# 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<sub>50</sub> value of  $21.91 \pm 1.8 µg/mL$ . 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 NFKB 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 °C with 250 mL of ethyl acetate. A rotary evaporator (Buchi, Switzerland) was used to concentrate the extract at a temperature of 40 °C. The extract was then air-dried and kept in a refrigerator at a temperature between 2-4 °C 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  $\mu$ g/mL. The IC<sub>50</sub> value was calculated using GraphPad Prism 6.0 software (USA). The percent cell viability was calculated by the following formula:

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<sub>50</sub> value obtained from the MTT assay of O. europaea extract was selected as the dose in this study i.e. 21.91  $\mu$ g/mL. The study was performed by the method proposed by Liu et al., (2015) with suitable modification, briefly, 5 x 10<sup>5</sup> 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 serumfree DMEM media following incubation. The plate was incubated for 24 hours at 37 °C with 5% CO<sub>2</sub>. 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

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Table 1: Chemical composition of ethyl acetate extract of <i>O. europaea</i> le	eaves
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1.     Propanci acid, ellyl ester     3.103     7.00     2.18       2.     Elhans, 1.1 - diehoxy     3.31     7.0     1.24       3.     Hexnal     3.876     797     0.16       5.     Decame     8.877     999     0.13       5.     Decame     8.887     999     0.13       6.     Berazyl alcohol     10.274     10.74     0.16       7.     cis-Linslyl oxide     10.781     1074     0.16       8.     2.Pherylethanol     13.36     113     0.25       10.     4.Methyl hundecene     11.940     1124     0.13       11.     H-Pyrrole-Zichone, Schult-Hunethyl-     12.107     1192     0.11       12.     Dadecane, Zi, 11-trimethyl-     13.820     1228     0.14       15.     Hydroxychronelial     13.820     1291     0.11       17.     Hydroxychronelia     13.828     1291     0.11       17.     Hydroxychronelia     13.828     1291     0.11       17.     Hydroxychronelia	S. No.	Name of chemical compound	RT	KI	% Composition
2.   Ethan, 1, 1-diethoy,   3,313   710   1,24     3.   Hexanal   3,76   77   0,16     4.   Camphene   7,066   953   0,16     5.   Berayl alcohol   10,274   1027   0,10     7.   cis-Linaly oxide   10,71   1074   0,13     8.   2.Phenylethanol   10,306   1132   0,13     9.   3.4-Omethyletnzyl alcohol   11,306   1113   0,25     10.   4-Methyl 1-indecene   11,306   1132   0,13     11.   1H-Pyrole2,5-dione,3-ethyl-4-methyl-   12,107   1192   0,11     12.   Dadeane   12,8271   1207   0,10     13.   Pulegone   12,871   1207   0,10     14.   Bornyl formate   13,802   1240   0,17     15.   Hydroxyltonolial   13,902   1240   0,17     16.   Dadeane, 2,6,14:methyl-   15,549   1290   0,17     17.   Thymol   15,078   1290   0,17     18.   Dadeane,2,6,14:methyl	1.	Propanoic acid, ethyl ester	3.103	700	2.16
3.   Hexanel   3.876   797   0.16     5.   Decame   8.687   999   0.13     6.   Benzyl alochol   10.274   1027   0.10     7.   cls-Lindyl oxide   10.781   1074   0.18     8.   2-Phenylchanol   10.906   1082   0.13     9.   3.4-Dimertylbranol   11.940   1124   0.13     10.   4-Metnyl Lundecene   11.940   1124   0.13     11.   11-H-Pyrole 2-Schone, 3-ethyl-4-metnyl-4   12.81   1199   0.19     12.   Dodecane   12.820   128   0.14     13.   Pulegone   12.81   1199   0.19     14.   Bornyl formatic   13.802   1228   0.14     15.   Dodecane, 2.6, 11-tinmethyl-4   14.495   1275   0.11     16.   Dodecane, 4.6-dimethyl   13.802   128   0.32     17.   Thymol   15.07   126   0.32   0.32     17.   Dindroceduan   15.16   1325   0.35     18.   Dindroceduan	2.	Ethane, 1,1-diethoxy	3.313	710	1.24
