AIM

The aim of this research is to estimate the biological activity of olive oil samples by developing of *in vitro* assays that reconstitute the *in vivo* activity. The experiments were performed both in colorectal cancer cells (DLD-1) and in Human Umbilical Vein Endothelial Cells (HUVECs), which are primary cells.

The research was conducted in the laboratory of Professor Fotsis in FORTH-Institute of Molecular Biology and Biotechnology/ Department of Biomedical Research.

ABSTRACT

A main component of the Mediterranean Diet (MD) is Extra-Virgin Olive Oil (EVOO) which contains phenolic compounds that are associated with the beneficials effects of EVOO consumption (Garcia-Martinez *et al.*, 2018; Serra-Majem *et al.*, 2019). Epidemiological studies indicate that EVOO rich in phenols has a cardioprotective effect (Boskou, 2008; Nocella *et al.*, 2017) and lower cancer incidence (Bartolí *et al.*, 2000; Calza *et al.*, 2001; Levi *et al.*, 1999; Schwingshackl *et al.*, 2018).

Regarding the fact that most studies were performed with compounds isolated from EVOO, we wanted to exert a more direct correlation of EVOO and its effect on cell growth, we directly treated cells using EVOO. We concluded that EVOO-enriched culture medium and direct addition of EVOO on cell culture specifically inhibit cancer cell growth without inhibiting primary endothelial cell growth. In addition, for the first time EVOOs from Greece have been analyzed regarding their phenolic concentration, which gives us the opportunity to investigate whether any effect on cell growth is due to known phenolic compounds or not.

MATERIALS & METHODS

Cells culture

DLD-1 cells were cultured in 10cm dishes at 37°C, 5% CO_2 using McCoy's 5A (HyClone) medium supplemented with 10% FBS (Gibco), 100U/mL Penicillin and 100mg/mL Streptomycin (Gibco). Cells were passaged every 2 days in a ratio of 1:4. During passaging, cells were washed with 10mL PBS (PAN-biotech) and incubated with Trypsin (Gibco) for 2-3 minutes at 37°C. Thereafter, cells were dissociated, resuspended in 10mL fresh medium, and transferred to new dishes.

HUVECs were cultured in 10cm dishes using M199 medium supplemented with 20% FBS, 100U/mL Penicillin, 100mg/mL Streptomycin, 0.05mg/ml Endothelial Cell Growth Extract (ECGS) and 5U/ml heparin. Dishes were preincubated with 4mL of collagen rat type I for 20 minutes in 37°C. Thereafter, these dishes were washed 2 times with 10mL of PBS. Passaging of the cells was conducted every 3 days in a ratio of 1 to 3. During passaging, cells were washed with 10mL PBS, incubated with Trypsin for less than 1 minute. Subsequently, they were dissociated, resuspended in 10mL fresh medium, and transferred to new dishes. All nutrients were filtered for retention of insoluble particles. Cells were used up to passage 5.

EVOO was added in cells using two different methods. The first one is the EVOO enriched cell culture medium. EVOO was added in serum free medium (Plain medium) in a ratio EVOO:medium 1:25. The mixture was vortexed for 20sec and an incubation at room temperature for 5 minutes. Cells were treated with each supernatant (Sup) carefully avoiding the oily phase on top of the mixture. 1:50 ratio was prepared from 1:25 by diluting 1:2. 1:100 ratio was prepared from 1:50 by diluting 1:2.

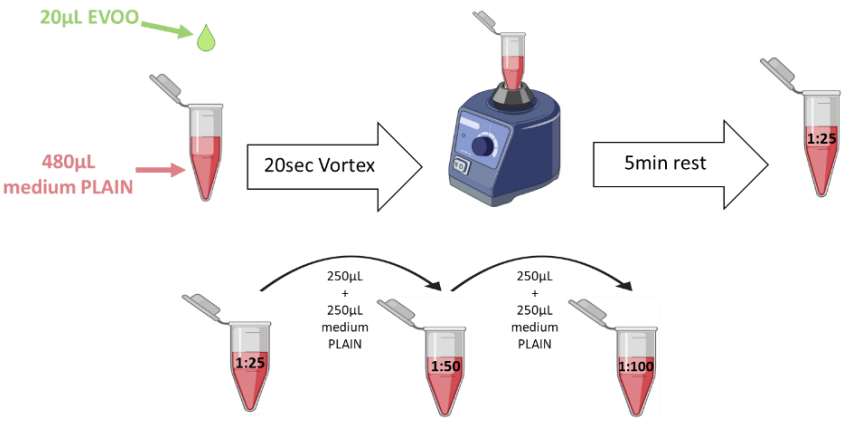


***Figure 1**: Schematic overview of production of EVOO (or Corn oil)-enriched culture medium and every dilution used in experiments.*

EVOO enriched medium contains the more hydrophilic components of EVOO, including the phenolic compounds.

The second one is the direct addition on EVOO in cells. EVOO was added directly in culture medium in a ratio of 1:25, 1:50 or 1:100 depending on the final volume of each plate, i.e. in 96-well plate, in a volume of 100ul 4ul of EVOO were added.

