



Research paper

Pretreatment with methanolic extract of *Pistacia lentiscus L.* increases sensitivity to DNA damaging drugs in primary high-grade serous ovarian cancer cells



Imane Charid^{a,*}, Mirjana Kessler^d, Silvia Darb-Esfahani^{c,l}, Tomasz Zemojtel^{e,f}, Salem Abobaker^{c,g}, Sandra Tyuarets^{h,i}, Stefanine Schrauwen^{h,i}, Dina Atmani-Kilani^a, Nadjet Benaida-Debbache^a, Reinhold Schäfer^l, Dan Cacsire Castillo-Tong^j, Djebbar Atmani^a, Farid Cherbal^b, Frederic Amant^{h,i,k}, Jalid Sehouli^{c,g}, Hagen Kulbe^{c,g,1}, Elena I. Braicu^{c,g,1}

^a Laboratoire de Biochimie Appliquée, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000, Algeria

^b Molecular Genetics Team, Laboratory of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Science and Technology "Houari Boumediene" Algiers, Algeria

^c Tumor bank Ovarian Cancer Network, Department of Gynecology, Charité Universitätsmedizin Berlin, Germany

^d Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany

^e Institute for Medical Genetics and Human Genetics, Charité Universitätsmedizin Berlin, Germany

^f Labor Berlin-Charité Vivantes GmbH, Humangenetik, Berlin, Germany

^g Department of Gynecology, Charité Universitätsmedizin Berlin, Germany

^h Department of Oncology, Gynecologic Oncology, KU Leuven (University of Leuven), 3000 Leuven, Belgium

ⁱ Department of Obstetrics and Gynecology, University Hospitals Leuven, 3000 Leuven, Belgium

^j Translational Gynecology Group, Department of Obstetrics and Gynecology, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria

^k Center for Gynaecologic Oncology Amsterdam (CGOA), Antoni van Leeuwenhoek-Netherlands Cancer Institute, 1066 CX Amsterdam, Netherlands

¹ Institute of Pathology, Charité Universitätsmedizin Berlin, Germany

ARTICLE INFO

ABSTRACT

Keywords:

Pistacia lentiscus
Mastic tee
Ovarian cancer
Primary cell line models
Apoptosis
Chemosensitivity
Therapeutics

Introduction: Ovarian cancer remains the most lethal gynecologic cancer in women. Despite achievements in surgical and systemic therapy, most patients develop platinum resistance. Thus, new strategies to increase or reverse the platinum sensitivity in ovarian cancer patients are urgently needed. In this study, we aimed to investigate the anti-cancer effect of the leaves of *Pistacia lentiscus L* medicinal plants used in Algerian traditional medicine on ovarian cancer cells *in vitro*.

Methods: Four newly established primary cell lines derived from ascites of patients with high-grade serous and clear cell ovarian cancer were used to test the anti-cancer effect of the leaves of *Pistacia lentiscus L*. An experimental study was performed to study the therapeutic effects of the plant leaves substances in patient derived models. The anti-proliferative activity of ethanolic, acetonic and methanolic plant leaves extracts was measured by cell viability assays, and the apoptotic effect assessed using flow cytometry analysis. The impact on constitutive active oncogenic pathways and cytokine release in cell culture supernatant were monitored by Western blotting and ELISA, respectively.

Results: Sequencing analysis confirmed the presence of mutations in several genes, such as *TP53*, *RB1*, *PIK3C*, which are commonly mutated in high-grade serous ovarian cancer. Obtained results indicated a cytotoxic effect of the methanolic extract of *P. lentiscus L* (MEPL) on primary cell line cultures, inhibiting PI3K/AKT and MAPK/ERK signaling pathways, and decreasing the release of IL6 and VEGF by the malignant cells. Moreover, treatment

Abbreviations: EOC, Epithelial ovarian cancer; HGSOC, High-grade serous ovarian cancer; CCC, Clear Cell Carcinoma; MEPL, Methanolic Extract of *Pistacia lentiscus*; RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco's Modified Eagle Medium; PBS, Phosphate-buffer saline; FCS, Fetal calf serum; PI, Propidium iodide; STR, Short Tandem Repeat; DAB, diaminobenzidine peroxide; PARP, Poly ADP-ribose Polymerases; MoA, Mechanism of action; VEGF, Vascular Endothelial Growth Factor; IL6, Interleukin6; TNF, Tumor necrosis factor; MMP, Matrix Metalloproteinase; DSB, Double Strand Breaks; MAPK, MAP kinase; EpCam, Epithelial Cell adhesion molecule; PI3K, Phosphoinositide 3 kinase; HNF1beta, Hepatocyte nuclear factor1-beta; SNVs, Single nucleotide variants; GATK, Genome Analysis ToolKit; GRP-78, Glucose-regulated protein -78; CHOP, C homologous protein

* Corresponding author.

E-mail address: charid.imane@gmail.com (I. Charid).

¹ Authors contributed equally to this work.

with MEPL enhanced the sensitivity to platinum-based chemotherapy in our primary cell lines of patients.
Conclusion: Methanolic extract of *P. lentiscus L* might be a promising candidate for novel therapeutic approaches in combination with classic chemotherapy for patients with high-grade serous ovarian cancer.

1. Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies worldwide [1]. To date, the development of acquired chemo resistance is a major obstacle in clinical management of ovarian cancer. The poor prognosis and high mortality rates show that current therapies often fail and novel approaches are urgently required in order to improve the prospect of recovery in ovarian cancer patients.

Epithelial ovarian cancer (EOC) is a histologically, clinically and molecularly diverse disease, with HGSOC being the most common subtype [2,3]. However, ovarian cancer is still treated as a single clinical entity, usually using platinum-based chemotherapy after primary cytoreductive surgery. HGSOC is often diagnosed at an advanced stage and is characterized by a remarkable degree of genomic instability compared with other solid cancer types [4]. Although most of the patients show high clinical response after first line platinum based chemotherapy, 70 % of the patients will relapse and develop resistance with a 5 years survival rate of less than 40 % [5].

Epidemiological studies have indicated that ovarian cancer is associated with chronic inflammation as revealed by the fact that anti-inflammatory agents such as aspirin reduce the risk of colon and ovarian cancer [6]. However, the underlying mechanisms that link inflammation to ovarian cancer are not well understood.

In the last decade, alternative medicine has achieved big steps in fighting fatal diseases such as cancer and neurodegenerative disorders [7,8]. Several studies showed anti-cancer activity of natural compounds, which arouse a great potential for cancer research and new therapeutic approaches [9,10]. Recent data supported the protective effect of fruits and vegetables intake with high antioxidant content [11,12]. In support of this data, it was demonstrated that dietary chemopreventive compounds induce inhibition of cell growth or apoptosis in human ovarian cancer cells [13,14].

Pistacia lentiscus L, commonly known as mastic tree (Anacardiaceae), grows widely in the Mediterranean basin ecosystems. All parts of this plant and especially its leaves have many use in traditional medicine in Algeria. The leaves of *P. lentiscus L*, have been reportedly used as decoctions and infusions for asthma and rheumatism [15] and for the treatment of brain and gastro-intestinal disorders [15].

Previous studies reported that the plant leaves extracts constitute a rich source of natural polyphenols compounds with a high concentration of flavonoids and phenolics acids [15–18] and validated the virtues of the plant as antioxidant [15], anti diabetic [16] anti-inflammatory and anti-tumoral [17]. In recent published study, we reported that leaves extracts of *P. lentiscus L* have a strong effect in aluminum induced neurotoxicity in mice [18].

Despite the fact that *P. lentiscus L* has not been widely used traditionally to treat or prevent cancer in Algeria, its strong antioxidant and anti-inflammatory potential suggests that it may have anti-cancer potential. By generating new patient-derived cell lines, therefore reflecting better patients' diversity and clinical reality, we aimed to delineate the mechanisms responsible for the anti-cancer properties of *P. lentiscus L* (MEPL) extracts.

