *T. chebula*, and *B. nigra*, while untreated cells were considered as the controls.

Following treatment for 48 hours, the cells were fixed using 4% paraformaldehyde, diluted in phosphate-buffered saline (PBS; pH, 7.4). After washing the cells twice with PBS, incubation was carried out in 3% H₂O₂ solution in methanol over 10 minutes; then, they were washed again with PBS. Cell incubation was performed for 10 minutes in a permeabilization solution on ice. After adding the reaction mixture (25 µL including TdT enzymes and nucleotides), they were incubated for 60 minutes at 37 °C. The slides were incubated for 40 minutes with 25 µL of streptavidin-peroxidase HRP solution (Roche, Germany) after rinsing twice with PBS. The cells were incubated with 3'-diaminobenzidine substrate after washing again (Sigma, Germany); a light microscope was used to examine the stained cells.

DNA fragmentation assay

DNA fragmentation is an important biochemical marker of apoptosis. Untreated and treated cells with *A. pyrethrum*, *T. chebula*, and *B. nigra* extracts were suspended in PBS (200 µL), proteinase K (10 µL), and RNase A (5 µL); then, they were incubated at 250°C for 20 minutes. Lysis buffer B (200 µL) was then added and incubated at 70°C for 15 minutes. After mixing the sample with dehydrated alcohol (200 µL), it was pipetted into a filter tube.

The flowthrough was discarded after centrifugating the mixture for 1 minute at 6000×g. As described earlier, the mixture was centrifuged after adding washing buffer I (600 µL) to the filter tube. Washing buffer II (700 µL) was also added and centrifuged for 2 minutes at 19 000×g, and the flowthrough was

discarded. After repeating the process of washing, a final spin at high speed (19 000×g) was done for 2 minutes, and warm elution buffer (100 µL) was added to the filter tube. After collecting the eluted DNA via centrifugation for 2 minutes at 19 000×g, it was electrophoretically examined on 2% agarose gel with 0.1% ethidium bromide. Ultraviolet illumination was used to visualize DNA band patterns.

Results

Examination of cell growth inhibition in human mouth epidermal carcinoma (KB) with *A. pyrethrum*, *T. Chebula*, and *B. nigra* extracts

In the present study, different concentrations of the herbal extracts were added to KB cell lines to examine their antiproliferative effects. The results showed that *A. pyrethrum* and *T. Chebula* extracts has potent cytotoxic effects on KB cell line, whereas *B. nigra* extracts exhibited no significant cytotoxic effects on the cell lines. IC₅₀ values from the MTT assay are presented in Table 1.

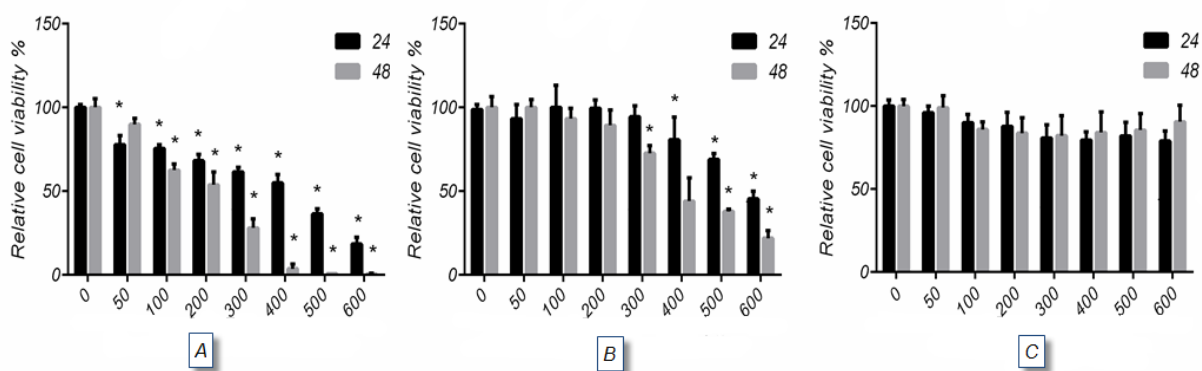


Figure 1. (A): Inhibition of cell viability of (A) *A. pyrethrum*, (B) *T. chebula*, and (C) *B. nigra*-treated cancer cells. KB cancer cells were treated with or without *A. pyrethrum*, *T. chebula*, and *B. nigra* extracts and incubated for 24 and 48 hours. MTT was added at the end of incubation, and cell viability was examined via MTT assay using quadruplicate samples. Growth inhibition was determined as percentage of inhibition, compared with the controls.

