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Possible induction of apoptotic and necrotic death pathways in lung cancer cells by *Clinopodium vulgare* L. and phenolic acids and flavonoids detection by LC-MS-MS

[Posibilidad de inducción de apoptósis y necrosis en células cancerosas mediante *Clinopodium vulgare* L. y ácidos fenólicos, y detección de flavonoides mediante LC-MS-MS]

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Abstract: The objective of this research was to ascertain the phenolic content and antiproliferation and inducing of apoptosis activities of methanol extract of *Clinopodium vulgare* (CVME) on lung cancer cells. Initially, the viability and proliferation of lung cancer cells treated with CVME were assessed using MTT staining. AnnexinV/Propodium iodide fluorescent staining was then utilized to scan apoptotic cells with a cell counting device. Furthermore, the mRNA levels of proapoptotic Bax and antiapoptotic Bcl-2 were assessed via real-time PCR. Additionally, LC-MS-MS was used to determine the phenolic content of CVME. Following the administration of varying doses of CVME, it was found that the viability and proliferation of lung cancer cells reduced. The Bax and Bcl-2 mRNA levels did not significantly alter, however it was demonstrated that the cells killed by the necrotic pathway rather than by apoptosis. The main components of flavonoids and phenolic acids in CVME were found to be resveratrol and caffeic acid, respectively. Consequently, it can be said that lung cancer cells are inhibited by CVME, an abundant source of phenolics, via the necrotic pathway.

Keywords: *Clinopudium vulgare*; Antiproliferation; Apoptosis; Necrosis; Phenolics

Resumen: El objetivo de esta investigación fue determinar el contenido fenólico, y la actividad de antiproliferación y apoptósica de los extractos metanólicos de *Clinopodium vulgare* (CVME) en células de cáncer de pulmón. Inicialmente, la viabilidad y proliferación de las células cancerosas tratadas con CVME fue evaluada usando tinción MTT. Posteriormente se utilizó tinción fluorescente de AnnexinV/yoduro de propodio para escanear células apoptósicas con un dispositivo de conteo de células. Luego, los niveles proapotptóticos Bax y antiapoptósicos Bcl-2 en ARNm se evaluaron en tiempo real mediante PCR. Además, LC-MS-MS se utilizó para determinar el contenido fenólico de CVME. Siguiendo con la administración de distintas dosis de CVME, se encontró qué la viabilidad y proliferación de células de cáncer pulmonar se redujo. Los niveles Bax y Bcl-2 en ARNm no cambiaron significativamente, no obstante, se demostró que las células fueron eliminadas por necrosis más que por apoptosis. El principal componente de los flavonoides y ácidos fenólicos del CVME fueron resveratrol y ácido caféico, respectivamente. En consecuencia, se puede afirmar que el CVME, una abundante fuente de fenoles, inhibe las células de cáncer pulmonar vía necrosis.

Palabras clave: *Clinopodium vulgare*; Antiproliferación; Apoptosis; Necrosis; Fenoles

INTRODUCTION

Apoptosis, programmed cell death, is a physiologically specific and highly effective cell suicide pathway used in the development and regulation of tissues in living organisms (Meier *et al*., 2000). Due to disruptions in apoptotic cell death, it causes cell accumulation in diseases such as cancer and cell reduction in diseases such as heart failure and neurodegeneration (Reed, 2000). Apoptosis consists of two basic molecular mechanisms, intrinsic and extrinsic (Favaloro *et al*., 2012). In the intrinsic pathway, also known as the mitochontrial pathway, the apoptotic proteins Bax (Bcl-2 Associated Protein X) and BAK (Bcl-2 Homologous Antagonist Killer) disrupt the balance of the antiapoptotic protein Bcl-2 (B-Cell Lymphoma 2) and factors such as cytocrome-C, SMAC, AIF are released from the mitochondrial matrix into the cytosol and caspase-dependent apoptosis works due to the activation of caspase 9 and finally caspase 3. In the extrinsic pathway, caspase-8 is activated depending on the DISC domain and the activated caspase-8 continues the cell suicide program either through the mitochondrial pathway by activating Bid (BH-3 Interacting Domain death agonist) or directly by activating caspase 3 (Favaloro *et al*., 2012). In diseases such as cancer, cells have developed mechanisms to escape apoptosis by mutations in apoptotic genes such as PUMA, P53, caspases, TRAILR2 (TNF-related apoptosis-inducing ligand receptor 2), overexpression of oncogenic microRNAs, mitochondrial changes in terms of energy and reactive oxygen species, methylation and post transcriptional modifications (Dandoti, 2021). Therefore, research is ongoing for natural chemotherapeutic agents to prevent escape from apoptosis and induce apoptosis (or other cell death pathways) in cancer cells.