2. Materials and methods

2.1. Plant extracts

Leaves of *P. lentiscus L* were collected from the forest of Azru' n Bechar located in the province of Amizour, Bejaia, (North east of Algeria). The identification of the plant was carried out in the

laboratory of Botany, University of Bejaia, Algeria according to a voucher herbarium specimen (N° 970704) disposed in the National Institute of Agronomy, Algiers, Algeria. Plant leaves were air-dried in darkness at room temperature and then ground and sieved to get a fine powder (< 63 µm) using a commercial grinder (KIKO Labortechnic, Staufen, Germany). The extract was prepared according to the method described by Atmani et al. (2009) [15]. The obtained powder was mixed separately in different organic solvents at 70 % (ethanol, methanol, and acetone) and placed under agitation for 24 h. After decantation and centrifugation, the reunified extractive liquid was evaporated by rotary evaporation. Obtained extracts were stored at –20 °C until use.

2.2. Ovarian cancer cells

Ovarian cancer cell lines A2780 and SKOV3 were cultured in RPMI 1640 (Sigma, Gibco, UK), 10 % FCS (Invitrogen) in a humidified incubator with 5 % CO₂ at 37 °C. Primary cells were isolated from freshly obtained ascites of EOC patients at the Department of Gynecology, Campus Virchow Clinic, Charité Medical University Berlin, Germany. Sample collection was permitted by the local ethics committee of the Charité Medical University Berlin (AVD-No. 2004-000034) and all individuals had given informed consent. Cell suspension was cultured at 37 °C and 5 % CO₂ in DMEM medium containing, 100 U / mL penicillin and 100 µg / mL streptomycin, 10 % FCS, 10 % ascites from each individual patient. All cells were routinely tested for mycoplasma contamination and have undergone STR authentication. Online STR analysis using the DSMZ database (<http://www.dsmz.de/>) revealed unique STR profiles of human origin for the in-house derived cell lines.

2.3. Cell viability assay

Cells were plated in triplicate at a density of 3 × 10³ cells/well in 96 wells plates incubated at 37 °C and treated 24 h later with different concentrations (10–100 µg/ml) of methanolic, ethanolic and acetonate extracts of leaves of *P. lentiscus L* or alternatively cells were treated with different concentrations of standards carboplatin (1–1000 µM) and paclitaxel (3–3000 nM). Cell viability was determined after 72 h, by adding 10 µL WST-1 reagent (Roche Applied Science, Mannheim, Germany) to each well followed by incubation for 2 h. The absorbance of the treated samples against a control was measured at 450 nm as the detection wavelength and 670 nm as the reference wavelength for the assay.

2.4. Cell cycle analysis

Cells were treated with 10 and 25 µg/mL of MEPL for 24 and 48 h. They were fixed with ethanol (70 %) at room temperature (RT) overnight and were stained with (PI) / RNase A staining buffer, containing 10 µg PI / ml PBS (Sigma-Aldrich, St. Louis, USA) and 20 µg RNase A / ml PBS (Sigma-Aldrich, St. Louis, USA). Cell cycle distribution was analyzed by flow cytometry by using a FACScan® flow cytometer (Becton Dickinson, San Jose, USA). Results were analysed by using FlowJo 7.6.5 software (Tree Star Inc., Ashland, USA).

2.5. Measurement of apoptosis

Apoptosis in ovarian cancer cells was determined by double staining with FITC-conjugated Annexin V and PI according to manufacturer's instructions. The percentage of apoptotic cells was analyzed using a

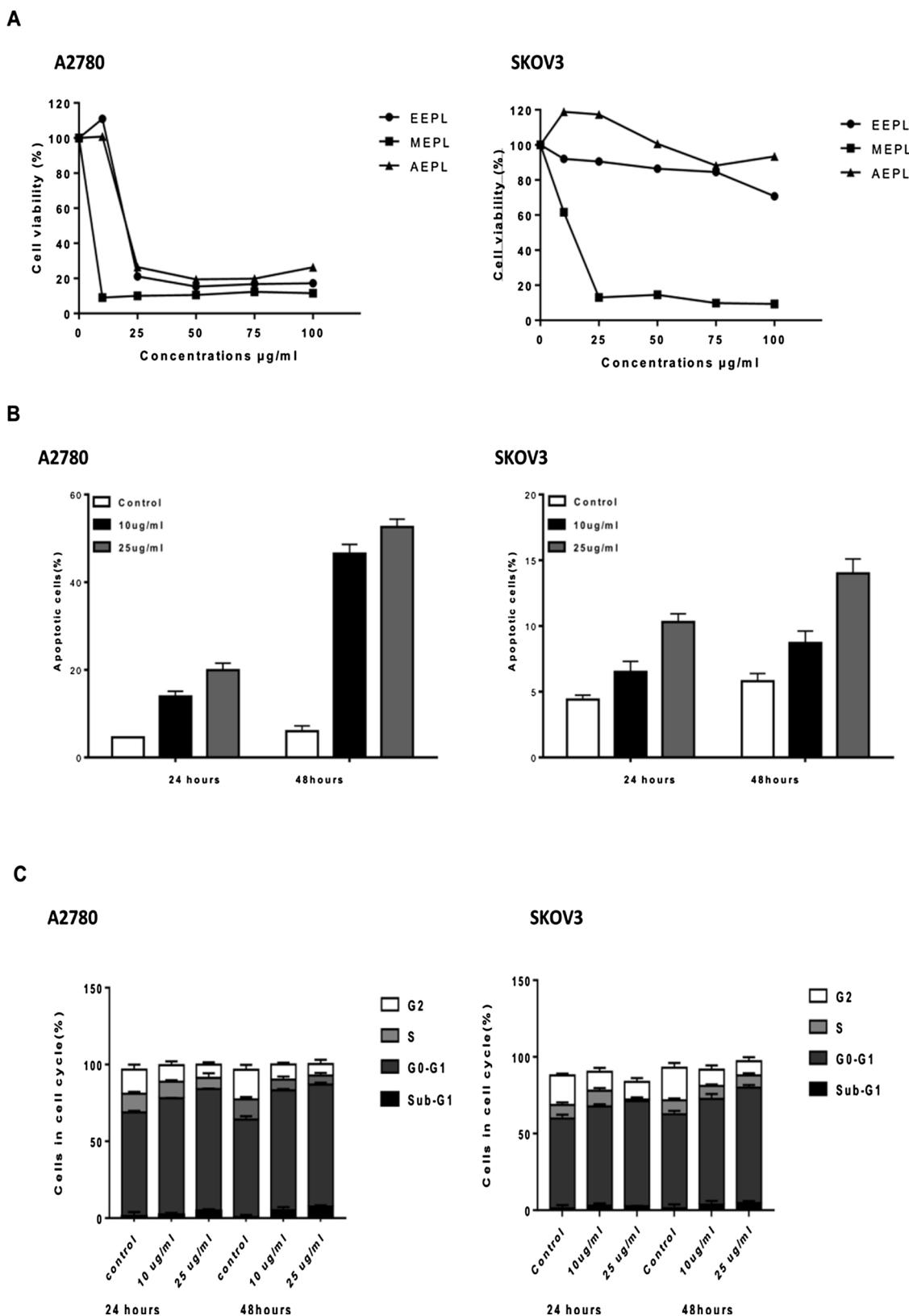


Fig. 1. Anti-cancer activity of plant extracts in ovarian cancer cells. A) Proliferative activity in A2780 and SKOV3 cells was determined after 72 h treatment with methanolic (MEPL), acetonnic (AEPL) and ethanolic (EEPL) extracts of *P. lentiscus* B) Representative analysis for induction of apoptosis, apoptotic cells were assessed after 24 and 48 h treatment with 10 and 25 $\mu\text{g}/\text{mL}$ of MEPL in A2780 and SKOV3 cells by PI/ Annexin V staining. C) Top, representative flow cytometry analysis for investigation of cell-cycle arrest. Bottom, summary of the results showing the induction of mitotic arrested cells in percent after treatment with MEPL.

FACScan® flow cytometer (Becton Dickinson, San Jose, USA).

2.6. Immunohistochemical staining (IHC)

Sections of surgical specimens were re-evaluated by a gynecological pathologist (SDE). Histological subtyping was performed according to the current WHO Classification of Tumors of the Female Reproductive Organs [19]. All immunohistochemical staining were performed using a Ventana Benchmark XT device (Ventana Medical Systems, Inc., Tucson, AZ, USA). 3,3'-diaminobenzidine peroxide (DAB) was used as a chromogen. Antibodies and dilutions were used as follows: p53 (1:50, Dako, Glostrup, Denmark), p16 (1:12, Ventana Medical Systems Inc., Tucson, AZ, USA), EpCAM (1:200, Thermo Scientific Inc., Waltham, MA USA), PAX8 (1:100, Proteintech Inc., Rosemont, IL, USA) and HNF1 beta (1:500, Sigma-Aldrich Chemie GmbH, Munich, Germany).