Cell Growth

For the growth capability of the cells, the IncuCyte® live imaging system (Sartorius) was used. IncuCyte® is a live imaging system which can be used for a variety of techniques as it can monitor cells while growing in an incubator. Parameters and analysis can be controlled through its dedicated software.

10,000 cells were seeded in a 96-well plate in duplicates or triplicates for each condition. For HUVECs, 96-well plates were coated with collagen rat type I, incubated in 37^{o}C, 5% CO_2 for 20 minutes and washed 2 times with PBS. After an overnight incubation of the cells, each treatment was performed, and the plate was placed in the IncuCyte®. Pictures were obtained every 2-4 hrs for 48 hrs using 10x lens.

Number of EVOO	12	13	14	15	16	17	18	19	20	21
EVOO	Corn Oil - No phenolics	Control Oil - No phenolics								
Variety	-	-	KORONEIKI	KORONEIKI	PIKOUAL	KORONEIKI	MANAKI	LIANOLIA-KORONEIKI	DOPIA ZAKYNTHOU	
Oleocanthal	-	-	232	318	180	180	290	217	302	376
Oleacein	-	-	231	322	132	89	107	197	267	348
Oleocanthal + Oleacein (index D1)	-	-	464	640	312	269	397	415	569	723
Listroside aglycon (monoaldehyde form)	-	-	93	83	74	69	32	132	55	58
Oleuropein aglycon (monoaldehyde form)	-	-	121	119	97	87	29	204	73	73
Listroside aglycon (dialdehyde form)	-	-	228	409	239	135	123	370	237	153
Oleuropein aglycon (dialdehyde form)	-	-	168	336	148	80	51	236	190	119
Total tyrosol derivatives	-	-	553	811	493	384	445	719	594	587
Total hydroxytyrosol derivatives	-	-	520	777	378	257	186	637	530	539
Total polyphenols analyzed	-	-	1073	1587	871	641	632	1357	1124	1126

Number of EVOO	22	23	24	25	26	27	28	29
EVOO								
Variety	KORONEIKI	MANAKI-KATSOULIERA		Chalkidikis	Lianolia-Koroneiki	Tsounati	Kalamon	Koroneiki-kypriaki
Oleocanthal	232	232		295	262	264	882	455
Oleacein	204	105		204	182	221	310	107
Oleocanthal + Oleacein (index D1)	436	337		499	444	485	1191	561
Listroside aglycon (monoaldehyde form)	65	37		83	83	179	37	25
Oleuropein aglycon (monoaldehyde form)	107	34		109	92	255	29	19
Listroside aglycon (dialdehyde form)	307	107		195	225	667	72	<5
Oleuropein aglycon (dialdehyde form)	224	48		95	148	370	51	<5
Total tyrosol derivatives	604	376		573	571	1110	990	480
Total hydroxytyrosol derivatives	535	187		408	422	846	390	126
Total polyphenols analyzed	1138	536		981	993	1956	1380	606

Table 1: Chemical analysis of each EVOO to identify phenolic compounds and their concentration