4.     Camphene     7.066     953     0.16       5.     Decane     867     999     0.13       6.     Benzyl alcohol     10.274     1027     0.10       7.     Als-Linghlycade     10.781     1074     0.13       8.     2Phenylethanol     10.306     1133     0.25       9.     3.4-Unnethylbenzyl alcohol     11.306     1113     0.25       10.     4-Methyl 1-modecane     12.871     1109     0.19       13.     Dodecane     12.871     1207     0.10       14.     Bornyl formate     13.802     1240     0.15       15.     Hydroxychronelia     13.902     1240     0.16       16.     Dodecane, 2.6, 11-tinnethyl-     14.495     1275     0.11       17.     Tridacane     15.398     1291     0.11       17.     Dindyrocelunan     15.398     1291     0.12       20.     Neoicopulegol     16.277     1309     6.22       21.     Dodecane, 4.6-dinethyl     17.8	3.	Hexanal	3.876	797	0.16
5.     Decare     5.667     999     0.13       6.     Bernyl atohoh     0.274     1027     0.10       7.     cis-Linayl oxide     10.781     1074     0.161       8.     2-Prenylethanol     10.906     1082     0.13       9.     3.4-Dimethylenzyl aloohol     11.306     1113     0.28       10.     4-Methyl-Lundecene     11.940     1124     0.13       11.     11-H-Pyricle 2-Schore, 2-ethyl-4-methyl-     12.107     1182     0.14       12.     Dedecane     2.851     11207     0.10       13.     Pulegone     12.871     1207     0.10       14.     Bonyl formate     13.820     1280     0.17       15.     Pydroxycironelial     15.398     1291     0.11       17.     Tymol     15.398     1291     0.11       18.     Dirydroxcirina     15.816     1382     0.82       21.     Dodcane, 2.6,11-trimethyl-     15.816     1386     0.85       22.     Dirydroxcinian	4.	Camphene	7.066	953	0.16
6.     Bengy lacbhol     10,274     1027     0.10       7.     cisLunaly coride     10,781     1074     0.18       8.     2-Phenylethanol     10,906     1082     0.13       9.     3,4-Dimethylbenzyl alcohol     11,306     1113     0.28       10.     4-Methyl t-nudecene     12,107     1192     0.11       11.     H-Pyrole-2,5-dione, 3-ethyl-4-methyl-     12,107     1192     0.11       12.     Dodecane     12,871     1207     0.10       13.     Pulegone     12,871     1207     0.10       14.     Bomyl formate     13,802     1228     0.117       17.     Trymol     15,079     1280     0.17       18.     Dirlytorekulan     15,388     1281     0.11       19.     Dirlytorekulan     15,398     1282     0.32       21.     Dodecane, 4,6-dimethyl     16,851     1386     0.55       22.     2,510-trimathyl     17,84     1376     0.12       24.     Dodecane, 4,6-di	5.	Decane	8.687	999	0.13
7.     cb.Linayl cxide     10.781     1074     0.161       8.     2.Phenylethanol     10.906     1082     0.13       9.     3.4-Dimethylenzyl alcohol     11.306     1113     0.25       10.     4-Methyl 1-undecene     11.940     1124     0.13       11.     11-HPyrole 2-Schone 3-ethyl-4-methyl-     12.107     1192     0.11       12.     Dedecane     12.871     1207     0.10       13.     Pulegone     12.871     1207     0.10       14.     Bornyl formate     13.820     1228     0.14       15.     Dedecane, 2, 11-trimethyl-     14.485     1275     0.11       17.     Thymol     15.079     1280     0.17       18.     Dihydroedulan     15.079     1280     0.12       21.     Dedecane, 4, 6-dimethyl     16.812     1382     0.35       22.     2,3-Dihydro-benzofuran     16.955     1388     0.55       23.     Eihyl (24,2) nona - 4,-dieneate     17.154     1376     0.12 <td< td=""><td>6.</td><td>Benzyl alcohol</td><td>10.274</td><td>1027</td><td>0.10</td></td<>	6.	Benzyl alcohol	10.274	1027	0.10