2.7. Confocal microscopy

Primary ovarian cancer cells were grown on cover slips and fixed with 2 % paraformaldehyde/PBS. Cells were permeabilized with 0.5 % Triton X-100, blocked and stained with antibodies against PAX8 (1:50, Proteintech, 10336-1-AP), EpCam (1:300, Cell signaling, UK), p53 (1:250, DO-7 Merck Millipore, Darmstadt, Germany), β-catenin (1:300, Sigma-Aldrich, Darmstadt, Germany) followed by a secondary antibody coupled to Alexafluor 488 (1:200, Molecular Probes/ MoBiTech) and Cy3 (1:300, Dianova, 711-166-152), respectively. Confocal images were recorded using a Leica TCS SP8 confocal microscope.

2.8. Capture of the targeted disease-related genome and Next-Generation Sequencing

A Sure SelectXT Automation Custom Capture Library (Agilent) target enrichment panel was designed. The enrichment panel comprised all coding exons of 121 genes associated with ovarian cancer (Supplementary Table 1). Capture was performed according to the

manufacturer's instructions using an NGS Workstation Option B (Agilent) for automated library preparation starting with 3 µg DNA per sample. Sequence reads were mapped to the haploid human reference genome (hg19) using BWA. Single nucleotide variants (SNVs) and short insertions and deletions (indels) were called using GATK [20]. Variant annotation was performed using Jannovar [21].

2.9. Measurement of cytokines by ELISA

Cells were plated at 3×10^5 cells / well, and cytokine concentrations were measured in cell culture supernatants after 48 h using Quantikine® ELISA kits (R&D Systems).

2.10. Western blotting analysis

Cell extract (15 µg) was run on a 10 % SDS acrylamide gel and transferred to a nylon membrane. The membrane was blocked for 1 h (4 °C in PBS with 0.1 % Tween and 10 % milk powder) and probed overnight using cleaved PARP (5625, Cell Signaling, UK), phospho-Akt473 (9271, Cell Signaling, UK), Akt-473 (4685, Cell Signaling, UK), phospho-ERK (9101, Cell Signaling, UK) and ERK (4659, Cell Signaling, UK) specific antibodies, respectively.

A horseradish peroxidase-conjugated secondary antibody was used for detection (1:2,000) dilution at RT for 1 h Protein concentration equivalence was confirmed by anti-β-actin antibody.

2.11. Statistical analysis

All experiments were repeated three times and the statistical analysis was evaluated using unpaired *t*-test with Welch correction (GraphPad Prism version 4 Software, San Diego, CA). The level of significance was accepted at $P < 0.05$.

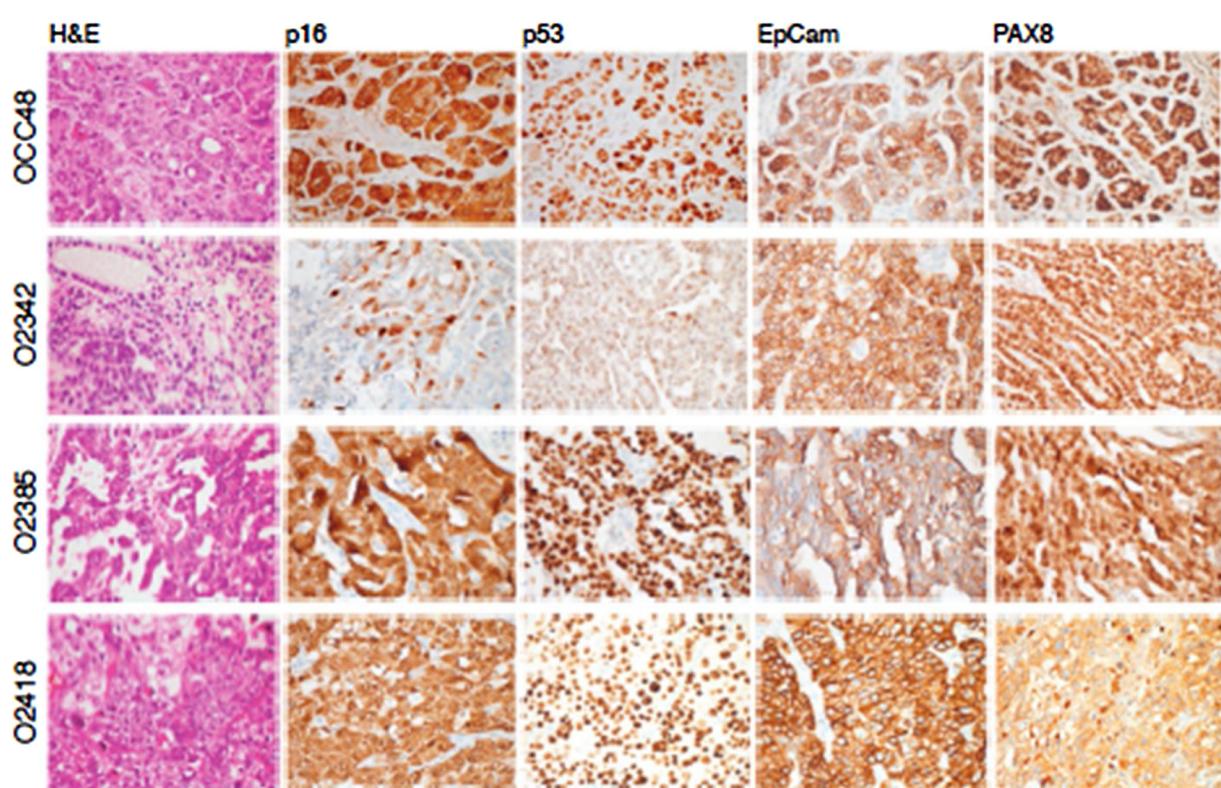


Fig. 2. Example of the pathological assessment of the disease by immunohistochemical analysis on tumor sections.