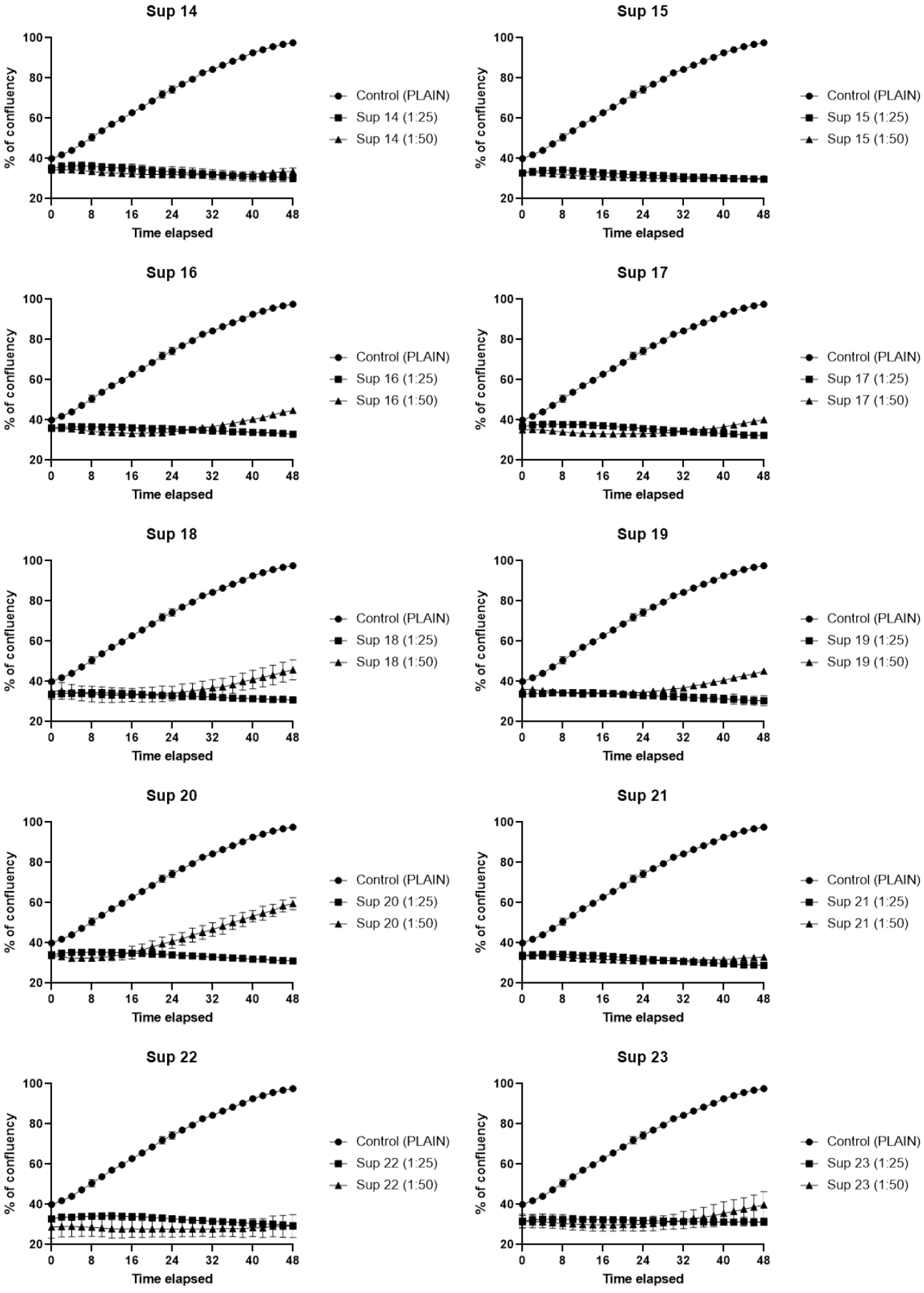
RESULTS

In order to investigate the cell growth of DLD-1 colorectal cancer cells, we treated the cells (direct or indirect) using EVOO and images were obtained every 4h using the IncuCyte. Results are shown in Figures 2 & 3.

As shown in Figure 2, EVOO-enriched culture medium strongly inhibits cell growth when dilution 1:25 was used. When cells were treated with the dilution 1:50, only EVOO 20 led to minor cell growth inhibition. Concerning the dilution 1:100, only EVOO 26 and 29 resulted in a minor inhibition in cell growth.

Corn oil and Ctl oil (EVOO poor in phenolics) had no effect in cell growth when used in the enriched culture medium.

From the experimental data we observe that the maximum inhibitory effect of EVOO in DLD-1 cancer cells is observed when cells are treated directly with EVOO (Figure 3).