As natural sources, medicinal and edible plants have important phytochemicals, such as phenolics, terpenes, Sulfur-containing compounds and alkaloids (Crozier *et al*., 2006). These chemicals play important roles in the biological activities of plants and are used in different fields such as medicine, pharmacology, perfumery and food. The plant family Lamiaceae has been used since ancient times for many purposes, especially for the treatment of diseases. The Lamiaceae is a large family of flowering plants with about 200 genera and 3300 species (Trease & Evans, 1983). Many species in Lamiaceae have essential oils and important phytochemicals, especially phenolics. One of the genera belonging to this family is *Clinipodium*. In previous studies, *Clinipodium* species were found to have important biological activities such as antitumoral (Dzhambazov *et al*., 2002), antimicrobial (Castilho *et al*., 2007), antifungal, insecticidal (Debbabi *et al*., 2020), anti-cardiotoxic (Beddiar *et al*., 2021). *Clinopodium vulgare* belongs to Lamiaceae and is divided into two subspecies, *C. vulgare* L. subsp. *vulgare* and *C. vulgare* subsp. *arundanum* (Hedge & Lamond, 1982). *C. vulgare* is a perennial aromatic herbaceous plant. It grows naturally in the temperate regions of the northern hemisphere at an altitude of about 2000-2500 m. *C. vulgare* subsp. *arundanum* taxon is distributed in South of Europe, West of Syria, North of Iraq, Transcaucasia, Iran and Afghanistan but has a wide range in Turkey (Davis, 1982).

In anticancer studies, it has been proven that plants suppress cancer cells, suppress inflammatory agents involved in the cancer process, induce cell death pathways, especially apoptotic, and suppress invasion and metastasis (Yumrutas *et al*., 2018; Ege *et al*., 2020, Cocelli *et al*., 2021, Yumrutas & Bozgeyik, 2023). Previous studies have reported anticancer activities of *Clinopodium* species (Benites *et al*., 2021; Sharifi *et al*., 2022). In addition, although there is antitumor activity of *C. vulgare*, there is limited information on the death pathway through which this activity is mediated. Therefore, in this study, the possible cytotoxic/antiproliferative effect of CVME rich in phenolic acids and flavonoids on lung cancer cells and the relationship between apoptotic and necrotic cell death were investigated. In addition, phenolic acids and flavonoids of CVME were determined by LC-MS-MS.

MATERIAL AND METHODS

C. vulgare was collected from natural habitats and it was identified by Assoc Prof Mustafa Pehlivan (Gaziantep-Islahiye, Huzurlu plateau, 02.07.2023, voucher number: MPH2023-1). It was washed with distilled water and was dried on blotting paper in the open air and in an environment away from sunlight. Then, it was ground in a mortar before the extraction. *C. vulgare* was exposed to 200 mL of methanol for 48 hours. The methanol solution filtered and was evaporated by rotary evaporator. The methanol extract was stored at +4°C until the assays start.

