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RESEARCH ARTICLE

Potential anticancer activity of chemically characterized extract of *Olea europaea* (Olive) leaves

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ABSTRACT

Olea europaea Linn. (Olive) is considered as essential component of Mediterranean diets. Olive leaves, fruits, and oil are traditionally known for several health benefits including diabetes, cardiac complications, cancer, etc. The objective of the present study is to determine the anticancer potential of chemically characterized *O. europaea* extract in MTT assay and EB/AO double staining method using Human lung cancer cell lines (A549). The chemical constituents present in the ethyl acetate extract of *O. europaea* leaves were characterized by GC-MS and its cytotoxic activity was assessed by MTT assay and EB/AO double staining method. The GC-MS analysis identified 63 chemical constituents, and neophytadiene (21.80%), zingiberenol (12.36%), and allohimachalol (5.49%) was found as major chemical constituents in ethyl acetate extract of *O. europaea* leaves. *O. europaea* produces a time and dose-dependent inhibition of cell proliferation of A549 cell lines. The cell viability of A549 cell lines after 24 hrs treatment with *O. europaea* ranged from 97.96 ± 3.44 to 18.95 ± 2.14 % for a concentration range of 0.5-500 µg/mL, respectively, with IC_{50} value of 21.91 ± 1.8 µg/mL. EB/AO double staining shows significant apoptosis in early and late apoptotic, and necrotic cells with increased volume and showed uneven orange-red fluorescence at their periphery. The study outcome shows that *O. europaea* extract significantly inhibited cell proliferation and apoptosis in human lung cancer (A549) cell lines, and it also explores the chemical composition of *O. europaea* leaves extract.

Keywords: Apoptosis; A549 cell lines; Cancer; GC-MS; MTT assay; Olea europea; Olive

INTRODUCTION

Olea europaea Linn. (Family: Oleaceae) is commonly known as Olive and Zaytoon, and its fruits and oil are predominantly used in the Mediterranean diet. The Olive plant is a major crop cultivated in the Mediterranean, and it is traditionally used as salad, cooking food products, and for skin and hair care (Abaza et al., 2015). The leaf of Olive is traditionally used for the treatment of diabetes (Mootoosamy and Mahomoodally, 2014). The Olive tree is emerged as special to mankind as it has several beneficial roles in maintaining human health and also Olive is reported throughout historical and religious texts (Kaniewski et al., 2012). Olive fruits and leaves have several pharmacological actions including cardioprotective, antidiabetic, hypolipidemic, neuroprotective, antioxidant, and hepatoprotective (Ahamad et al., 2019; Janahmadi et al., 2015; Andreadou et al., 2006; Hadrich et al., 2016; Barbaro et al., 2014; Al-Azzawie and Alhamdani, 2006). Olive has been reported as a beneficial agent for the treatment of different forms of cancer (De Marino et al., 2014; Hernández-Corroto et al., 2018; Castejón et al., 2000; Antoniou and Hull, 2021).

The most common cancer-related death worldwide is now lung cancer. Lung cancer is thought to be mostly caused by smoking. According to Collins et al. (2007), there are three types of lung cancer: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Treatment options for lung cancer range from chemotherapy to surgical resection based on the type and stage of the tumor that has been found. Severe negative consequences have been linked to both chemotherapy and surgical resection. Palliative therapy is, therefore, necessary in addition to chemotherapy for the treatment of lung cancer (Jones and Baldwin, 2018). Cancer and other chronic human diseases are reported to be treated by medicinal plants (Gezici and ekerolu, 2019).

Olive extracts and its bioactive compounds have shown potential anticancer activity in many *in-silico, in-vitro*, and

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in-vivo studies using different cancer cell lines (Antoniou, and Hull, 2021; Castejón et al., 2020; Imran et al., 2018; Qais et al., 2022). In a study conducted by Gallazzi et al., (2020) on lung cancer cell lines using an extract of Olive Mill Wastewater (OMWW), the study finding shows that OMWW downregulates growth, adhesion, and invasion in lung cancer cells. In another study on lung cancer, Olive leaf extract ameliorates benzo (a) pyrene-induced lung cancer through Nrf2 and NFκB pathway (Majumder et al., 2021). The current study involves the standardization of Olive leaf extract by GC-MS and then standardized extracts' anticancer potential was determined by MTT assay and EB/AO double staining method using Human lung cancer cell lines (A549).