Sup 14
% of confluency
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Control (PLAIN)
Sup 14 (1:25)
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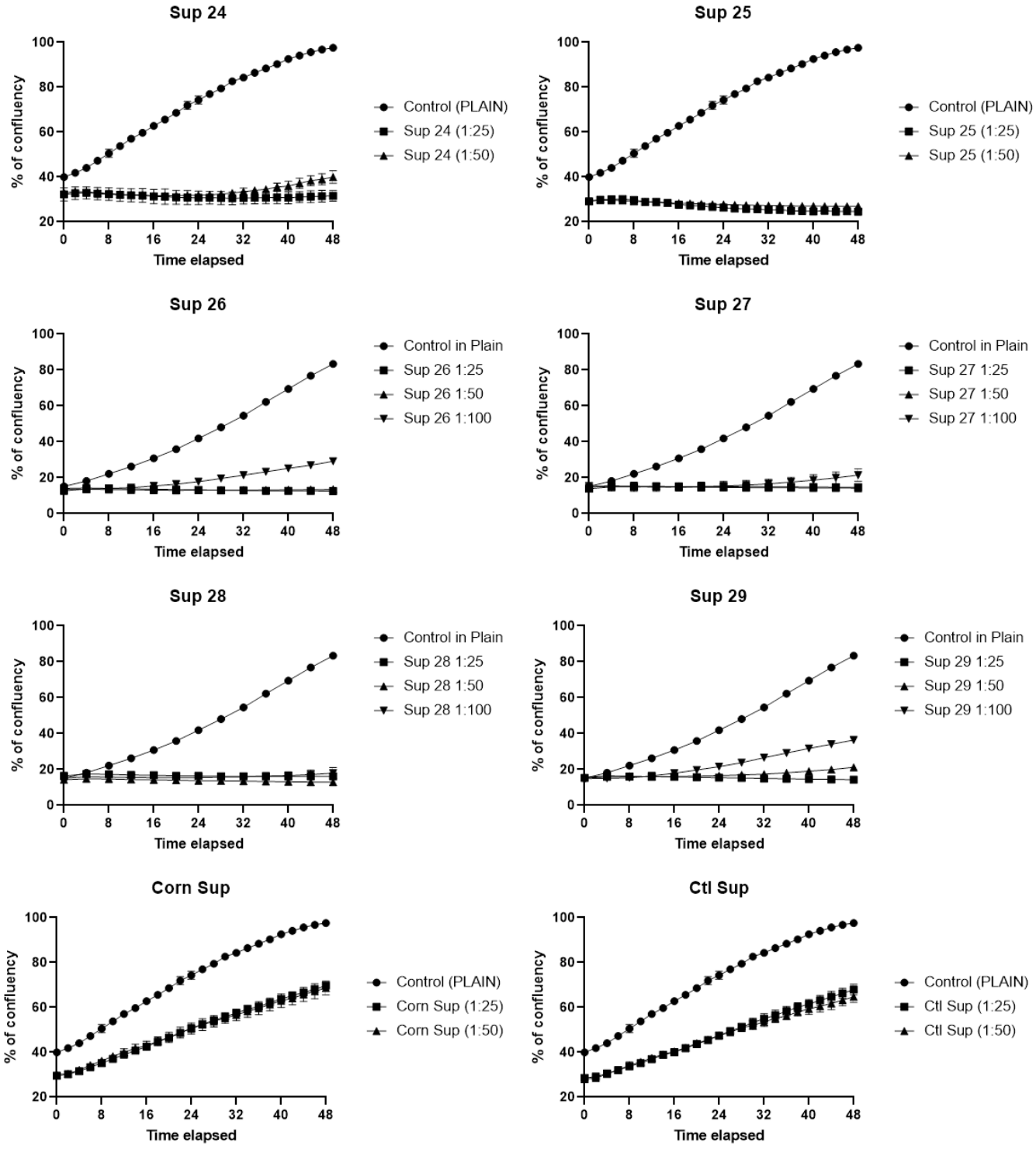
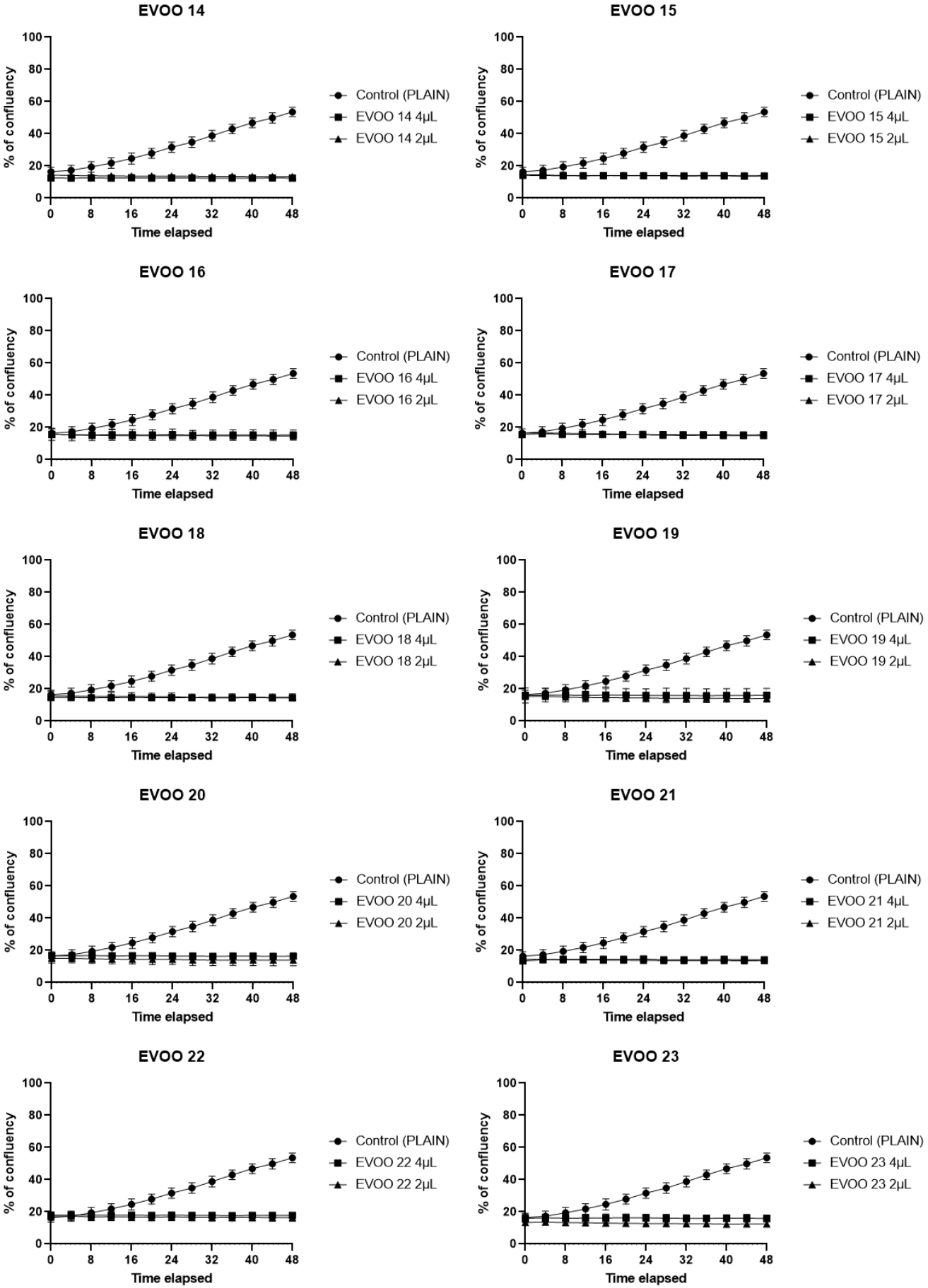