Determination of anti-proliferation activity

After preserving lung cancer cells (A549) in SF for 24 hours, 12-well plates containing 70-80% confluent of lung cancer cell cultures were treated for 24 hours with various dilutions of CVME (25, 50, 100, 200 µg/mL). Positive control consisted of cells cultured in 10% FCS. MTT was utilized to evaluate cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). Replaced the culture media with SF medium containing 1 mg/mL MTT (Sigma) and incubated at 370°C for 15 minutes. The cells were then treated with MTT solution and dimethyl sulfoxide (DMSO, Sigma). The color shift will be measured at 550 nm with a colorimetric reader (Ege *et al*., 2020).

Determination of apoptosis induction by AnnexinV and Propodium iodide (PI)

For the determination of apoptosis induction activity of CVME, lung cancer cells were seeded with a $1x10^6$/mL density to 6-well plates and lowest (25µg/mL) and highest (200 µg/mL) doses of CVME were applied for a period of 24 hours. Annexin V/PI apoptosis detection kit was used to measure cellular apoptosis and manufacturer' recommended protocols were followed. Results were measured in Becton-Dickinson flow cytometer (Cocelli *et al*., 2021).

Determination of BAX and BCL-2 levels by Real-Time PCR

Lung cells ($5x10^6$) were exposed to 25 and 200 mg/L concentrations of CVME for 24 hours. Then, the supernatant discarded and 700 µL of QIAzol Lysis Reagent was added. The total RNA was extracted with TRIpure total RNA extraction reagent (ELK Biotechnology, Wuhan). Then, total RNA reverse trascripted into cDNA by using reverse transcriptase according to the kit's instructions specifications. RT-PCR was performed on a Rotor Gene Q (Qiagen, Germany). cDNA template for each primer with SYBR green master mix reacted at 95°C for 15 min, 40 cycles 95°C for 20 s, 60°C for 30s, 72°C for 30 s. The relative gene expression for Bax (forward: GTCGCCCTTTTCTACTTTGCC, reverse: GTCGCCCTTTTCTACTTTGCC) and Bcl-2 (F: GTCGCCCTTTTCTACTTTGCC, reverse: TCACTTGTGGCCCAGATAGG) genes was determined with normalization to GAPDH (forward: GATCATCAGCAATGCCTCCT, reverse: TGTGGTCATGAGTCCTTCCA). mRNA expression levels are given as $2^{-\Delta\Delta CT}$.

Determination of phenolic compounds in CVME by LC-MS-MS

The phytochemical analysis of CVME were prepared by dissolving samples in methanol and then filtrated with 0.22 µM filter. The LC-MS-MS apparatus of Nexera UHPLC (Shimadzu) with LC-20AD two pumps, DGU-20A3R degasser, CTO-10ASVP column furnace and SIL-20AC autosampler was used for the study. C18 Intersil ODS-4 analytical colon (3.0 mm x 100 mm, 2 µm) was used. The injection volume was 2 µl and flow rate 0.3 mL/min. Mobile phase A (Water and 0.1% Formic acid) and mobile phase B (Methanol and 0.1% Formic acid) were used in a linear gradient flow and column temperature was set at 40°C initially.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 8.0.2. program. Dunnet's test was used for statistical evaluation for antiproliferative effect, Bax and Bcl-2 mRNA expressions, and Tukey test was used for apoptosis induction. For all statistics, $p<0.05$ accepted as statistically significant.

RESULTS

Screening of phenolic acids and flavonoids in CVME by LC-MS-MS

Table N° 1 presents the quantified levels of phenolic acids and flavonoids identified in CVME samples, which were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS-MS) for a total of 24 substances. Fifteen phenolic compounds were identified as a consequence of the conducted study. The main component among flavonoids was revealed to be resveratrol, with a concentration of 135.64 mg/mL. Among other phenolic acids, caffeic acid was found to be the main compound, with a concentration of 122.83 mg/mL.

Determination of antiproliferation activity of CVME on lung cancer cells

Using the MTT assay, the antiproliferative effect of various dosages of CVME on the lung cancer cell line A549 was found and the results are presented in Figure N° 1. As can be seen Figure N° 1, the viability of lung cells decreased in a dose dependent manner and the proliferation of the cells was suppressed statistically significantly especially at 200 µg/mL

dose (p=0.0245).