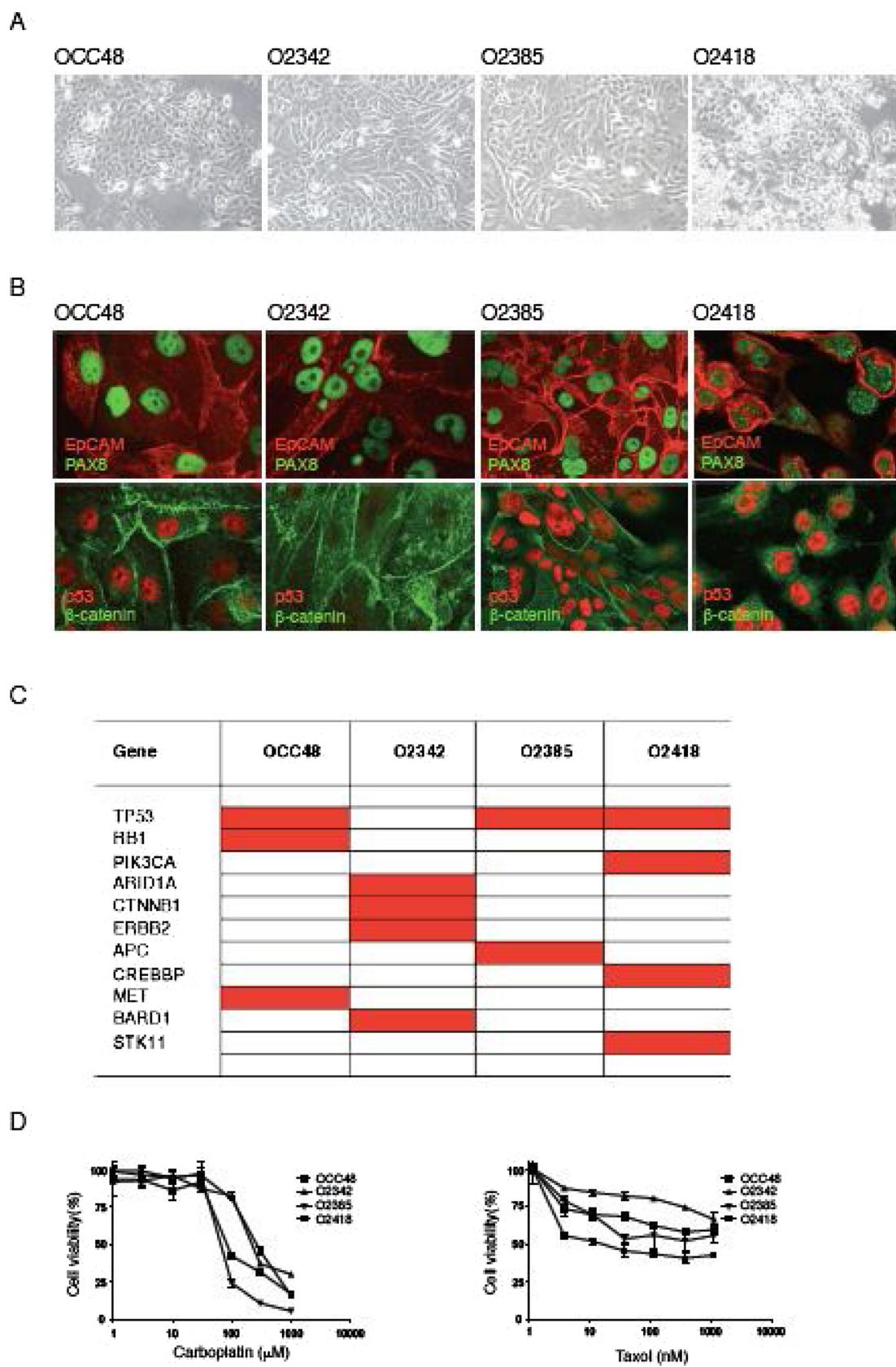


Fig. 3. Characterization of primary ovarian cancer cell lines derived from ascites. A) Morphology of cells by bright field microscopy. B) Immunofluorescence staining of epithelial marker EpCAM and β -catenin as well as transcription factors p53 and PAX8 in primary ovarian cancer cells. C) Summary of gene mutations (in red) identified by Next-Generation Sequencing of 121 target genes commonly mutated in ovarian cancer. D) Cell viability of 4 primary cell lines after 72 h treatment with various concentrations of carboplatin and paclitaxel, respectively.

3. Results

3.1. Methanolic extracts of *P. lentiscus* (MEPL) exhibit cytotoxic effect in ovarian cancer cells: A2780 and SKOV3

Previous studies showed the presence of flavonoids, phenolic acids, and tannins in the leaves of *P. lentiscus L* exhibiting anti-proliferative activity against solid cancers (breast, melanoma and colon) [17,18]. In order to evaluate the anti-proliferative activity of methanolic, ethanolic and acetonic leaves extracts of *P. lentiscus L* in ovarian cancer cells, Cells viability assays were performed using two commercially available cell lines A2780 and SKOV3. Treatment of A2780 cells showed reduction of proliferation over a period of 72 h in response to all extracts used but only the methanolic extracts of *P. lentiscus L* (MEPL) exhibited significant anti-proliferative activity in both A2780 and SKOV3 cells with IC₅₀ values 10 and 18 µg/mL respectively (A). Therefore MEPL was selected for follow-up experimental analysis in more detail.

3.2. Cell cycle analysis and apoptosis

In order to explore the underlying mechanisms of the anti-proliferative activity, the effect on apoptosis and cell cycle arrest was assessed by flow cytometric analysis. Annexin V and PI expression by cells after 24 and 48 h treatment with 10 and 25 µg/mL MEPL was determined. Results showed induction in early and late apoptosis in both ovarian cell lines with greater potency in A2780 compared with SKOV3 cells (Fig. 1B), in line with the results of the cytotoxic assays.

Furthermore, the effect of MEPL on cell cycle arrest was assessed under the same treatment conditions of time and concentrations. As shown in Fig. 1C, cell cycle arrest was induced in phase G1 in both cell lines after 24 and 48 h treatment. Therefore, we noticed an increase in the G1 population after 24 and 48 h treatment with 10 and 25 µg/mL of MEPL. We detected increases in the expression of sub-G1 indicating an increase in cell death, which is consistent with the apoptosis results (Fig. 1B).

3.3. Establishment of patient derived cell lines

Utility of SKOV3 and A2780 cell lines as *in vitro* models for HGSOC has been recently disputed [22,23] since their genetic background and long time in culture greatly differs from the profile observed in tumors from HGSOC patients. Therefore we established four primary ovarian cancer cell lines derived from ascites of EOC patients. All patients were chemotherapy naïve. Median age at diagnosis was 57 years. Three primary tumors (OCC48, O2385, and O2418) were characterized as HGSOC and one (O2342) as CCC. All patients were diagnosed in advanced stage. After receiving platinum based chemotherapy, follow-up and response data were gathered. One of the patients (O2342), was intermediary platinum sensitive, but developed resistance after second line platinum based chemotherapy. Further patients' characteristics are presented in Supplementary Table 2.

3.4. Immunohistochemical characterization of the primary tumors

Immunohistochemical analysis of primary tumors based on EpCAM, p16, p53 and PAX8 is shown in Fig. 2. All tumors proved to be EpCAM positive confirming their epithelial origin, in line with the expected phenotype of EOC (Fig. 2). The morphology of OCC48 and O2385 tumors was rather typical for HGSOC with a papillary-solid growth pattern. Tumor cells showed a diffuse nuclear and cytoplasmic expression of p16 and a p53 mutational pattern, characterized by strong over expression in the majority of nuclei. EpCAM showed membranous expression in most cells. A strong PAX8 staining was evident in tumor cell nuclei confirming Müllerian origin of the samples.

On the other hand, O2342 was identified as a clear cell adenocarcinoma (CCC) with a tubule-cystic and papillary growth pattern.

Most cells belonging to this cell line showed characteristic features such as a clear cytoplasm and distinctive cell membranes. P16 was focally expressed in a patchy pattern (though negative) and p53 showed a variable staining intensity suggestive of a wild type status. EpCAM was strongly expressed on cell membranes and PAX8 showed a diffuse expression in tumor cell nuclei. Further characterization of clear cell carcinoma was carried out by immunohistochemical assessment of frequently expressed transcription factor HNF1β, for which strong nuclear staining was eminent throughout the whole tumor section (Supplementary Fig. 1).

Furthermore, O2418 cell line showed ubiquitous solid growth of rather large tumor cells with big nuclei that were either hyper chromatic or clear with eosinophilic nucleoli. Mitotic rate was very high, frequent apoptotic bodies were seen. No glandular or papillary pattern was seen. Its morphology was qualified as an undifferentiated carcinoma. Occasionally, squamous metaplasia was noted suggesting that this might be related to the poorly differentiated endometrioid subtype. P16 was expressed diffusely while p53 showed a mutational staining pattern. Strong EpCAM expression was visible, in contrast to only a faint cytoplasmic nuclear PAX8 expression was seen.

3.5. Characterization of primary patient derived cell lines by confocal microscopy

Fibroblasts were reduced and finally eliminated by repeating selective trypsinization, until pure tumor cell cultures were obtained and passaged more than 10 times. The unique identity and purity of the cell populations were verified using STR profile analysis. All cell lines had a polymorphic appearance and high nucleus to cytoplasm ratio. Three cell lines (OCC48, O2342 and O2385) grew as adherent, epithelial-like cells in monolayer (Fig. 3A). On the other hand, O2418 did not develop fully adherent cell aggregates, which appeared as light-colored/bright masses (Fig. 3A).

Detailed characterization of the primary cell cultures using confocal microscopy revealed high expression of epithelial markers EpCAM and β-catenin in the cell membrane and positive PAX8 staining restricted to the nucleus in OCC48, O2342 and O2385 cells. Regarding O2418 cell line, the rather faint and diffuse nuclear PAX8 expression confirms the results obtained by IHC on the corresponding tumor sections. We further observed a strong p53 expression pattern in all three primary cell lines of high-grade serous origin. No nuclear signal was observed in primary ovarian clear cell line O2342. Primary cell lines from OCC48, O2385 and O2418 are derived from type II tumors characterized by: HGSOC, advanced stage, genetically unstable and high frequency of TP53 mutations.