*Figure 2: **Effect of EVOO-enriched medium on DLD-1 cell growth.** DLD-1 cells were treated with the 2 different EVOO-enriched plain culture medium in 3 different ratios EVOO:medium, 1:25, 1:50 and 1:100. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software*


EVOO 14
% of confluency
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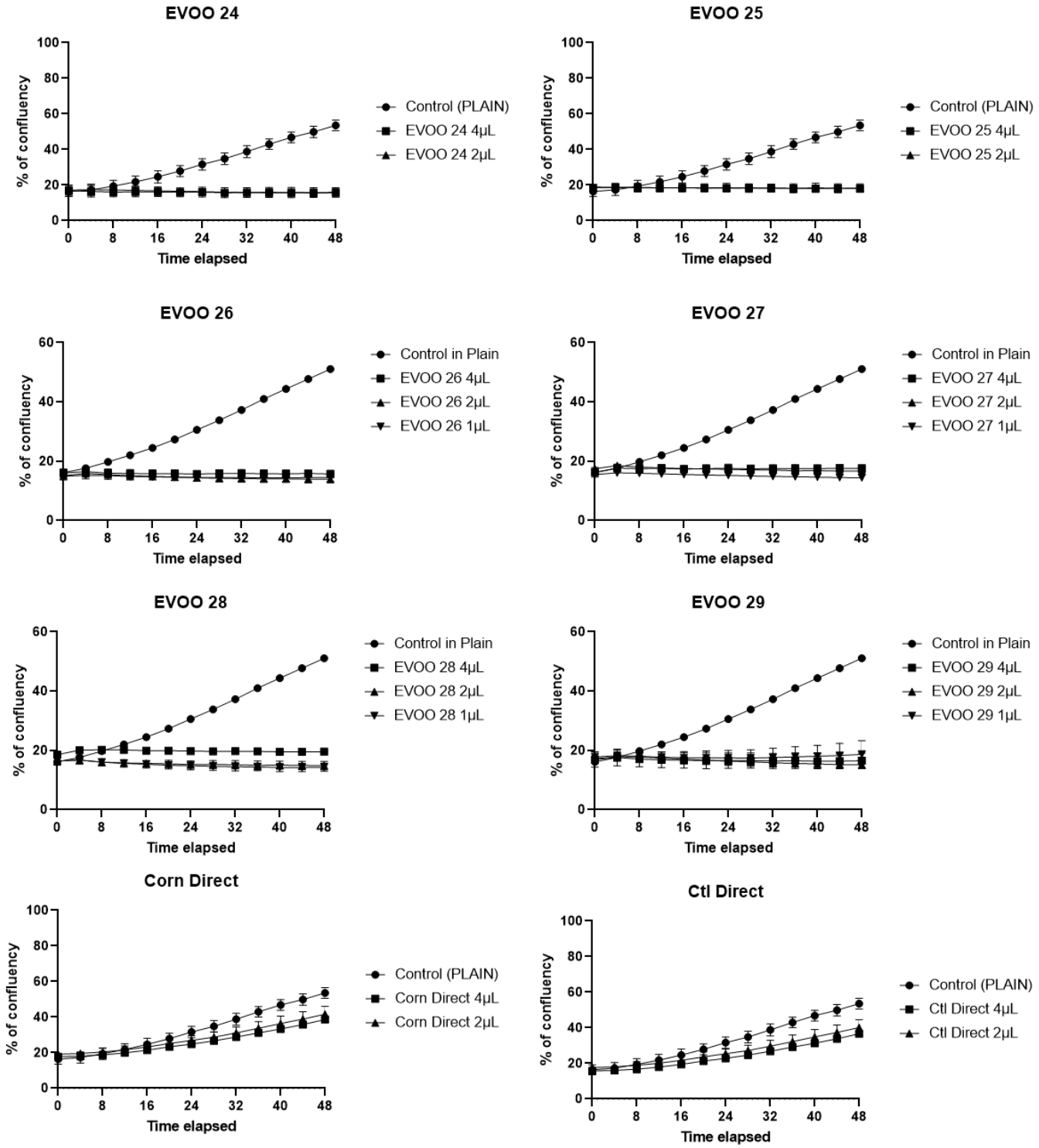

*Figure 3: **Effect of EVOOs on DLD-1 cell growth after direct addition in culture medium.** DLD-1 cells were treated directly with the 2 different EVOOs. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software.*

To estimate the effect on tumour angiogenesis, we examined whether proliferation of human primary endothelial cells were also affected by EVOOs, four EVOOs were used to treat HUVECs. When HUVECs were treated with EVOO in full culture medium, no inhibition in cell growth occured. On the contrary, cells reached a plateau in their growth without reggression (Figure 4).