Table No. 1
Flavonoids and phenolic compounds found in CVME

No	Compounds	
1	Acetohydroxamic Acid	3,193
2	Catechinhyrate	Nd
3	Vanillic Acid	Nd
4	Syringic Acid	11,241
5	Thymoquinone	Nd
6	Resveratrol	135,646
7	Myricetin	21,845
8	Kaempferol	29,344
9	Fumaric Acid	54,555
10	Gallic Acid	3,01
11	Protocatechuic Acid	29,045
12	4-Hydroxybenzoic Acid	9,218
13	Caffeic Acid	122,831
14	Salycilic Acid	2,517
15	Phloridzindyhrate	12,137
16	2-Hydoxycinnamic Acid	Nd
17	Oleuropein	Nd
18	2-hyroxy1,4 naphthaquinone	Nd
19	Naringenin	3,473
20	Silymarin	Nd
21	Quercetin	3,288
22	Luteolin	30,292
23	Alizarin	Nd
24	Curcumin	Nd

Nd: not determined

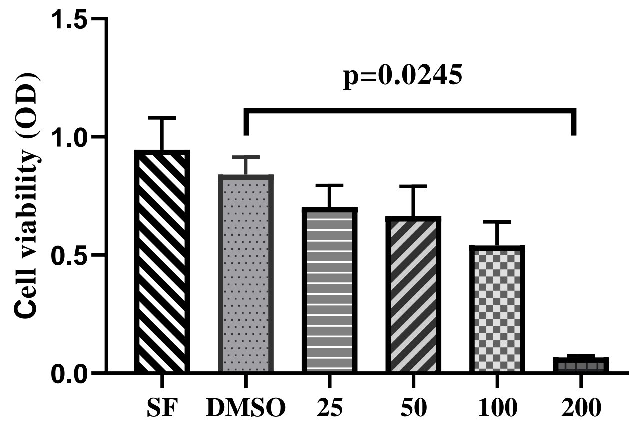

Figure No. 1
Antiproliferative effect of CVME on A549 cells. CVME doses were compared with DMSO

Determination of apoptosis inducing activity of CVME on lung cancer cells

To observe the apoptotic changes occurring in lung cancer cells after the application of 25 and 200 µg/mL doses of CVME, the cells were stained with AnnexinV/PI and scanned with a cell counting device. When the Figure No. 2 is examined, while no change was observed in the number of apoptotic cells at both the doses of 25 and 200 µg/mL, a significant increase in the number of necrotic cells (19.7%) was observed, especially at the dose of 200 µg/mL.