Furthermore, the cell line O2418 harbored a pathogenic mutation, c.3140A > G, in PIK3CA. Type I tumors, including low-grade serous, low-grade endometrioid, clear cell, mucinous carcinomas and Brenner tumors, are characterized by gene mutations, in specific signaling pathways mainly KRAS, BRAF, ERBB2, CTNNB1, PTEN PIK3CA, ARID1A, and PPPR1A. In particular O2342 cells carried a characteristic mutational profile of clear cell carcinomas with mutations in ARID1A, CTNNB1, ERBB2 and wild type p53 status.

The full detailed sequencing information is presented in Supplementary Table 3.

3.6. Chemosensitivity in primary ovarian cancer cells *in vitro*

All novel cell lines were responsive to carboplatin with OCC48 and O2385 cells being more sensitive than O2342 and O2418 cells (Fig. 3D). Furthermore, Taxol had shown considerable efficiency (IC₅₀ < 100 µM) on all three HGSOC cell lines whereas the CCC cell line O2342 was highly resistant (IC₅₀ > 1000 nM) (Fig. 3D).

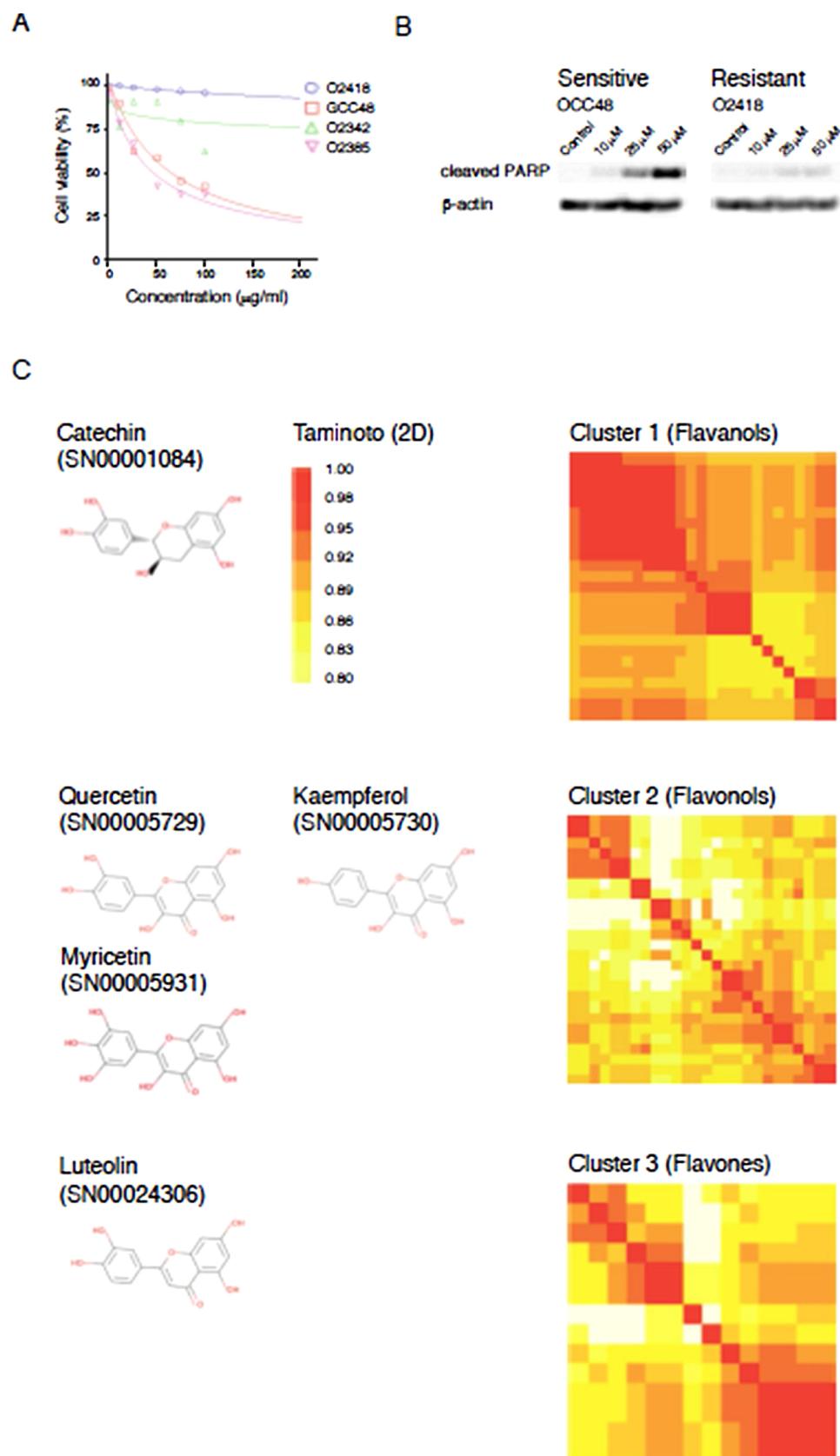


Fig. 4. Biological activity of MEPL in primary ovarian cancer cell lines A) Proliferative activity in four primary ovarian cancer cell lines was determined after 72 h treatment with MEPL using the WST-1 assay. B) Apoptotic activity was assessed using cleaved-PARP as a marker of apoptosis by Western blot analysis in two representative HGS ovarian cancer cell lines after 48 h treatment. C) Chemical structure of biological active compounds in MEPL and their cluster based on structural similarity using the Supernatural II database (Taminoto coefficient, 2D similarity).

3.7. Anti-proliferative action of MEPL by induction of apoptosis in primary ovarian cancer cells in vitro

To evaluate the anti-proliferative effect of MEPL, cell viability assays (WST-1) were performed with different concentrations of the extract over a period of 72 h treatment. The results demonstrated that MEPL reduced proliferation in two HGSOC cell lines (OCC48 and O2385), in a dose-dependent manner with IC₅₀ at 45 µg/mL and 50 µg/mL, respectively (Fig. 4A), but showed no cytotoxic activity on the third HGSOC cell line (O2418). Treatment of the clear cells derived from patient O2342 showed a modest reduction on proliferation when treated with MEPL.

Western blot analysis of cleaved PARP, as a marker of apoptosis was used to determine the apoptotic activity of MEPL in two HGSOC cell lines. The results demonstrate an induction of apoptosis in MEPL-sensitive OCC48 cells after treatment with MEPL in a dose-dependent manner in contrast with MEPL-resistant O2418 cells (Fig. 4B). These findings are in line with the observed anti-proliferative effect of MEPL in these HGSOC cell lines.

3.8. Identification of mechanism of action using the Super Natural II database

Super Natural II is a freely available web-based database of natural products (NPs) (<http://bioinformatics.charite.de/supernatural>) [24]. It contains information on over 300.000 natural compounds regarding their chemical structures, and their known biological activities including known mechanism of action (MoA) and associated pathway information. We previously have been shown by HPLC and RMN that MEPL contains flavonoids like catechin, quercetin, myricetin, kaempferol and luteolin [18,25]. Based on the database of Super Natural II, these flavonoids are natural active substances with anti-inflammatory properties.

In order to investigate the MoA of MEPL in ovarian cancer cells, we used the Super Natural II database to identify similar natural active compounds with known mechanistic biological processes and pathways. Similar compounds were selected by cluster analysis based on structural similarity using Taminoto coefficient with high similarity (Taminoto 2D = 1) (Fig. 4C) such as Rhamnini (SN00006007), datin (SN00271788) and Bois d'arc (SN00006042). A summary of these compounds according to the database and some of their known effects are shown in Table 1. The identified biological processes included cell cycle arrest, apoptosis and cytokine–receptor interaction with effects on oncogenic pathways like p53, PI3K/AKT and MAPK signaling pathways. Furthermore, the reported anti-inflammatory properties in

previous studies could be confirmed and the impact on TNF and VEGF signaling pathways were identified in this analysis.

3.9. Anti-cancer effects of MEPL on PI3K/AKT and MAPK signaling pathways

To validate the effects of MEPL on oncogenic PI3K/AKT and MAPK signaling pathways, the phosphorylation status of their known substrates (AKT-Ser473 and ERK1/2-Thr202/Tyr204, respectively) was monitored by Western blot analysis. As shown in Fig. 5A, a decrease in PI3K/AKT activity was observed in high-grade serous cells OCC48 and O2418 after treatment with MEPL. However, the effect was more predominant in MEPL sensitive OCC48 compared with O2418 cells. In cells derived from O2418 higher constitutive AKT activity was detected corresponding to the existence of a pathogenic PIK3CA mutation. The opposite effect on MAPK signaling pathway was found between sensitive and resistant cells in the same experimental setting (Fig. 5A).