8.     2-Phenylethanol     10.906     1082     0.13       9.     3-4-Dinetylhenyl alcohol     11.940     1124     0.13       11.     1H-Pyrole-2.5-dione, 3-ethyl-4-methyl-     12.107     1122     0.11       12.     Dedecane     12.47     1207     0.10       13.     Pulegone     12.871     1207     0.10       13.     Pulegone     12.871     1207     0.10       15.     Hydroxyltronella     13.902     1240     0.15       16.     Dodecane, 2.6.11-trimethyl-     14.495     1275     0.11       17.     Thymol     15.079     1280     0.32       10.     Tridecane     15.490     1290     0.32       21.     Dodecane, 4.6-dimethyl     15.615     1325     0.35       22.     2.5.01-bydro-benzolaran     15.615     1325     0.35       22.     2.5.01-bydro-benzolaran     15.87     1388     0.82       23.     Dodecane, 2.6.10-trimethyl     7.88     1382     0.28       24.	7.	cis-Linalyl oxide	10.781	1074	0.16
9.   3.4-Dimetry/benzy/lachol   11.366   11.31   0.25     10.   4-Metry/1-undecene   11.940   1124   0.13     11.   11-Prynole-2.5-dione, 3-ethyl-4-metryl-   12.107   1192   0.11     12.   Dodecane   12.871   1207   0.10     13.   Pulegone   12.871   1207   0.10     14.   Bory/Iormate   13.802   1240   0.15     15.   Hydroxycitonelial   13.802   1240   0.15     16.   Dodecane, 2.6,11-trimethyl-   14.465   1275   0.11     17.   Thymol   15.079   1290   0.12     18.   Dihydroedulan   15.409   1291   0.11     19.   Tridecane   15.409   1290   0.32     21.   Dodecane, 4.6-dimethyl   16.516   1328   0.35     22.   2.3-Dilydro-benzduran   16.457   1368   0.52     23.   Indydroechindolide   18.512   1368   0.82     24.   Dodecane, 2.6,10-trimethyl   7.86   1363   0.56     25.	8.	2-Phenylethanol	10.906	1082	0.13
10.   4.Methyl 1-undecene   11.40   11.42   0.13     11.   11.Helyrole-2.5-clione, 3-ethyl-4-methyl-   12.107   11.92   0.11     12.   Dodecane   12.891   11.99   0.19     13.   Pulegone   12.871   1207   0.10     14.   Bornyl formate   13.802   1228   0.14     15.   Hydroxycitronelial   13.802   1240   0.15     16.   Dodecane, 2.6,11-trimethyl-   14.405   1275   0.11     17.   Thynod   15.079   1290   0.32     20.   Neisopulegol   16.277   1309   1.62     21.   Dodecane, 2.6,10-trimethyl   16.816   1325   0.35     22.   2.3-Dihydro-benzofuran   16.865   1368   0.55     23.   Ethyl (2.42)-none2.4-dienaate   17.154   1376   0.12     24.   Dodecane, 2.6,10-trimethyl   17.888   1382   2.88     25.   1-Tetradecene   18.507   1472   0.21     26.   Isceurenol   18.512   148   0.36	9.	3,4-Dimethylbenzyl alcohol	11.306	1113	0.25
11.   14-Proribe-2,5-dione, 3-ethyl-4-methyl-   12,107   1192   0.11     12.   Dodecane   12,591   1192   0.19     13.   Pulegone   12,871   1207   0.10     14.   Bornyl formate   13,802   1240   0.15     15.   Hydroxycironellal   13,802   1240   0.15     16.   Dodecane, 2,6,11-trimethyl-   14,468   1275   0.11     17.   Thymol   15,079   1290   0.17     18.   Dihydroedulan   15,398   1291   0.11     19.   Tridecane   16,477   1309   1.62     21.   Dodecane, 4,6-dimethyl   16,516   1325   0.35     22.   2,3-Dihydro-benzofuran   16,851   1376   0.12     24.   Dodecane, 2,6,10-trimethyl   17,154   1376   0.12     25.   1.Fortadecane   18,250   1479   0.16     26.   Isoeugonol   18,512   1429   0.27     27.   o-curcumene   18,503   1479   0.16     28.   o-fortorume	10.	4-Methyl 1-undecene	11.940	1124	0.13
12.   Dodecane   12.671   1207   0.19     13.   Pulegone   12.671   1207   0.10     14.   Bornyl formate   13.820   1228   0.14     15.   Hydroxycitronella   13.902   1240   0.15     16.   Dodecane.   2.611-trimethyl-   14.485   1275   0.11     17.   Trymol   15.079   1290   0.12     18.   Dihydroedulan   16.538   1299   0.32     20.   Neoisopulegol   16.277   1309   1.62     21.   Dodecane.   2.61-trimethyl   16.856   1368   0.55     22.   2.30/bydro-benzofuran   16.856   1368   0.55     23.   Ethyl (2E.42)-nons2.4-denoate   17.154   1376   0.12     24.   Dodecane. 2.6.10-trimethyl   17.868   1382   2.86     25.   1-Teradecene   18.512   1429   0.27     27.   o-Curoumene   18.525   1479   0.16     28.   Dibrdroxderidoloide   19.285   1634   0.29 <t< td=""><td>11.</td><td>1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-</td><td>12.107</td><td>1192</td><td>0.11</td></t<>	11.	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	12.107	1192	0.11