Following, we treated HUVECs using 3 different EVOOs with varying concentrations in phenolics (EVOO 27, 28 & 29 with 1956, 1380 & 606 mg/mL phenolic compounds respectively). When HUVECs were treated with these EVOOs in either experimental protocol (EVOO-enriched culture medium and direct addition - Figures 5 and 6) in full (20% FBS) culture medium or in 5% FBS medium, no effect on cell growth was observed. protocol.

As for the corn and the ctl oil, it is remarkable the fact that when commercial olive oil poor in phenolic compounds (Ctl Oil) caused no inhibition on the cell growth. On the contrary, especially when it was administered directly in HUVEC, led to an increase in cell growth compared to untreated cells. Corn Oil had no effect in cell growth of HUVECs (Figure 4, 5 & 6).

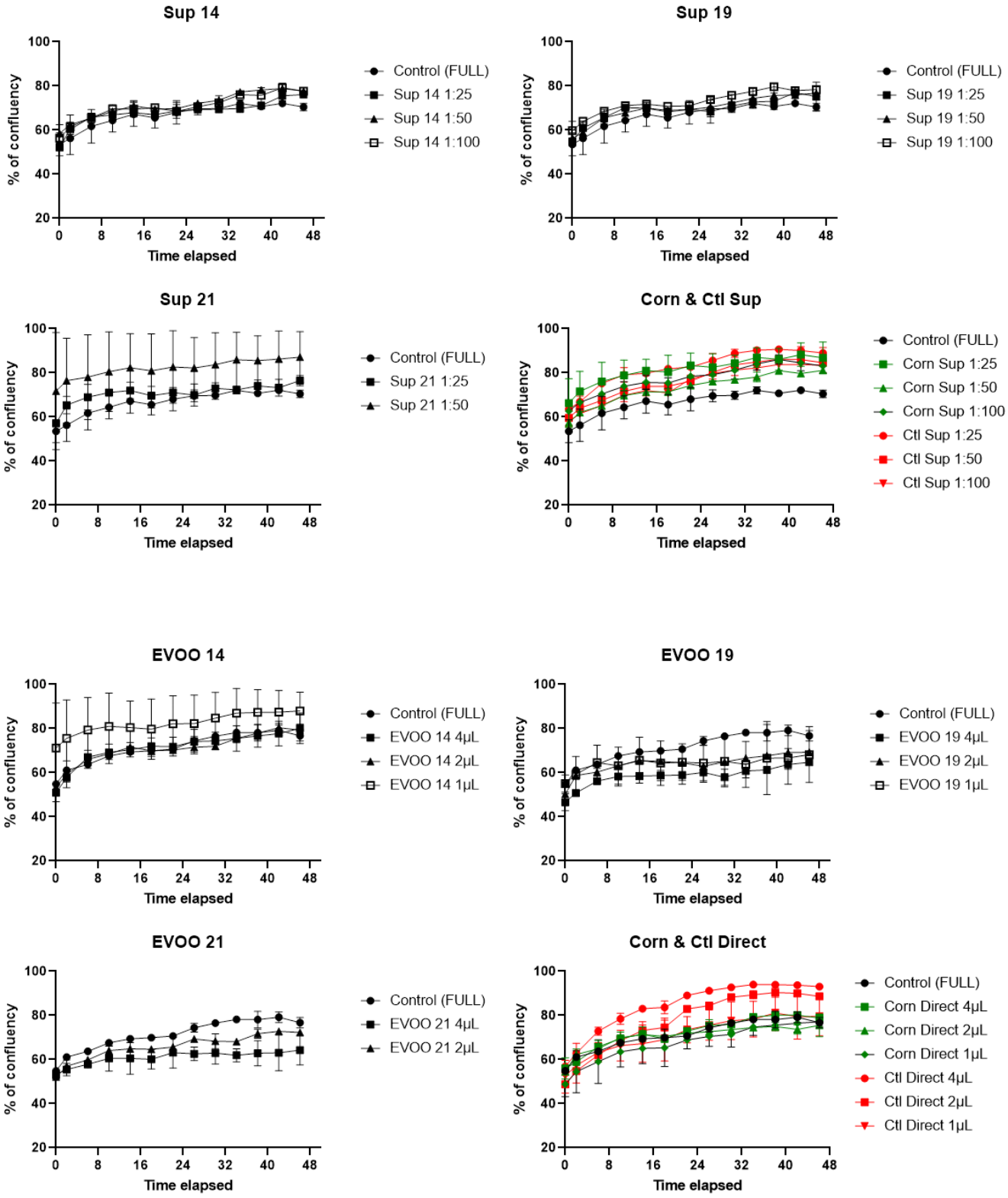

*Figure 4: **Effect of EVOO-enriched culture medium or direct addition of EVOOs on HUVECs cell growth.** HUVE cells were treated with EVOO-enriched, Corn oil-enriched medium, and Ctl oil-enriched full culture medium in 3 different ratios, 1:25, 1:50 or directly in Full FBS. Cell growth was monitored using IncuCyte for 48h taking images every 6h. Percentage (%) of confluency is calculated using IncuCyte software*