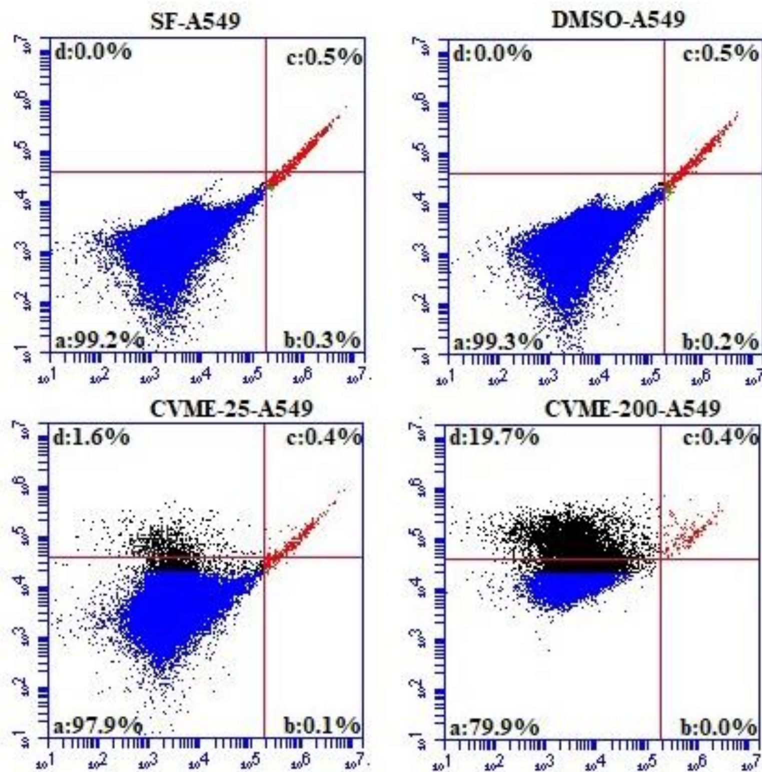

Figure No. 2
Determination of apoptotic and necrotic cells in CVME-treated cells by Annexin-V/PI staining

Determination of Bax and Bcl-2 levels in A549 cells after CVME treatment

Figure No. 3 presents the results of the testing performed on lung cancer cells to evaluate changes in Bax and Bcl-2 expression. Only 25 and 200 mg/mL concentrations of CVME were examined in this assay. When the results were analyzed, no significant change in Bax expression was observed. The BCL-2 level, however, was increased at 200 mg/mL dose but not statistically significant ($p>0.05$).

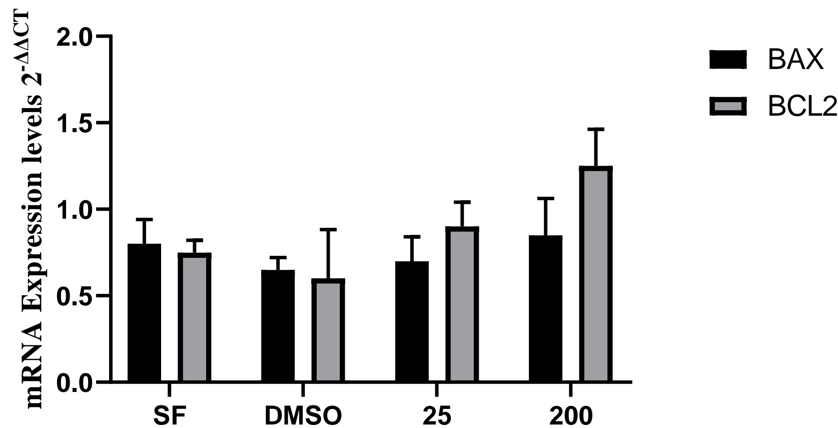

Figure No. 3
Changes in Bax and Bcl2 expression in A549 cells following CVME treatment

DISCUSSION

Cancer is one of the primary causes of death, with different prevalence rates in men and women. For both sexes, lung cancer ranks among the most common cancer types, along with breast and prostate cancer (Siegel *et al.*, 2023). In the United States alone, some 240,000 people are diagnosed a lung cancer diagnosis each year, and about 127,000 of them pass away (Siegel *et al.*, 2023). Considering the number of cases worldwide, it is thought that many more people will die from cancer in the near future. Therefore, it is very important to accelerate research and to identify new chemotherapeutic agents for the prevention and treatment of lung cancer and other cancers. Therefore, the present work aimed to molecularly explore the anticancer activities of *C. vulgare*, a significant therapeutic plant. The main focus of this study was to evaluate the antiproliferative efficacy of CVME on lung cancer cells. Based on the obtained results, it can be asserted that CVME exhibited a dose-dependent inhibition of lung cancer cell proliferation. Studies demonstrating the antiproliferative effects of several *C. vulgare* extracts on different cancer cells have been reported concurrently with our findings. Dzhambazov *et al.* (2002), determined the cytotoxic effects of *C. vulgare* aqueus extract on A2058 (human metastatic melanoma), HEp-2 (epidermoid carcinoma, larynx, human) and L5178Y (mouse lymphoma) cell) cells at IC_{50} doses of 20, 10 and 17.8 μg/mL, respectively. In a different study, it was demonstrated that the aqueous extract of *C. vulgare* reduced the viability and growth of human cervical adenocarcinoma HeLa, human colorectal adenocarcinoma HT-29, and human breast cancer cells MCF-7, particularly at concentrations of 250 and 1000 µg/mL. (Petrova *et al.*, 2023). Furthermore, acidic, basic and neutral water extracts of *C. vulgare* were found to exhibit antiproliferative activity in CaOV (human testicular cystadenocarcinoma), HeLa (human cervical adenocarcinoma), HT-29 (human colorectal adenocarcinoma) cells at doses between 200-400 µg/ml (Batsalova *et al.*, 2017). As a result of our review of the scientific literature, no study showing the effect of *C. vulgare* on the proliferation of lung cancer cells was found. In this respect, the results of the antiproliferation activity of CVME used in this study on lung cancer cells are thought to be the first report.