13.   Puegone   12.871   1207   0.10     14.   Bornyl formate   13.820   1228   0.14     15.   Hydroxyttonelial   13.920   1240   0.15     16.   Dodecane, 2,6,11 timethyl-   14.495   1275   0.11     17.   Thymol   15.079   1280   0.17     18.   Dihydroedulan   15.398   1291   0.11     19.   Tidecane   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-Dihydrobenzoturan   16.516   1325   0.55     23.   Ethyl (2E,42)-nona-2,4-dienoate   17.154   1376   0.12     24.   Dodecane, 2,6,10-timethyl   17.888   1382   0.88     25.   1.Tetradecene   18.512   1479   0.16     27.   eOuroumene   18.502   1472   0.21     28.   o.Morphemes   19.101   1485   0.35     30.   (Horporanilyl atob	12.	Dodecane	12.591	1199	0.19
14.   Bory formate   13.820   128   0.14     15.   Hydroxycitronellal   13.902   1240   0.15     16.   Dodecane, 2.6, 11-trimethyl-   14.495   1275   0.11     17.   Thymol   15.079   1280   0.17     18.   Dihydrochulan   15.98   1291   0.32     20.   Neoisopulegol   16.277   1309   1.62     21.   Dodecane, 4.6-dimethyl   16.516   1325   0.35     22.   2.3-Dihydrochenzotran   16.955   1386   0.55     23.   Ethyl (2E,42)-nona-2,4-dienoate   17.54   1382   0.48     24.   Dodecane, 2,6,10-trimethyl   17.88   1382   0.82     25.   1-Tetradocene   18.512   1429   0.27     27.   o-Curcumene   18.590   1472   0.21     28.   Isocuponilene   19.285   1486   0.29     39.   c-Morphemes   19.101   1485   0.56     30.   (-)-Eremophilene   19.285   1486   0.29     31.   Dimeth	13.	Pulegone	12.871	1207	0.10
15.   Hydroxychronellal   13.902   12.40   0.15     16.   Dodecane, 2,6,11-trimethyl-   14.495   1275   0.11     17.   Thymol   15.079   1280   0.17     18.   Dhydroedulan   15.079   1280   0.32     20.   Neoisopulegol   16.277   1309   1.62     21.   Dodecane, 4,6-dimethyl   16.616   1325   0.35     22.   2,3-Dinydro-benzofuran   16.955   1368   0.55     23.40   Ethyl (2E,42)-nona-2,4-dienoate   17.184   1376   0.12     24.   Dodecane, 2,6,10-trimethyl   17.884   1382   2.88     25.   1-Tetradecane   18.512   1479   0.16     27.   -c-Curcumene   18.501   1479   0.16     28.   Diodychactinidiolide   18.675   1479   0.16     29.   -c-Curcumene   19.285   1503   0.56     31.   Dimethyl 3,4-pyridinedicarboxylate   19.285   1503   0.56     32.   (-H)Eremophilene   19.485   1554   0.16	14.	Bornyl formate	13.820	1228	0.14
16.   Dodecane, 2,6,11-trimethyl-   14,495   1275   0.11     17.   Thymol   15,079   1290   0.17     18.   Dihydroedulan   15,390   1290   0.17     19.   Tridecane   15,490   1299   0.32     20.   Neoisopulegol   16,277   1309   0.32     21.   Dodecane, 4,6-dimethyl   16,516   1325   0.35     22.   2,3-Dihydro-benzofuran   16,955   1368   0.55     23.   Ethyl (2,4-)ronac,2,4-dienoate   17,154   1376   0.12     24.   Dodecane, 2,6,10-trimethyl   17,888   1382   2,88     25.   1-Tetradecene   18,512   1429   0,27     27.   α-Curcumene   18,512   1429   0,27     28.   Isoeugenol   18,512   1429   0,27     29.   α-Morphemes   19,010   1485   0,35     30.   (+)-Eremophilene   19,235   1486   0,29     31.   Dimethyl 3,4-byrdinedicarboxylate   19,851   1533   0,56     33.	15.	Hydroxycitronellal	13.902	1240	0.15