In order to confirm and extend these findings, we sought to determine the impact of MEPL on cytokine–receptor interaction regarding, TNF and VEGF signaling. The constitutive release of cytokines like TNF and IL6, and the growth factor VEGF was measured in primary cell culture supernatants after treatment with the compound using ELISA. IL6 release was significantly reduced in two of the primary ovarian cancer cell lines (Fig. 5B). We also observed a significant decrease of amounts of VEGF levels in the supernatant of all cell lines after treatment with MEPL.

3.10. MEPL enhances platinum-based chemosensitivity in high-grade serous cells

Since MEPL inhibits the activation of the PI3K/AKT signaling pathways, a major pathway involved in chemoresistance [26]. We hypothesized that pretreatment of the primary cells with MEPL would sensitize them to DNA-targeted anticancer drugs such as carboplatin. Therefore primary ovarian cancer cells were incubated with 10 µg/mL of MEPL, 10 µM carboplatin or in combination. Cell viability was assessed by cell counts after 72 h of treatment. A significant increase in response to carboplatin was seen in OCC48 and O2385 cells in this experimental setting (Fig. 5C). Nevertheless, no difference impact was noticed on cell viability in O2342 and O2418 cell lines. However, combined pre-treatment of HGSOC cells with MEPL and the higher concentration of carboplatin over a longer period of time (data not shown) showed a significant increase in chemosensitivity. The accentuated chemosensitivity was reflected by a high constitutive active phospho-AKT, which modulates the function of numerous substrates

Table 1

Summary of similar compounds to natural active substances in MEPL. Listed are the compounds identified in MEPL and their similar compounds according to the Super Natural II database (<http://bioinformatics.charite.de/supernatural>) with some of their known effects.

Classification	Compounds	Super natural ID	Similar Compounds	Mechanism of action (MoA)
Flavanols	Catechin	SN00001084	SN00402291 SN00345333 SN00340500 SN00324452	PI3K/AKT signaling pathway MAPK signaling pathway VEGF signaling pathways TNF signaling pathway
Flavonols	Quercetin	SN00005729	SN00335321 SN00373928 SN00003317	Apoptosis Cell cycle
	Myricetin	SN00005931	SN00013625 SN00013637 SN00005931	P53 signaling pathways PI3K/AKT signaling pathway MAPK signaling pathway
	Kaempferol	SN00005730	SN00307254 SN00013638 SN00014144	Cytokine–cytokine interaction VEGF signaling pathways TNF signaling pathway
Flavones	Luteolin	SN00024306	SN00006979 SN00013712 SN00389159 SN00356527	Cell cycle PI3K/AKT signaling pathway VEGF signaling pathways

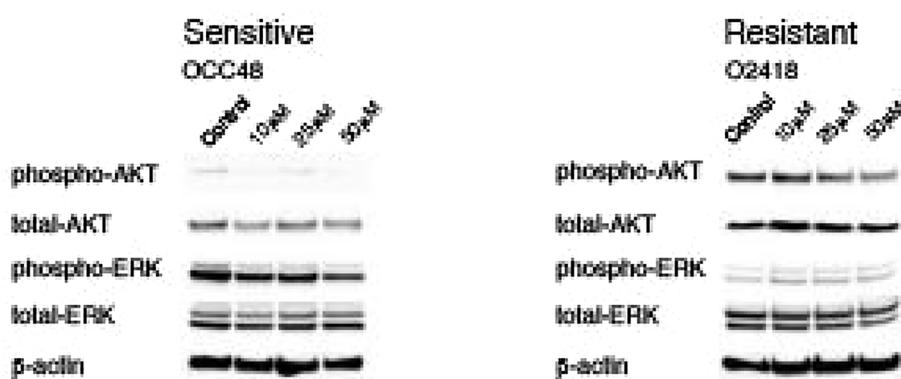
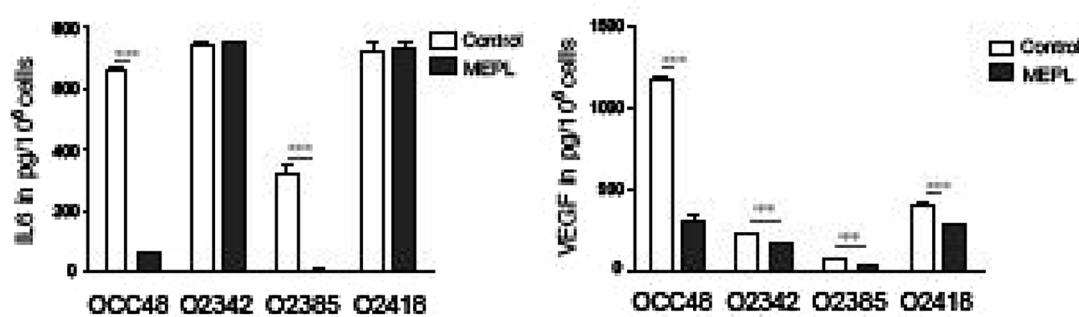
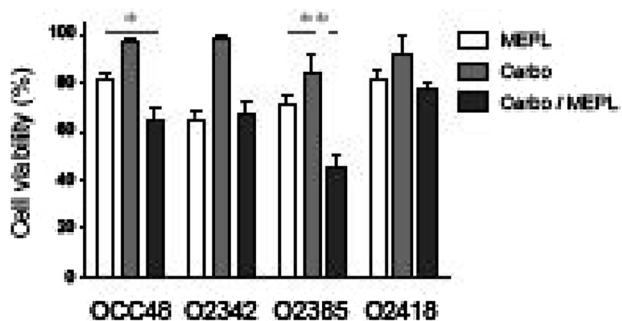
A**B****C**

Fig. 5. Effects of MEPL on oncogenic pathways, cytokine expression and chemosensitivity A) Western blot analysis of PI3K/AKT and MAPK signaling pathways by monitoring phospho-AKT and phospho-ERK activity after 48 h treatment with MEPL in two representative primary HGS cell lines. B) Interleukin 6 and growth factor expression levels were measured in cell culture supernatants after 48 treatment with 50 µg/mL of MEPL (mean ± SEM, ***, P < 0.001, **, P < 0.01). C) Effect of MEPL on chemosensitivity to carboplatin after treatment with 10 µg/mL of MEPL, 10 µM of carboplatin or in combination. Cell viability was assessed by cell counts after 72 h treatment, respectively (mean ± SEM, **, P < 0.01, *, P < 0.05).

involved in the regulation of cell survival, cell cycle progression and cellular growth. This has not been observed in the clear cell line O2342, which exhibited resistance to MEPL, as mentioned earlier.

4. Discussion

Phytochemicals, including flavonoids, present in many plants have received much attention in recent years due to their health benefits, including antioxidant, anti-inflammatory and cytotoxic activities [27]. In our study, we investigated the anti-cancer effects and underlying molecular mechanism of *P. lentiscus L.* on HGSOC cell lines. The different extracts have been obtained from dry leaves of *P. lentiscus L.*, using organic solvents methanol, acetone and ethanol. The yield of extraction of phenolic compounds depends on chemical and physical parameters, method and duration of extraction, solvents polarity to dissolve phenolic compounds and vegetal matrix. There is no standard method for extraction of biological active compounds. To date, reports showed that no difference was found in concentrations of total phenolic compounds in mangosteen peel (*Gacinia mangostana L.*) using different organic solvent methanol, ethanol or acetone for extraction [28].