In this study, the apoptotic and necrotic effects of CVME, whose antiproliferation activity was determined on lung cancer cells, were also measured. In apoptotic cells, phosphotidylserine in the inner membrane of the cell passes to the outer membrane (Segawa & Nagata, 2015). Then, thanks to

the phosphatidylserine, which passes into the outer membrane, the "eat me" signal occurs and the cells are digested by macrophages (Segawa & Nagata, 2015). Dyes such as annexin stain the phosphotidyl serine in the cell outer membrane fluorescently and the possible apoptotic effect of an anticancer substance is determined (Liu *et al*., 2009). In our study, Annexin staining demonstrated that following CVME treatment, lung cancer cells exhibited necrotic death rather than apoptotic death. Furthermore, at the mRNa level, proapoptotic Bax and antiapoptotic Bcl-2 levels were ascertained. In light of the information obtained, there was no discernible alteration in the levels of Bax and Bcl-2 following treatment with CVME. According to an evaluation combining the results of mRNa and Annexin staining, CVME treatment did not cause apoptosis in A549 lung cancer cells. In previous studies, necrotic cell death was commonly perceived as an unregulated process. Nevertheless, in recent years, it has been discovered that the regulation of the necrotic death pathway is additionally controlled by genetic factors (Moujalled *et al*., 2013; Chen *et al*., 2022). Since the observed tendency of cellular necrotic death following the application of CVME in our study, it is advisable to explore potential necrotic markers, such as RIPK1 and RIPK3, in future studies.

The effects of phenolic compounds on cancer cells have been reported in many studies (Luo *et al*., 2011; Abotaleb *et al*., 2020; Yumrutas & Yumrutas, 2022). In our study, the phenolic content of CVME was determined by LC-MS-MS and resveratrol and caffeic acid were identified as major compounds for flavonoids and phenolic acids, respectively. Bruno *et al*. (2019), reported that flavonoids were more dominant in quantity and quercetin was the main flavonoid by HPLC analysis of *C. vulgare*. Zheleva-Dimitrova *et al*. (2019), reported that rosmarinic acid was dominant in the *C. vulgare* water extract, and also soluble cafeic acid oligomers were present in the water extract. In another study, it was reported that *C. vulgare* hot water and methanol extracts had rich phenolic content and 16 phenolic compounds were determined. Among the phenolics obtained, rosmarinic and ellagic acid were determined to be the main compounds (Bektašević *et al*., 2022). It is thought that the difference in the phenolics and their amounts may be due to the difference in collection time, extraction and screening methods.

In conclusion, the phenolic acids and flavonoids of CVME, its antiproliferation activity and its relationship with apoptotic and necrotic death pathway were determined in this study. In the light of the data obtained, it can be said that *C. vulgare* has a rich phenolic content, suppresses the viability and proliferation of lung cancer cells and induces necrosis instead of apoptosis. In terms of all the data obtained, this article is thought to be the first report on the anticancer activity of *C. vulgare* on lung cancer. It is suggested that future studies should focus on caspases involved in the intrinsic and extrinsic apoptotic pathway and factors such as RIPK1 and RIPK3 involved in the necrotic pathway.

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