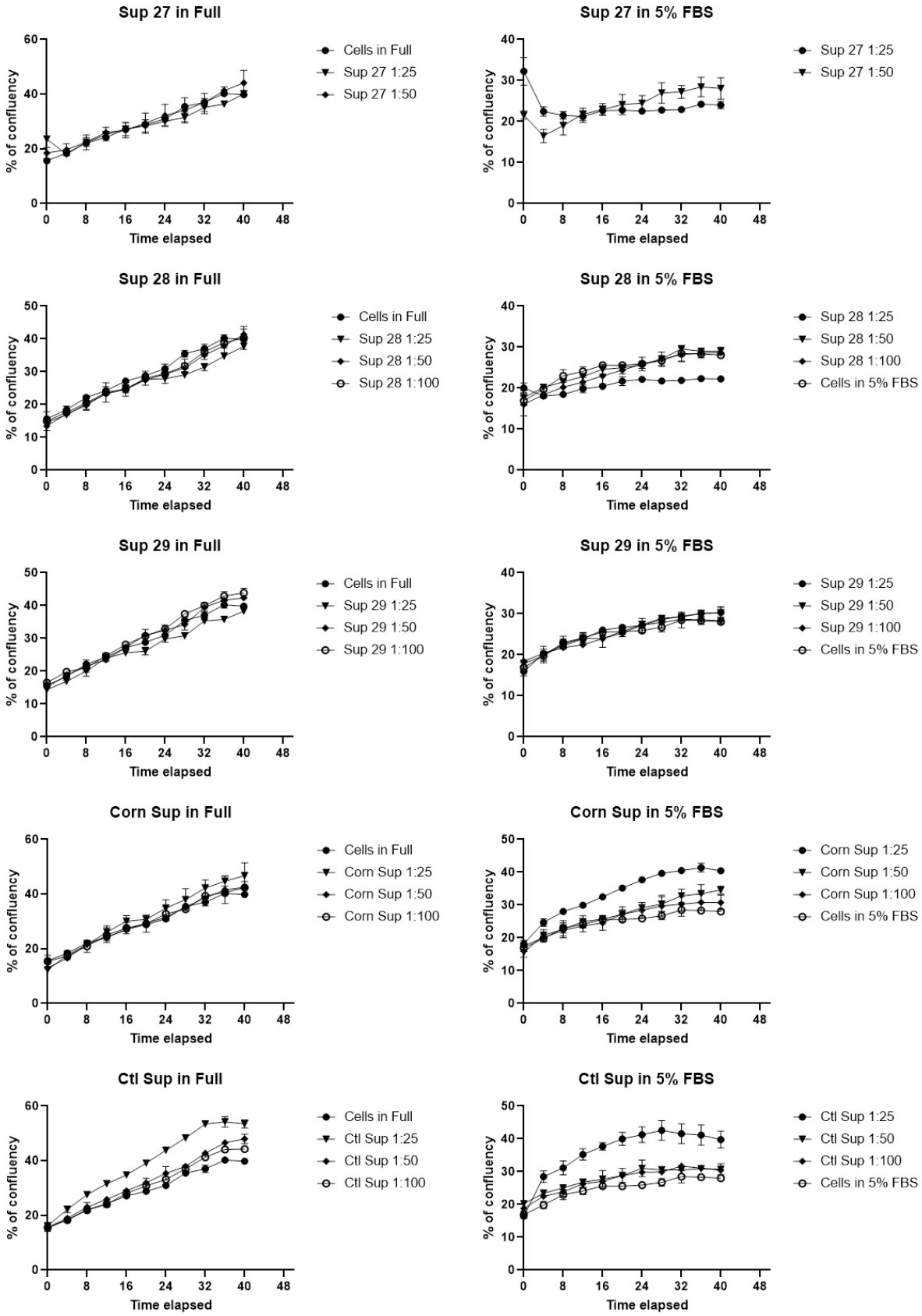

*Figure 5: **Effect of EVOO-enriched culture medium on HUVECs cell growth.** HUVE cells were treated with EVOO-enriched, Corn oil-enriched medium, and Ctl oil-enriched either Full (20%) or 5% FBS, in 3 different ratios, 1:25, 1:50. Cell growth was monitored using IncuCyte for 48h taking images every 6h. Percentage (%) of confluency is calculated using IncuCyte software*