17.   Tnymol   15.079   1290   0.17     18.   Dirydrocdulan   15.398   1291   0.11     19.   Tridecane   15.398   1291   0.11     19.   Tridecane   15.398   1291   0.32     20.   Neisopulegol   16.277   1309   1.62     21.   Dodecane, 4.6-dimethyl   16.955   1568   0.55     22.   2.3-Dirydro-benzofuran   16.955   1368   0.55     23.   Ethyl (2E.42)-none.2.4-dienoate   17.154   1376   0.12     24.   Dodecane, 2.6, 10-trimethyl   17.888   1382   2.88     25.   1-Tetradecene   18.512   1429   0.27     26.   Isoeugenol   18.675   1479   0.16     29.   c.Morphemes   19.101   1485   0.35     30.   (+)-Eremophilene   19.353   1603   0.66     31.   Dimethyl 3.4-pyridinedicarboxylate   19.821   1537   0.37     32.   (+)-Eremophilene   19.353   1603   0.26     33.   Johon	16.	Dodecane, 2,6,11-trimethyl-	14.495	1275	0.11
18.   Dilydroedulan   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-benzoturan   16.955   1368   0.55     23.   Ethyl (2E,42)-nona-2,4-dienoate   7.154   1376   0.12     24.   Dodecane, 2,6,10-trimethyl   17.888   1382   2.88     25.   1-Tetradecene   18.512   1429   0.27     27.   α-Curcumene   18.590   1472   0.21     28.   Dihydroachindiolide   18.675   1479   0.16     29.   α-Morphemes   19.101   1485   0.35     30.   (+)Eremophilene   19.235   1486   0.29     31.   Dimethyl 3-4-pridineidicarboxylate   19.285   1534   0.16     32.   (+)Eremophilene   19.353   1503   0.56     33.   Homovanilyl alcohol   19.604   164   164     34.<	17.	Thymol	15.079	1290	0.17
19.   Tradecane   15.490   1299   0.32     20.   Neoisopulegol   16.277   1309   1.62     21.   Dodecane, 4.6-dimethyl   16.855   1368   0.55     22.   2.3-Dihydro-benzofuran   16.955   1368   0.55     23.   Ethyl (2E, 42)-nona-2,4-dienoate   17.154   1376   0.12     24.   Dodecane, 2,6,10-trimethyl   17.881   1382   2.88     25.   1-fetradecene   18.254   1388   0.82     26.   Isoeugenol   18.512   1479   0.21     27.   ca-Curoumene   18.675   1479   0.16     29.   ca-Morphemes   19.101   1485   0.35     30.   (+)-Eremophilene   19.235   1486   0.29     31.   Dimethyl 3.4-pyrdinedicarboxylate   19.863   1503   0.56     32.   (+)-Eremophilene   19.435   1534   0.16     34.   Nerolical   19.864   1548   1.04     35.   3.4-Dihydroxybenzoicaid   19.864   15457   0.37     36. <td>18.</td> <td>Dihydroedulan</td> <td>15.398</td> <td>1291</td> <td>0.11</td>	18.	Dihydroedulan	15.398	1291	0.11
20.   Neoisopulegol   16.277   1309   1.62     21.   Dodecane, 4.6-dimethyl   16.516   1325   0.55     22.   2.3-Dihydro-benzofuran   16.516   1325   0.12     24.   Dodecane, 2.6, 10-trimethyl   17.888   1382   2.88     25.   1-Tetradecene   18.512   1429   0.27     27.   a-Curcumene   18.512   1429   0.27     27.   a-Curcumene   18.590   1472   0.21     28.   Dihydroactinidiolide   18.675   1479   0.16     29.   a-Morphemes   19.101   1485   0.35     30.   (-Fremophilene   19.235   1486   0.29     31.   Dimetryl 3.4-pyridinedicarboxylate   19.455   1534   0.16     32.   (+)Eremophilene   19.353   1503   0.56     33.   Homovanily lacohol   19.604   1548   1.04     35.   3.4-Dihydroxybenzoic acid   19.821   1557   0.37     36.   2-Propenal, 3-(2-turanyl)   20.041   1582   0.26	19.	Tridecane	15.490	1299	0.32
21.   Dodeone, 4,6-dimethyl   16.516   1325   0.35     22.   2,3 Dihydro-benzoturan   16.955   1368   0.55     23.   Ethyl (2: 42)-nona - 2,4-diencate   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   18.650   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   19.285   1534   0.16     33.   Homovanillyl alcohol   19.455   1534   0.16     34.   Nerolidol   19.604   1548   1.04     35.   3.4-Dihydroxybenzoic acid   19.821   157   0.37 <tr< td=""><td>20.</td><td>Neoisopulegol</td><td>16.277</td><td>1309</td><td>1.62</td></tr<>	20.	Neoisopulegol	16.277	1309	1.62