MATERIALS AND METHODS

Plant materials and chemicals

The *Olea europaea* fresh leaves (500 g) were collected from Erbil, Iraq in the month of March 2021. The plant is identified by Dr. Raad A. Kaskoos, Pharmacognosist, Faculty of Pharmacy, Hawler Medical University, Erbil, Iraq, and a voucher specimen (PRL/2021/03) was kept in the Department of Pharmacognosy, Faculty of Pharmacy, Tishk International University, Erbil, Iraq.

The human lung cancer cell lines (A549) was procured from NCCS, Pune, India. Phosphate Buffered Saline (PBS) salts, Penicillin-Streptomycin, ETBr, and Acridine orange were acquired from (Sigma Aldrich, USA), whereas Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA, Fetal Bovine Serum (FBS), and Penicillin/Streptomycin Antibiotic Solution were purchased from (Gibco USA). Dimethyl sulfoxide (DMSO), 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), and 1X PBS were acquired from Sigma-Aldrich in the United States and Himedia in India, respectively. Tarson (India) supplied 96 well tissue culture plates and wash beakers. Other chemicals and solvents were all of an analytical grade.

Preparation of *O. europaea* extract

O. europaea fresh leaves were first dried in the shade with enough air before being ground into a coarse powder in a mixer. *O. europaea* coarse powder (50 g) was extracted using an ultrasonicator (Elma, Germany). Drug powder was placed in a stoppered conical flask and extracted using an ultrasonicator at 200 W power for 30 minutes at 40 ºC with 250 mL of ethyl acetate. A rotary evaporator (Buchi, Switzerland) was used to concentrate the extract at a temperature of 40 ºC. The extract was then air-dried and kept in a refrigerator at a temperature between 2-4 ºC till usage.

GC-MS analysis of *O. europaea* extract

The chemical composition of the ethyl acetate extract of *O. europaea* was determined by the GC-MS method. For the analysis of the chemical composition, we used our previous method published elsewhere (Ahamad et al., 2020). The compounds were identified by mass fragmentation obtained from mass spectra obtained by GC-MS analysis with those stored in the spectrometer database of NIST, NBS 54 K.L, WILEY8 libraries and published literature (Adams, 2007; Ali, 2001; Kaskoos et al., 2009; Ahamad et al., 2020).

MTT assay to assess cytotoxicity

The cytotoxic activity of *O. europaea* was assessed by MTT assay against human lung cancer cell lines (A549). The assay was performed by the method described by Marquez et al., (2020). *O. europaea* ethyl acetate extract was assessed for anticancer activity in MTT assay for concentrations ranging from 0.5 to 500 µg/mL. The IC_{50} value was calculated using GraphPad Prism 6.0 software (USA). The percent cell viability was calculated by the following formula:

$$\text{Cell viability}(\%) = \frac{\text{OD test}}{\text{OD control}} \times 100$$

Assessment of apoptosis by dual AO/EB method

Dual ethidium bromide (EB)/acridine orange (AO) staining was performed to study the effects of the olive extract on the apoptosis of lung cancer cell lines (A549). The IC_{50} value obtained from the MTT assay of *O. europaea* extract was selected as the dose in this study *i.e.* 21.91 µg/mL. The study was performed by the method proposed by Liu et al., (2015) with suitable modification, briefly, 5 x 10^5 cells/mL of A549 cells were plated in a 96-well tissue culture plate and incubated for 24 hr in a DMEM growth medium. The cells were then exposed to 21.91 g/mL of *O. europaea* ethyl acetate extract in serum-free DMEM media following incubation. The plate was incubated for 24 hours at 37 ºC with 5% CO_2. 10 L of 1 mg/mL acridine orange and ethidium bromide were added to the wells after incubation and gently mixed. The plate was then centrifuged at 800 rpm for 2 minutes, and reviewed within an hour, and at least 100 cells were observed using an Olympus fluorescence microscope and a fluorescent filter.