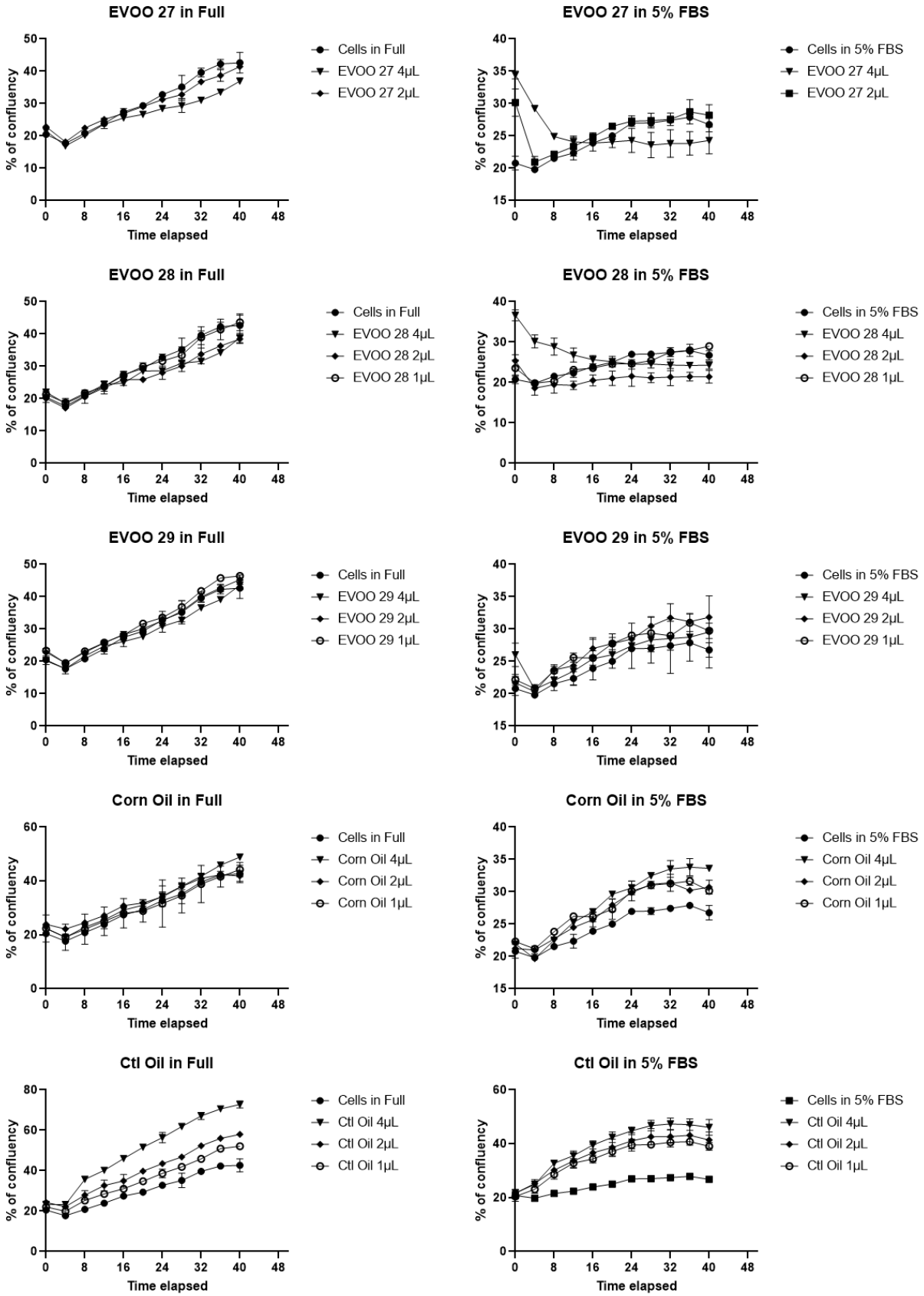

*Figure 6: **<u>Effect of EVOOs on HUVECs cell growth after direct addition in culture medium.</u>** HUVECs were treated directly with the different EVOOs in Full medium (20%) or in 5% FBS medium. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software.*

DISCUSSION

In the frame of the present study, we investigated the antiproliferative effect of EVOOs on cancer cells. For that reason, we treated colorectal adenocarcinoma cells (DLD-1) with different EVOOs using 2 different experimental procedures, as it is described above.

When DLD-1 cells were treated using the EVOO-enriched cell culture medium, cell growth was strongly inhibited inly dilution 1:25 was used. On the contrary, either dilution 1:50 or 1:100 didn't lead to a strong inhibition in cell growth of DLD-1 colorectal cancer cells. Moreover, direct addition of EVOO culture medium in all concentrations (4, 2, and 1 uL) resulted in cell growth inhibition. As negative control, Corn oil and commercially available olive oil (Ctl Oil) were used due to their low concentration in phenolic compounds. As it was expected, these oils didn't result in any cytostatic effect. At this point, it should be noted that EVOO effect in DLD-1 cancer cells was independent of the known concentration in phenolic compounds. In particular, EVOOs rich in phenolic compounds (oleocanthal, oleacein etc.) had comparable effect with EVOOs that weren't that rich in phenolics.

Regarding the HUVECs, when treated with EVOO either in full culture medium (20%) or in 5% FBS medium, cell growth was not inhibited. On the contrary, they reserved a plateau of their growth without reggression.

In brief, inhibitory effect of EVOO in 2-dimensional cell cultures is only observed in DLD-1 cancer cells and not in primary Human Umbilical Vein Endothelial Cells (HUVECs).

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