22.   2,3-Dihydro-benzofuran   16.955   1368   0.55     23.   Ethyl (2E,42)-non-2,4-dienoate   17.154   1376   0.12     24.   Dodecane, 2,6,10-trimethyl   17.154   1382   2.88     25.   1-Tetradecene   18.254   1388   0.82     26.   Isoeugenol   18.512   1429   0.27     27.   a-Curcumene   18.550   1479   0.16     28.   Dihydroactinidiolide   18.675   1479   0.16     29.   a-Morphemes   19.101   1485   0.35     30.   (-)-Eremophilene   19.235   1486   0.29     31.   Dimethyl 3,4-pyridinedicarboxylate   19.353   1503   0.56     33.   Homovanilyl alcohol   19.645   1548   1.04     35.   3,4-Dihydroxybenzoic acid   19.821   1557   0.37     36.   2,4-Propenal,3-(2-furanyl)   20.016   1619   0.29     37.   Isosgathulenol   20.768   1633   2.33     34.   Nerolido   20.160   1613   3.49	21.	Dodecane, 4,6-dimethyl	16.516	1325	0.35
23.   Ethyl (2E, 42)-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.   Isceugenol   18, 12   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, 353   1503   0.56     32.   (+)-Eremophilene   19, 353   1503   0.66     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, 768   1673   5.49	22.	2,3-Dihydro-benzofuran	16.955	1368	0.55
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.   a-Curcumene   18.590   1472   0.21     28.   Dihydroactinidolide   18.675   1479   0.16     29.   a-Morphemes   19.101   1485   0.35     30.   (+)-Eremophilene   19.235   1486   0.29     31.   Dimethyl 3.4-pyridinedicarboxylate   19.283   1502   0.21     32.   (+)-Eremophilene   19.353   1503   0.56     33.   Homovanillyl alcohol   19.455   1534   0.16     34.   Nerolidol   19.864   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   20.476   1635   12.36     38.   Zingiberenol   20.0768   1673   5.49     40.	23.	Ethyl (2E,4Z)-nona-2,4-dienoate	17.154	1376	0.12
25.   1-Tetradecene   18.254   1388   0.82     26.   Isoeugenol   18.512   1429   0.27     27.   ac-Ourcumene   18.590   1472   0.21     28.   Dihydroactinidiolide   18.675   1479   0.16     29.   ac-Morphemes   19.101   1465   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   19.821   1557   0.37     36.   2-Propenal, 3-(2-furanyl)   20.041   1582   0.26     37.   Isospathulenol   20.768   1673   5.49     40.   4.1ohimachalol   20.768   1673   5.49     41.   9-Octadecene   21.092   1676   0.64     41.   9-Octadecene   21.829   1816   1.51     43.   Neophytadiene <td>24.</td> <td>Dodecane, 2,6,10-trimethyl</td> <td>17.888</td> <td>1382</td> <td>2.88</td>	24.	Dodecane, 2,6,10-trimethyl	17.888	1382	2.88
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   19.604   1548   1.04     34.   Nerolidol   19.604   1548   1.04     35.   3.4-Dihydroxybenzoic acid   19.821   1557   0.37     36.   2-Propenal, 3-(2-furanyl)   20.0476   1635   12.36     37.   Isospathulenol   20.476   1635   12.36     38.   Zingiberenol   20.476   1635   12.36     39.   Allohimachalol   20.788   1673   5.49     40.   4-Hydrox-β-ionone   21.692   1676   0.64     41.   <	25.	1-Tetradecene	18.254	1388	0.82
27.   α-Curcumene   18.590   1472   0.21     28.   Dihydroactiniciolide   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.   Homovanilyl alcohol   19.455   1534   0.16     34.   Nerolidol   19.851   1557   0.37     35.   3,4-Dihydroxybenzoic acid   19.821   1557   0.37     36.   2-Propenal, 3-(2-furanyl)   20.041   1582   0.26     37.   Isospathulenol   20.476   1635   12.36     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.   <	26.	Isoeugenol	18.512	1429	0.27
28.     Dihydroactinidiolide     18.675     1479     0.16       29.     α-Morphemes     19.101     1465     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-ionen     21.092     1676     0.64       41.     9-Octadecene     21.606     1803     2.33       42.	27.	α-Curcumene	18.590	1472	0.21
29.   c-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   20.476   1635   12.36     38.   Zingiberenol   20.476   1635   12.36     39.   Allohimachalol   20.768   1673   5.49     40.   4-Hydroxy-Fjeinone   21.606   1803   2.33     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	28.	Dihvdroactinidiolide	18.675	1479	0.16