RESULTS AND DISCUSSION

GC-MS analysis

The chemical constituents present in the ethyl acetate extract of *O. europaea* leaves were determined by the GC-MS method and the results were presented in Table 1. Sixty-three chemical constituents were identified in the ethyl acetate extract of *O. europaea* leaves which represents about 78.06% of total chemical compounds

Table 1: Chemical composition of ethyl acetate extract of *O. europaea* leaves

S. No.	Name of chemical compound	RT	KI	% Composition
1.	Propanoic acid, ethyl ester	3.103	700	2.16
2.	Ethane, 1,1-diethoxy	3.313	710	1.24
3.	Hexanal	3.876	797	0.16
4.	Camphene	7.066	953	0.16
5.	Decane	8.687	999	0.13
6.	Benzyl alcohol	10.274	1027	0.10
7.	*cis*-Linalyl oxide	10.781	1074	0.16
8.	2-Phenylethanol	10.906	1082	0.13
9.	3,4-Dimethylbenzyl alcohol	11.306	1113	0.25
10.	4-Methyl 1-undecene	11.940	1124	0.13
11.	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	12.107	1192	0.11
12.	Dodecane	12.591	1199	0.19
13.	Pulegone	12.871	1207	0.10
14.	Bornyl formate	13.820	1228	0.14
15.	Hydroxycitronellal	13.902	1240	0.15
16.	Dodecane, 2,6,11-trimethyl-	14.495	1275	0.11
17.	Thymol	15.079	1290	0.17
18.	Dihydroedulan	15.398	1291	0.11
19.	Tridecane	15.490	1299	0.32
20.	Neoisopulegol	16.277	1309	1.62
21.	Dodecane, 4,6-dimethyl	16.516	1325	0.35
22.	2,3-Dihydro-benzofuran	16.955	1368	0.55
23.	Ethyl (2E,4Z)-nona-2,4-dienoate	17.154	1376	0.12
24.	Dodecane, 2,6,10-trimethyl	17.888	1382	2.88
25.	1-Tetradecene	18.254	1388	0.82
26.	Isoeugenol	18.512	1429	0.27
27.	α-Curcumene	18.590	1472	0.21
28.	Dihydroactinidiolide	18.675	1479	0.16
29.	α-Morphemes	19.101	1485	0.35
30.	(+)-Eremophilene	19.235	1486	0.29
31.	Dimethyl 3,4-pyridinedicarboxylate	19.288	1502	0.21
32.	(+)-Eremophilene	19.353	1503	0.56
33.	Homovanillyl alcohol	19.455	1534	0.16
34.	Nerolidol	19.604	1548	1.04
35.	3,4-Dihydroxybenzoic acid	19.821	1557	0.37
36.	2-Propenal, 3-(2-furanyl)	20.041	1582	0.26
37.	Isospathulenol	20.160	1619	0.29
38.	Zingiberenol	20.476	1635	12.36
39.	Allohimachalol	20.768	1673	5.49
40.	4-Hydroxy-β-ionone	21.092	1676	0.64
41.	9-Octadecene	21.606	1803	2.33
42.	Phytane	21.829	1816	1.51
43.	Neophytadiene	22.091	1827	21.80
44.	6,10,14-Trimethylpentadecan-2-one	22.709	1842	0.99
45.	Nonadecane	23.563	1900	1.17
46.	Heptadecane, 2,6,10,15-tetramethyl	23.812	1914	3.61
47.	Ethyl 9-hexadecenoate	24.016	1955	0.89
48.	Vinyl palmitate	24.151	1985	0.92
49.	Eicosane	25.137	2000	0.55
50.	Tetradecyl bromoacetate	25.260	2086	0.25
51.	Methyl oleate	25.873	2103	0.34
52.	Ethylene dibenzoate	26.315	2108	0.20
53.	Z-Phytol	26.591	2114	0.20
54.	Ethyl oleate	28.490	2171	0.91
55.	Ethyl stearate	30.098	2181	0.63

(Contd...)