Seventy-five percent of EOC are of the high-grade serous type. Furthermore, HGSOC is the most aggressive, making this subtype a very important subject of research. Experimental models play an important role to study the cellular and molecular mechanisms of different histological types of ovarian cancers and their response to therapeutic approaches. However, recent studies questioned the established cell lines as good models of HGSOC and as such many therapeutic compounds, even with promising results in preclinical studies, often failed in the clinical setting. Therefore, models with defined pathological indications for preclinical studies are required and primary cell lines with well-annotated clinical data representing the histological and genetic heterogeneity of the tumor are more accurate. In that context, we established a new cell lines from ascites of patients with primary EOC which would better reproduce the clinical reality. Three cell lines were derived from patients with HGSOC and one from a patient with a clear cell carcinoma. All cell lines were established before chemotherapy, HGSOC cell lines were harboring a unique *TP53* mutation corresponding to that of the original tumor, whereas CCC cells were *p53* wild-type. Furthermore, we detected mutations in *ARID1A*, *CTNNB1*, and *ERBB2* in clear cell ovarian cancer cells, according to published data [29]. The present findings indicate that MEPL induces apoptosis, inhibits proliferation and increases the sensitivity to carboplatin based chemotherapy in high-grade serous ovarian cancer cells lines, but its effect depends on the genetic background of the tumor. In the study published by Mingxin Ren et al., 2015 [30], it was reported that flavonoids compounds such as quercetin inhibited proliferation and induced apoptosis in ovarian cancer SKOV-3 cells. In the present study, MEPL induces apoptosis and inhibits angiogenesis in HGSOC through inhibition of VEGF expression, which could be also explained by the presence of Kaempferol as flavone compound. Kaempferol has been reported to inhibit VEGF expression and *in vitro* angiogenesis in EOC cells lines A2780 and SKOV3 through a novel ERK-NF κ B-cMyc-p21 pathway. Myricetin present in MEPL is abundant in nature and can be found in walnuts, vegetables and fruits. It has been reported that it induces cell death of human cancer colon cells via a BAX/BCL2-dependent pathway, upregulates the level of GRP78 and CHOP in a time dependent manner, and induces ER stress-associated apoptosis in SKOV3 cells [31]. Myricetin treatment was revealed to inhibit viability and induces apoptosis through ER stress and DNA DSBs in SKOV3 human ovarian cancer cells [32]. Yuanda et al., 2015 reported that luteolin inhibits cell migration and invasion and suppresses the expression of two matrix metalloproteinases (MMPs), MMP-2 and MMP-9 in ovarian cancer cell line ES-2 [33,34]. Moreover, Luteolin has been shown to have anti-inflammatory properties with effects on constitutive cytokine expression levels in the malignant cells. We have shown recently how these cytokines have paracrine actions on angiogenesis and

the immune cell infiltrate in both murine xenograft models and HGSOC, with an impact on overall survival [34–38]. Hence, we can assume that the anti-tumor activity of MEPL will not only be due to the pro-apoptotic effect on the malignant EOC cells, but also through the inhibition of the tumor promoting interaction within the tumor microenvironment. Currently, the combination of carboplatin and Taxol is the standard first line therapy for primary EOC. In our study, treatment with MEPL in combination with carboplatin increased the sensitivity to chemotherapy in our primary HGSOC cell lines and the effect was the strongest in cell lines where IL6 levels were significantly inhibited [39,40]. To date, IL6 and the downstream STAT3 signaling pathway have been shown to mediate chemoresistance in cancer cells [41,42].

There are potential limitations of our current study which should be considered. We tested the anti-cancer effects of the leaves of *P. lentiscus L.* in a small number of primary cell lines generated from ascites of patients with HGSOC. Studies *in vivo* are needed in primary cell lines of patients with HGSOC and mice model with HGSOC to test the effect of the phenolic components of the leaves of *P. lentiscus* (MEPL) determined by HPLC and H-NMR in combination with chemotherapy.

5. Conclusion

In the present study, we demonstrated for the first time the anti-cancer effects of the leaves of *P. lentiscus L.* in newly established primary high-grade serous ovarian cancer cells. We have established four primary cell lines, from ascites of patients with HGSOC *in vitro*. Our present data showed that MEPL appeared to increase the sensitivity in the DNA damaging drug in combination with chemotherapy through inhibition of the PI3K/AKT and MAPK/ERK oncogenic signaling pathways and the effect was the strongest in cell lines where IL6 levels were significantly inhibited. MEPL might be a promising novel therapeutic approach in HGSOC patients to enhance platinum response. Further studies *in vivo* are needed to determine the effects of MEPL in combination with chemotherapy in mice model with high-grade serous ovarian cancer.

Authorship contributions

I.C., F.C. and H.K. designed research; **M.K., S.D-E., T.Z.** characterized new cell lines; **I.C., S.A., S.S., S.T., and H.K.** performed research; **D.A-K., N.B-D., and A.D.** collected, characterized and contributed the plant extracts; **I.C., S.A., S.S., and H.K.** analyzed data; **R.S. and D.CC-T.** helped us setting up some crucial experiments; **F.A** provided intellectual input, advice on experiments and helped with manuscript editing; **J.S. and E.I.B.** provided patient samples, patient information and intellectual input; and **I.C., M.K., S.A., and H.K.** wrote the paper. **I.C., F.C., E.I.B., and H.K.** writing review and editing.

Funding

This work was supported by grants from European Community's Seventh European program under grant agreement No. 279113-2 and The Algerian Ministry of Higher Education and Scientific Research through a scholarship for I.Charid (PNE 2015/2016, Program National Exceptional for training abroad). El.Braicu is a participant in the Charité Clinical Scientist Program funded by the Charité Universitätsmedizin Berlin and the Berlin Institute of Health (BIH). S.Tuyaert is financially supported by the Anticancer Fund (www.anticancerfund.org) and F.Amant is a senior researcher for the Researcher Fund Flanders (F.W.O).

Declaration of Competing Interest

The authors declare no conflict of interest

Acknowledgements

We would like to thank the patients for participation in this study.