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     19.654     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     20.476     1635     12.36       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. <td< td=""><td>29.</td><td>α-Morphemes</td><td>19.101</td><td>1485</td><td>0.35</td></td<>	29.	α-Morphemes	19.101	1485	0.35
1     Dimethyl 3,4-pyridinedicarboxylate     19.288     1502     0.21       31.     Dimethyl 3,4-pyridinedicarboxylate     19.353     1503     0.56       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     22.091     1827     21.80       43.     Neophytadiene     22.709     1842     0.99       45. <td>30.</td> <td>(+)-Eremophilene</td> <td>19.235</td> <td>1486</td> <td>0.29</td>	30.	(+)-Eremophilene	19.235	1486	0.29
2.   (+)-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   23.563   1900   1.17     45.   Nonadecane   23.513   1900   1.17     46.   Heptadecane, 2,6,10,15-tetramethyl   23.812   1914   3.61     47.	31.	Dimethyl 3.4-pyridinedicarboxylate	19.288	1502	0.21
Answer     Answer     Answer     Answer       33.     Homovanillyi 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     20.768     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     23.563     1900     1.17       46.     Heptadecane, 2,6,10,15-tetramethyl     23.812     1914     3.61       47.     Ethyl 9-he	32.	(+)-Fremophilene	19.353	1503	0.56
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   21.37   2000   0.55   0.92	33.	Homovanillyl alcohol	19.455	1534	0.16
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   21.802   1803   2.33     42.   Phytane   22.091   1827   21.80     43.   Neophytadiene   22.091   1822   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   25.260   2086   0.25     50.   Tetradecyl bromoacetate   25.873   2103   0.34     52.   Et	34.	Nerolidol	19.604	1548	1.04
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37.Isospathulenol20.16016190.2938.Zingiberenol20.476163512.3639.Allohimachalol20.76816735.4940.4-Hydroxy-β-ionone21.09216760.6441.9-Octadecene21.60618032.3342.Phytane21.82918161.5143.Neophytadiene22.091182721.8044.6,10,14-Trimethylpentadecan-2-one22.70918420.9945.Nonadecane23.56319001.1746.Heptadecane, 2,6,10,15-tetramethyl23.81219143.6147.Ethyl 9-hexadecenoate24.01619550.8948.Vinyl palmitate24.15119850.9249.Eicosane25.26020860.2550.Tetradecyl bromoacetate25.26020860.2551.Methyl oleate25.87321030.3452.Ethylene dibenzoate26.31521080.2053.Z-Phytol26.59121140.2055.Ethyl oleate28.49021710.91	36.	2-Propenal, 3-(2-furanvl)	20.041	1582	0.26
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39.Allohimachalol20.76816735.4940.4-Hydroxy-β-ionone21.09216760.6441.9-Octadecene21.60618032.3342.Phytane21.82918161.5143.Neophytadiene22.091182721.8044.6,10,14-Trimethylpentadecan-2-one22.70918420.9945.Nonadecane23.56319001.1746.Heptadecane, 2,6,10,15-tetramethyl23.81219143.6147.Ethyl 9-hexadecenoate24.01619550.8948.Vinyl palmitate24.15119850.9249.Eicosane25.26020860.2550.Tetradecyl bromoacetate25.87321030.3452.Ethyl oleate26.31521080.2053.Z-Phytol26.59121140.9155.Ethyl oleate28.49021710.91	38.	Zingiberenol	20.476	1635	12.36
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41.9-Octadecene21.60618032.3342.Phytane21.82918161.5143.Neophytadiene22.091182721.8044.6,10,14-Trimethylpentadecan-2-one22.70918420.9945.Nonadecane23.56319001.1746.Heptadecane, 2,6,10,15-tetramethyl23.81219143.6147.Ethyl 9-hexadecenoate24.01619550.8948.Vinyl palmitate24.15119850.9249.Eicosane25.13720000.5550.Tetradecyl bromoacetate25.26020860.2551.Methyl oleate25.87321030.3452.Ethylene dibenzoate26.31521080.2053.Z-Phytol26.59121140.2054.Ethyl oleate28.49021710.91	40.	4-Hvdroxy-β-ionone	21.092	1676	0.64