Table 1: (*Continued*)

S. No.	Name of chemical compound	RT	KI	% Composition
56.	2-Methyltetracosane	32.341	2460	0.11
57.	(8)-Gingerdione	32.864	2560	1.12
58.	15-Tetracosenoic acid, methyl ester	33.013	2680	0.18
59.	Dioctyl phthalate	34.623	2682	1.21
60.	Glyceryl monooleate	35.081	2714	1.27
61.	p-Methoxybenzoic acid, pentadecyl ester	35.820	2786	1.34
62.	Supraene	36.504	2817	1.15
63.	Vitamin E	36.840	3100	0.36

where, KI: Kovats index, and RT: retention time

(Table 1). Neophytadiene (21.80%), zingiberene (12.36%), allohimachalol (5.49%), heptadecane, 2,6,10,15-tetramethyl (3.61%), dodecane, 2,6,10-trimethyl (2.88%), 9-octadecene (2.33%), and propanoic acid, ethyl ester (2.16%) was identified as major chemical compounds in ethyl acetate extract of *O. europaea* leaves. The ethyl acetate extract of *O. europaea* also contains neoisopulegol (1.62%), *p*-methoxybenzoic acid, pentadecyl ester (1.34%), ethane, 1,1-diethoxy (1.24%), glyceryl monooleate (1.27%), dioctyl phthalate (1.21%), phytane (1.51%), nonadecane (1.17%), supraene (1.15%), (8)-gingerdione (1.12%), and nerolidol (1.04%) as minor chemical compounds. Chemical compounds less than 1% are also presented in Table 1. The main focus of this analysis to characterize fatty acid components present in leaves of *O. europaea*. Several studies were previously published about the chemical composition of *O. europaea* oil (Ahamad et al., 2020). The present study leads identification of phytochemicals present in leaves by GC-MS analysis, and the main fatty components identified are neophytadiene (21.80%), zingiberenol (12.36%), and allohimachalol (5.49%).

Anticancer activity of *O. europaea*

MTT assay

An MTT assay was performed to measure the cytotoxicity of *O. europaea* ethyl acetate extract against A549 (Human lung cancer cells) cell lines. The results of the MTT assay are shown in Table 2 and Figs. 1 & 2. *O. europaea* ethyl acetate extract produces a time (24 hrs) and dose (0.5-500 µg/mL) dependent inhibition of cell proliferation against A549 cell lines. The cell viability of A549 cell lines after 24 hrs treatment with *O. europaea* ethyl acetate extract ranged from 97.96±3.44 to 18.95±2.14 % for a concentration range of 0.5-500 µg/mL, respectively (Table 2). At 24 hrs, the IC_{50} value of *O. europaea* ethyl acetate extract was 21.91±0.18 µg/mL for A549 cell lines. MTT is a water-soluble substance that the live cell can take up. For calorimetric measurement, a water-insoluble blue formazan that is the reduction product of MTT must be dissolved. The untreated A549 cells kept

Table 2: Cytotoxicity (% cell viability) produced by ethyl acetate extract of *O. europaea* leaves

Conc. (µg/mL)	Cell viability (%)
Control	100
0.5	97.96±3.44
1	76.75±3.27
5	71.45±0.99
10	64.61±0.86
50	59.91±2.35
100	52.21±2.95
200	43.86±3.82
300	35.36±4.94
400	25.70±3.85
500	18.95±2.14
IC_{50} value (µg/mL)	21.91±0.18

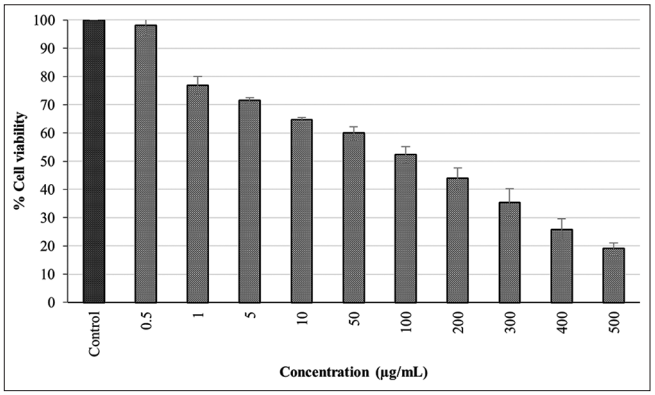

Fig 1. MTT assay for measurement of cytotoxicity (% cell viability) produced by ethyl acetate extract of *O. europaea* leaves (Data were presented as mean of triplicate determinations ± SD)

their original morphology and close contact with one another even when the incubation time was extended to 24 hours, as seen in Fig. 2. In the treatment groups the A549 cells, on the other hand, began to resemble their pre-treatment form 24 hours later. The elongated spindle-shaped morphology of the A549 cell lines was no longer present. Suspension cells (dead cells) were found when the incubation was continued for 48 hours, and more suspension cells were seen at 48 hours (Fig. 2 b-g). In order to evaluate the anticancer potential of medicinal