Table 1. IC₅₀ values of *A. pyrethrum*, *T. chebula*, and *B. nigra* extracts against KB cell line

IC ₅₀ (µg/µL)		
Time	24 h	48 h
<i>Anacyclus pyrethrum</i>	331.6	161.8
<i>Terminalia chebula</i>	583.2	398.2
<i>Brassica nigra</i>	-	-

A distinct biochemical marker of apoptosis is DNA degradation into multiple internucleosomal fragments (200 bp). TUNEL assay and agarose gel electrophoresis were applied to confirm whether the effects induced by *A. pyrethrum*, *T. Chebula*, and *B. nigra* extracts involve DNA fragmentation in KB cells. Based on the TUNEL assay, exposure to extracts, except *B. nigra* extract, resulted in apoptosis. In comparison of untreated and treated cells, KB cells treated with *A. pyrethrum* and *T. Chebula* showed that cell nuclei turned brown after 2 days of receiving IC_{50} of *A. pyrethrum* and *T. Chebula*.

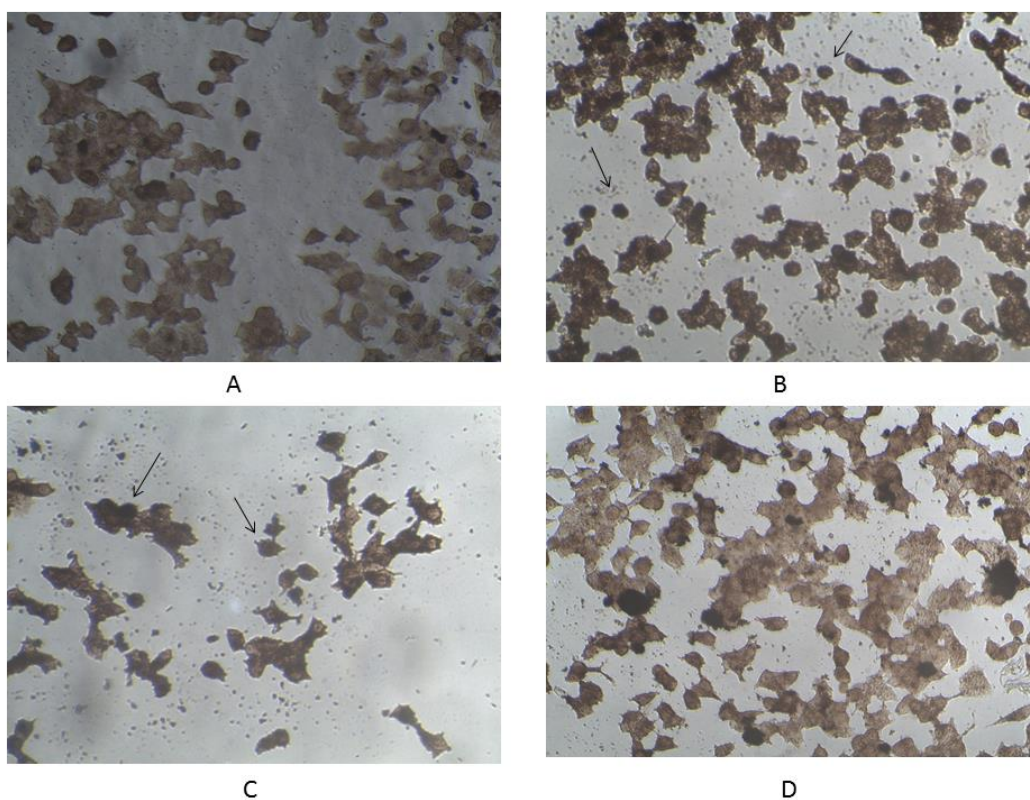


Figure 2. Changes in TUNEL reactivity induced by *A. pyrethrum*, *T. Chebula*, and *B. nigra* extracts: (A) KB cell control (untreated cells); (B) KB cells treated with *A. pyrethrum*; (C) KB cells treated with *T. Chebula*; (D) KB cells treated with *B. nigra*.

DNA fragmentation

For DNA fragmentation assay, the nuclear DNA was separated from the cells through agarose gel electrophoresis; then, it was stained with ethidium bromide. Formation of a typical ladder was reported upon 48 hours of treatment with *A. pyrethrum*, *T. Chebula*, and *B. nigra* extracts in KB cells, while no such observation was reported for the untreated cells (Figure 4).

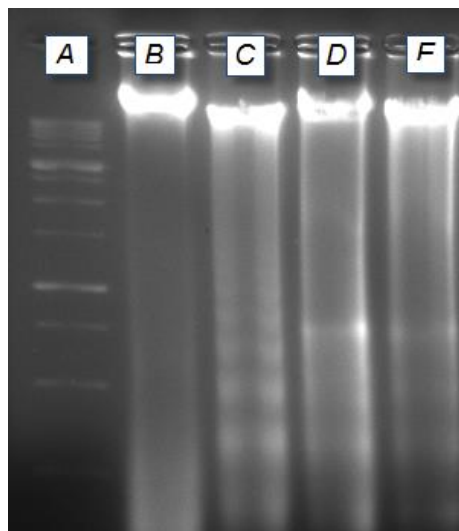


Figure 3. Agarose gel electrophoresis of DNA extracted from *A. pyrethrum*, *T. chebula*, and *B. nigra* treated and untreated KB cells. Cells were treated for 48 hours with IC₅₀ dose of the extracts. As described in the method section, RNase A and proteinase K were used to digest the cells (3×10^6), and 1.5% agarose gel electrophoresis was performed on crude DNA mixtures.