42.Phytane21.82918161.5143.Neophytadiene22.091182721.8044.6,10,14-Trimethylpentadecan-2-one22.70918420.9945.Nonadecane23.56319001.1746.Heptadecane, 2,6,10,15-tetramethyl23.81219143.6147.Ethyl 9-hexadecenoate24.01619550.8948.Vinyl palmitate24.15119850.9249.Eicosane25.13720000.5550.Tetradecyl bromoacetate25.26020860.2551.Methyl oleate25.87321030.3452.Ethylene dibenzoate26.31521080.2053.Z-Phytol26.59121140.2054.Ethyl oleate28.49021710.9155.Ethyl oleate20.09821810.62	41.	9-Octadecene	21.606	1803	2.33
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   0.62	42.	Phytane	21.829	1816	1.51
44.6,10,14-Trimethylpentadecan-2-one22.70918420.9945.Nonadecane23.56319001.1746.Heptadecane, 2,6,10,15-tetramethyl23.81219143.6147.Ethyl 9-hexadecenoate24.01619550.8948.Vinyl palmitate24.15119850.9249.Eicosane25.13720000.5550.Tetradecyl bromoacetate25.26020860.2551.Methyl oleate25.87321030.3452.Ethylene dibenzoate26.31521080.2053.Z-Phytol26.59121140.2054.Ethyl oleate28.49021710.91	43.	Neophytadiene	22.091	1827	21.80
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   26.591   2114   0.20     53.   Z-Phytol   26.591   2114   0.91     54.   Ethyl oleate   28.490   2171   0.91	44.	6.10.14-Trimethylpentadecan-2-one	22,709	1842	0.99
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	45	Nonadecane	23 563	1900	1 17
47.Ethyl 9-hexadecenoate24.01619550.8948.Vinyl palmitate24.15119850.9249.Eicosane25.13720000.5550.Tetradecyl bromoacetate25.26020860.2551.Methyl oleate25.87321030.3452.Ethylene dibenzoate26.59121140.2053.Z-Phytol26.59121140.2054.Ethyl oleate30.09821810.62	46	Hentadecane 2.6.10.15-tetramethyl	23 812	1914	3.61
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   26.591   2114   0.20     53.   Z-Phytol   26.591   2114   0.20     54.   Ethyl oleate   30.098   2181   0.62	47	Ethyl 9-bexadecenoate	24 016	1955	0.89
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   26.591   2114   0.20     53.   Z-Phytol   26.591   2114   0.20     54.   Ethyl oleate   30.098   2181   0.62	48	Vinyl palmitate	24 151	1985	0.92
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	49	Ficosane	25 137	2000	0.55
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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	51	Methyl oleate	25.873	2103	0.34
53. Z-Phytol 26.591 2114 0.20   54. Ethyl oleate 28.490 2171 0.91   55. Ethyl stearate 30.098 2181 0.62	52	Ethylene dibenzoate	26 315	2108	0.04
54. Ethyl oleate 28.490 2114 0.20   55. Ethyl stearate 30.098 2181 0.63	53	Z-Phytol	26 591	2114	0.20
55 Ethyl stearate 30 008 2181 0.62	54	Ethyl oleate	28.490	2174	0.20
	55	Ethyl stearate	30,098	2181	0.63

(Contd...)

Table 1. (Commund)							
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. (Continued)

(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- $500 \,\mu g/mL$ ) 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  $0.5-500\,\mu g/mL$ , respectively (Table 2). At 24 hrs, the IC<sub>50</sub> value of *O. europaea* ethyl acetate extract was  $21.91\pm0.18\,\mu$ g/mL for A549 cell lines. MTT is a water-soluble substance that the live cell can take up. For calorimetric measurement, a waterinsoluble 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 <sub>50</sub> 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 spindleshaped 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 Najibullah, et al.



**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  $\mu$ g/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  $\mu$ g/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	e apoptotic and	I necrotic index
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Control	Early apopt	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:	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  $\mu$ g/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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