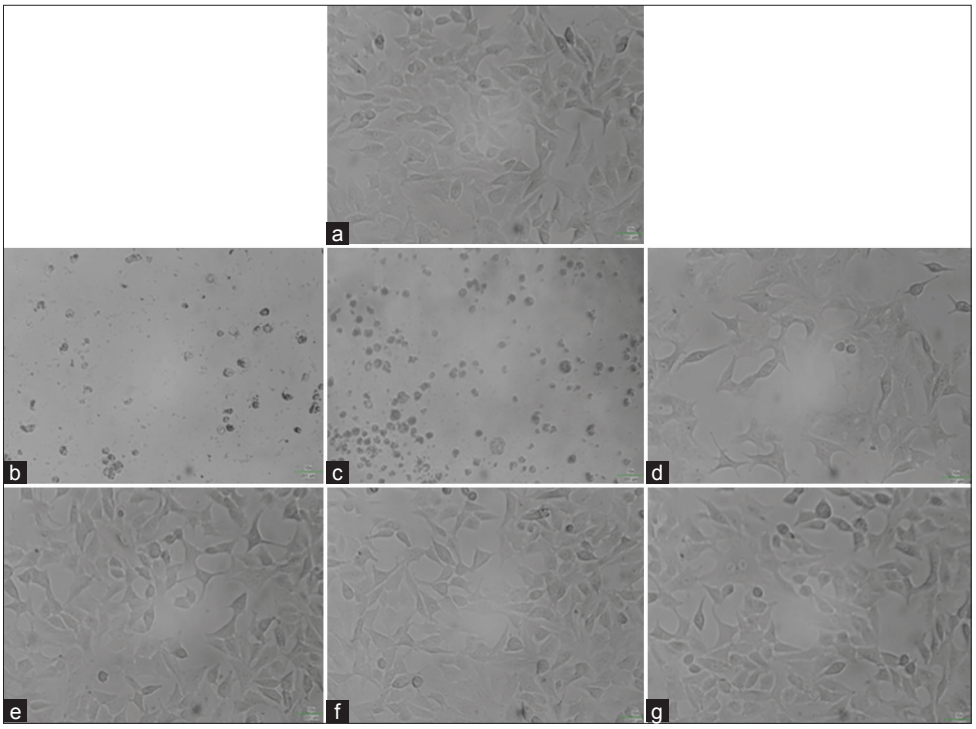

Fig 2. Cell cytotoxicity produced by ethyl acetate extract of *O. europaea* against Human lung cancer A549 cell lines. where, figure (a): Control cells; and figure b to g: *O. europaea* ethyl acetate extract (b: 500 µg/mL, c: 300 µg/mL, 100 µg/mL, 50 µg/mL, 10 µg/mL, and 0.5 µg/mL).

plants, cell cytotoxicity by MTT test has frequently been performed (Ahamad et al., 2019).

EB/AO double staining

The results of EB/AO double staining analysis were presented in Table 3 and Figs. 3a, b. The treatment with olive extract (21.91 µg/mL) caused a significant reduction of the number of viable cells in EO/AO double staining analysis lung cancer (A549) cell lines in early and late apoptotic cells (Fig. 3b), and there was no significant apoptosis detected in the negative control group (Fig. 3a). Besides that, several cells had apoptotic signs such as plasma membrane blabbing. The number of red-stained cells (necrotic cells) did not raise. This shows that the majority of the cells were not necrotic and that cell death was predominantly caused by apoptosis. The results of the early apoptotic (EA), late apoptotic (LA), and total apoptotic (total necrosis) cell populations were expressed as percentages of apoptosis and presented in Fig. 3b and Fig. 4.