Discussion and Conclusion

Squamous cell carcinoma accounts for at least 90% of all oral malignancies [1]. The World Health Organization has predicted a global rise in the occurrence of OSCCs in the coming decades. Considering the high mortality and morbidity rates, there has been a growing interest in finding suitable drugs for the treatment of OSCC. Medicinal plants are an important source of therapeutic substances, and various plants seem to contain bioactive and anticancer constituents [20-23].

Many chemicals, such as tannin, gallic acid, and chebulic acid from herbal plants show major cytostaticity and cytotoxicity. In this regard, the inhibitory effects of *Urtica dioica* extract on breast adenocarcinoma cells were reported in a recent study by G2/M phase arrest and apoptosis (24, 25). In the current study, active constituents of *A. pyrethrum*, *T. Chebula*, and *B. nigra* extracts were investigated. *A. pyrethrum* and *T. Chebula* exerted more cytotoxic effects on KB cells and could be promising agents, although further in vivo research is required. DNA fragmentation confirmed the occurrence of apoptosis. Moreover, TUNEL assay showed that *A. pyrethrum* and *T. Chebula* extracts induced apoptosis in KB cells [24-26].

According to the MTT assay of *A. pyrethrum* and *T. Chebula*, more significant cytotoxic effects could be induced by increasing the extract concentration. Nonetheless, the greatest cytotoxic effects were reported within 48 hours rather than 24 hours, and extended incubation increased the IC₅₀ values.

Therefore, these extracts seem to have a half-life, and repeated doses should be used to reestablish the effects at 24-hour intermissions.

According to the present study, ethanol extracts of *A. pyrethrum* and *T. chebula* could exert apoptosis-inducing effects in KB cell line both dose- and time-dependently.

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