References

- [1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, et al., Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, *Int. J. Cancer* 136 (5) (2015) E359–86.
- [2] W.G. McCluggage, Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis, *Pathology* 43 (5) (2011) 420–432.
- [3] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2015, *CA Cancer J. Clin.* 65 (1) (2015) 5–29.
- [4] C.G.A.R. Network, D. Bell, A. Berchuck, M. Birrer, J. Chien, D.W. Cramer, et al., Integrated genomic analyses of ovarian carcinoma, *Nature* 474 (2011) 609–615.
- [5] P.M. Webb, S.J. Jordan, Epidemiology of epithelial ovarian cancer, *Best Pract. Res. Clin. Obstet. Gynaecol.* 41 (2017) 3–14.
- [6] B. Trabert, R.B. Ness, W.H. Lo-Ciganic, M.A. Murphy, E.L. Goode, E.M. Poole, L.A. Brinton, et al., Aspirin, nonaspirin nonsteroidal anti-inflammatory drug, and acetaminophen use and risk of invasive epithelial ovarian cancer: a pooled analysis in the Ovarian Cancer Association Consortium, *JNCI* 106 (2) (2014) djt431.
- [7] L. Bourrine, S. Bensalem, P. Peixoto, A. onzalez, F. Maiza-BenabdessaM, F. Bedjou, et al., Evaluation of the cytotoxic and cytostatic activities of alkaloid extracts from different parts of *Peganum harmala L.* (Zygophyllaceae), *Eur. J. Integr. Med.* 9 (2017) 91–96.
- [8] E. Ben-Arye, E. Lev, E. Schiff, Complementary medicine oncology research in the Middle-East: shifting from traditional to integrative cancer care, *Eur. J. Integr. Med.* 3 (1) (2011) 29–37.
- [9] O. Ait-Mohamed, V. Battisti, V. Joliot, L. Fritsch, J. Pontis, S. Medjkane, et al., Acetonic extract of *Buxus sempervirens* induces cell cycle arrest, apoptosis and autophagy in breast cancer cells, *PLoS One* 6 (9) (2011) e24537.
- [10] L. Bourrine, S. Bensalem, P. Peixoto, A. Gonzalez, F. Maiza-BenabdessaM, F. Bedjou, J.N. Wauters, M. Tits, M. Frédéric, V. Castronovo, A. Bellahcène, Revealing the anti-tumoral effect of Algerian Glaucium flavum roots against human cancer cells, *Phytomedicine* 20 (13) (2013) 1211–1218.
- [11] X. Hua, L. Yu, R. You, Y. Yang, J. Liao, D. Chen, L. Yu, Association among dietary flavonoids, flavonoid subclasses and ovarian cancer risk: a meta-analysis, *PLoS One* 11 (3) (2016) 1–15.
- [12] P.R. Dandawate, D. Subramaniam, P.B. Padhye, S. Anant, Bitter melon: a panacea for inflammation and cancer, *Chin. J. Nat. Med.* 14 (2) (2016) 81–10013.
- [13] M. Shi, Q. Cai, L. Yao, Y. Mao, Y. Ming, Antiproliferation and apoptosis induced by curcumin in human ovarian cancer cells, *Cell Biol. Int.* 30 (3) (2006) 221–226.
- [14] V. Haghshenas, S. Fakhari, S. Mirzaie, M. Rahmani, F. Farhadifar, S. Pirzadeh, A. Jalili, Glycyrrhetic acid inhibits cell growth and induces apoptosis in ovarian cancer a2780 cells, *Adv. Pharm. Bull.* 4 (Suppl 1) (2014) 437.
- [15] D. Atmani, N. Chaher, M. Barboucha, K. Ayouni, H. Lounis, H. Boudaoud, et al., Antioxidant capacity and phenol content of selected Algerian medicinal plants, *Food Chem.* 112 (2009) 303–309.
- [16] C. Mehenni, D. Atmani-Kilani, S. Daumarçay, D. Perrin, P. Gérardin, D. Atmani, Hepatoprotective and antidiabetic effects of *Pistacia lentiscus* leaf and fruit extracts, *J. Food Drug Anal.* 24 (3) (2016) 653–669.
- [17] S. Remila, D. Atmani-Kilani, S. Delemasure, J.L. Connat, L. Azib, T. Richard, et al., Antioxidant, cytoprotective, anti-inflammatory and anticancer activities of *Pistacia lentiscus* (Anacardiaceae) leaf and fruit extracts, *Eur. J. Integr. Med.* 7 (3) (2015) 74–286.
- [18] L. Azib, N. Debbache-Benaïda, G. Da Costa, D. Atmani-Kilani, N. Saidene, K. Ayouni, et al., *Pistacia lentiscus L.* leaves extract and its major phenolic compounds reverse aluminium-induced neurotoxicity in mice, *Ind. Crops Prod.* 137 (2019) 576–58.
- [19] R.J. Kurman, M.L. Carcangi, C.S. Herrington, R.H. Young, WHO Classification of Tumours of the Female Reproductive Organs (IARC WHO Classification of Tumours), 4th ed., World Health Organization, 2014 N06.
- [20] A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, et al., Genome analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, *Genome Res.* 20 (9) (2010) 1297–1303.
- [21] M. Jäger, K. Wang, S. Bauer, D. Smedley, P. Krawitz, P.N. Robinson, Jannovar: AJava library for exome annotation, *Hum. Mutat.* 35 (5) (2014) 548–555.
- [22] S. Domcke, R. Sinha, D.A. Levine, C. Sander, N. Schultz, et al., Evaluating cell lines as tumour models by comparison of genomic profiles, *Nat. Commun.* 4 (1) (2013) 1–10.
- [23] C.M. Beaufort, J.C. Helmijr, A.M. Piskorz, M. Hoogstraat, K. Ruigrok-Ritstier, N. Bessink, Ovarian cancer cell line panel (OCCP): clinical importance of *in vitro* morphological subtypes, *PLoS One* 9 (9) (2014).
- [24] P. Banerjee, J. Erehman, B.O. Gohlke, T. Wilhelm, R. Preissner, M. Dunkel, et al., Super natural II a database of natural products, *Nucleic Acids Res.* 43 (D1) (2015) D935–D939.
- [25] C. Rodríguez-Pérez, R. Quirantes-Piné, N. Amesis-Ouchemoukh, K. Madani, A. Segura-Carretero, A. Fernández-Gutiérrez, A metabolite-profiling approach allows the identification of new compounds from *Pistacia lentiscus* leaves, *Pharm. Biomed. Anal.* 77 (2013) 167–174.
- [26] J.Q. Cheng, X. Jiang, M. Fraser, M. Li, H.C. Dan, M. Sun, M.K. Tsang, Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway, *Drug Resist. Updates* 5 (3–4) (2002) 131–146.
- [27] S.S. Kamble, R.N. Gacche, Evaluation of anti-breast cancer, anti-angiogenic and antioxidant properties of selected medicinal plants, *Eur. J. Integr. Med.* 25 (2019) 13–19.
- [28] M. Plainsirichai, N. Prasomthong, P. Bussaman, M. Wongsawas, Methanol, ethanol, and acetone result in non different concentration of total phenolic content in Mangosteen (*Garcinia mangostana L.*) peel, *J. Agric. Sci.* 7 (2) (2015) 131.
- [29] S.Y. Kwan, X. Cheng, Y.T. Tsang, J.-S. Choi, S.Y. Kwan, D.I. Izaguirre, et al., Wong, loss of ARID1A expression leads to sensitivity to ROS-inducing agents in ovarian cancer cells, *AACR* (2016).
- [30] M.X. Ren, X.H. Deng, Ai, G.Y. Yuan, H.Y. Song, Effect of quercetin on the proliferation of the human ovarian cancer cell line SKOV-3 *in vitro*, *Exp. Ther. Med.* 10 (2) (2015) 579–583.
- [31] M.E. Kim, T.K. Ha, H.J. Yoo, J.S. Lee, Myricetin induces cell death of human colon cancer cells via BAX/BCL2-dependent pathway, *Anticancer Res.* 34 (2) (2014) 701–706.
- [32] Y. Xu, Q. Xie, S. Wu, D. Yi, Y. Yu, S. Liu, et al., Myricetin induces apoptosis via endoplasmic reticulum stress and DNA double-strand breaks in human ovarian cancer cells, *Mol. Med. Rep.*, 13 (3) 2094–2100.
- [33] Y. Du, Y. Du, J. Feng, R. Wang, H. Zhang, J. Liu, Effects of flavonoids from *Potamogeton crispus* on proliferation, migration, and invasion of human ovarian cancer cells, *PLoS One* 10 (6) (2015).
- [34] C.C. Wu, C.Y. Fang, H.Y. Hsu, H.Y. Cuang, H.J. Cheng, J.Y. Chen, et al., EBV reactivation as a target of luteolin to repress NPC tumorigenesis, *Oncotarget* 7 (14) (2016) 18999.
- [35] H. Kulbe, P. Chakravarty, D.A. Leinster, K.A. Charles, J. Kwong, R.G. Thompson, et al., A dynamic inflammatory cytokine network in the human ovarian cancer microenvironment, *Cancer Res.* 72 (1) (2012) 66–75.
- [36] J. Coward, H. Kulbe, P. Chakravarty, D. Leader, V. Vassileva, D.A. Leinster, et al., Interleukin-6 as a therapeutic target in human ovarian cancer, *Clin. Cancer Res.* 17 (18) (2011) 6083–6096.
- [37] D.A. Leinster, H. Kulbe, G. Everitt, R. Thompson, M. Perretti, F.N. Gavins, et al., The peritoneal tumour microenvironment of high-grade serous ovarian cancer, *J. Pathol.* 227 (2) (2012) 136–145.
- [38] F. Barbieri, A. Bajetto, T. Florio, Role of chemokine network in the development and progression of ovarian cancer: a potential novel pharmacological target, *J. Oncol.* 2010 (2010).
- [39] D. Zhang, H.L. Piao, Y.H. Li, Q. Qiu, D.J. Li, M.R. Du, et al., Inhibition of AKT sensitizes chemoresistant ovarian cancer cells to cisplatin by abrogating S and G2/M arrest, *Exp. Mol. Pathol.* 100 (3) (2016) 506–513.
- [40] S.Y. Shin, H. Jung, S. Ahn, D. Hwang, H. Yoon, J. Hyun, et al., Polyphenols bearing cinnamaldehyde scaffold showing cell growth inhibitory effects on the cisplatin-resistant A2780/Cis ovarian cancer cells, *Bioorg. Med. Chem.* 22 (6) (2014) 1809–1820.
- [41] H. Kojima, T. Inoue, H. Kunimoto, K. Nakajima, IL-6-STAT3 signaling and premature senescence, *Jak-stat* 2 (4) (2013) e25763.
- [42] R. Gharaei, H. Akrami, S. Heidari, H. Asadi, A. Jalili, The suppression effect of *Ferula gummosa* Boiss. extracts on cell proliferation through apoptosis induction in gastric cancer cell line, *Eur. J. Integr. Med.* 5 (3) (2013) 241–247.