A wide range of natural compounds have been discovered to have the ability to cause apoptosis in human tumor cells (Mathur et al., 2009; Shiezadeh et al., 2013). The substances are made up of different chemical entities, and many of them can be found in medicinal plants as well as fruits and vegetables that are widely ingested by humans. So, it is important to screen apoptotic inducers from plants, either

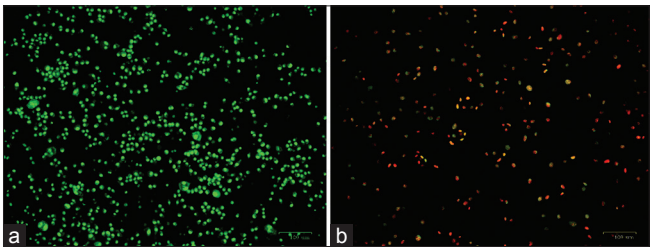

Fig 3. (a) Negative control group (normal cells): the circular nucleus uniformly distributed in the center of the cell. (b) Experimental groups treated with Olive extract (21.91 µg/mL): (1) Early apoptotic cells: nucleus showed yellow-green fluorescence by acridine orange (AO) staining and concentrated into a crescent or granular that located in 1 side of cells. (2) Late apoptotic cells: the nucleus of cell showed orange fluorescence by EB staining and gathered in concentration and located in bias. (3) Necrotic cells: The necrosis cells' volume was increased, showing uneven orange-red fluorescence.

in the form of crude extract or as components, isolated from (Taraphdar et al., 2001). There are a variety of *in-vitro* methods for detecting apoptotic cell death. The use of a fluorescent microscope in apoptosis detection methods offers several notable benefits. For detecting apoptosis, double staining approaches (EB/AO) produce consistent and repeatable results. As a result, differentiating clearly between apoptotic cell subpopulations (early or late apoptotic cells) (Baskic et al., 2006). The most common number of apoptotic cells found in 21.91 µg/mL of olive extract-treated cells and late apoptotic cells were considerably elevated in the EB/AO staining assay.

Table 3: Comparison of the number of early, late apoptotic and necrotic index

Control	Early apoptosis index (%)		Late apoptosis index (%)		Necrosis index (%)	
	0	1	3	3	0	0
Treated with Olive extract	11	15	76	82	3	4
Mean values	13=26/2		79=158/2		3.5=7/2	

where, data is presented in mean±SD; Early apoptotic cells: No. of cells which appeared yellow-green fluorescence/100 cells, Late apoptotic cells: No. of cells which appeared orange nuclear fluorescence/100 cells, Necrotic cells: No. of cells which appeared orange-red fluorescence/100 cells

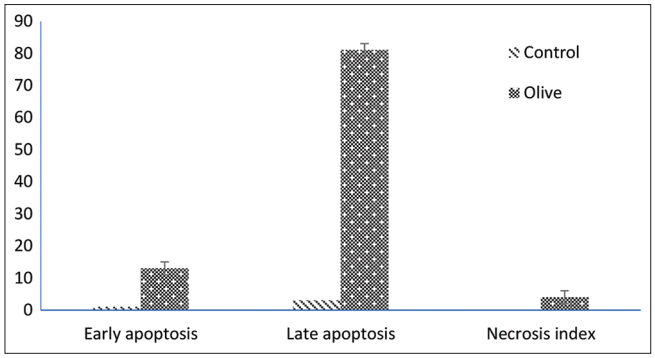

Fig 4. Treatment with Olive extract (21.91 µg/mL) in lung cancer cell lines in Dual EB/AO staining shows significant apoptosis in early and late apoptotic cells; and necrotic cells compared to control cells.

CONCLUSION

O. europaea is an important component of Mediterranean diets and it has several pharmacological actions such as antidiabetic, cardioprotective, and neuroprotective, etc. In the present study chemical constituents of ethyl acetate extract of *O. europaea* leaves were determined by GC-MS. The GC-MS analysis shows the presence of neophytadiene, zingiberenol, and allohimachalol as major chemical constituents. *O. europaea* produces dose-dependent inhibition of human lung cancer cell lines. The results of EB/AO double staining show significant apoptosis produced by *O. europaea* extract. The study outcome shows that *O. europaea* extracts significantly inhibited cell proliferation and apoptosis in human lung cancer (A549) cell lines. The present also study expands knowledge about the chemical composition of *O. europaea* leaf extract.

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CONFLICT OF INTEREST

The authors declared no conflicts of interest with this research work.

Authors contribution

SNMN: laboratory work and writing; JA: project design, laboratory work, data analysis, and proofreading; SS: editing and literature; and SU: data analysis and editing.

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