RESEARCH ARTICLE

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Euphol from *Tapinanthus sp.* Induces Apoptosis and Affects Signaling Proteins in Glioblastoma and Prostate Cancer Cells

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Abstract

Background: Plants play an important role in cancer therapy. They are source of natural molecules which can induce apoptosis in cancer cells by affecting molecular mechanisms implicated in cancer progression. The MAP Kinase/ERK1/2 and PI3K/AKT signaling pathways are two classical signaling pathways implicated in cancer progression and constitute therapeutic targets against cancer. This study aimed to evaluate the effect of euphol on MAP Kinase/ERK1/2 and PI3K/AKT signaling pathways in glioblastoma and prostate cancer cells. Euphol is a tetracyclique triterpene alcohol isolated from *Tapinanthus sp.* which is a hemi parasitic plant belonging to Loranthaceae family. **Methods:** Plant powder was extracted by maceration and euphol was isolated and described using respectively column chromatography separation on silica gel and spectroscopic data. Cytotoxic effect of euphol was evaluated using XTT assay and its effect on MAP Kinase/ERK1/2 and PI3K/AKT protein expression was investigated by Western immunoblot analysis. Apotosis was analyzed by evaluating caspase-3/7 activity. **Results:** Our investigations demonstrated that this compound has an important cytotoxic effect on C6 and U87 MG glioblastoma (GBM) cells and PC-3 prostate cancer cells. Furthermore, euphol-induced apoptosis revealed by elevated caspase 3/7 activity, was correlated with a significant inhibition of MAP kinase/Erk 1/2 and PI3K/Akt signaling pathway in glioblastoma U87 MG cells. The reverse effect was observed in C6 glioblastoma cells, where apoptosis was correlated with a long-lasting activation of Erk 1/2. In PC-3 cells, euphol had no or limited effect on Erk 1/2 and Akt activity. **Conclusion:** These results indicate that euphol induces cell death in glioblastoma and prostate cancer cells and regulates significantly Erk1/2 and Akt activity in glioblastoma cells.

Keywords: Euphol- Tapinanthus sp.- cytotoxic effect- Erk1/2 and Akt protein expression- apoptosis

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Introduction

Among the many signaling pathways activated in cancer cells, the mitogen-activated protein kinases (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway regulate a wide range of processes such as cell growth and differentiation, cell proliferation, cell survival and apoptosis, gene expression, mitosis, cell motility, metabolism and angiogenesis (Brotelle and Bay, 2016; Rai et al., 2019). These pathways are activated either at the plasma membrane (receptors with tyrosine kinase activity) or intracellularly with transformed or overexpressed proteins involved in signal transduction (Brotelle and Bay, 2016).

Extracellular signal-regulated kinase (Erk, Erk1 and Erk2) and protein kinase B (PKB/Akt) are proteins of the MAPK and PI3K pathways, respectively. Erk1/2 MAPKs can be activated transiently or sustainably and generally promotes cell survival, but under certain conditions Erk1/2 can have pro-apoptotic functions (Yue and Lopez, 2020).

In cancers, studies have detected overexpression and hyperactivation of Akt in a wide range of human tumors, and this is linked with poor prognosis (Song et al., 2017; Sun et al., 2018). All both Erk1/2 and Akt are involved in the development and progression of several human cancers including glioblastoma and prostate

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cancer (Jiang et al., 2020). The fundamental importance of the PI3K/Akt pathway led to the development of Akt inhibitors for cancer therapy (Popova and Jucker, 2021). Targeting the MAPK /Erk1/2 and PI3K/Akt pathways in GBM and prostate cancer could be a promising therapeutic strategy.

Plants are an infinite source of bioactive compounds capable to inhibit the main molecular components of signaling pathways implicated in cancer progression. They have played important role in cancer therapy or prevention. Many anticancer drugs are derived from plants or have natural origin. Paclitaxel, vinblastine, vincristine, etoposide etc, are some of powerful anticancer agents isolated from plants (Cragg and Pezzuto, 2016) and used in clinic. The aim of this study is to evaluate the effect of euphol on Erk1/2 and Akt protein expression in glioblastoma and prostate cancer cells. Euphol is one of the compounds isolated in our previous phytochemical study on Tapinanthus sp. which led to the isolation of one new flavonoid glycoside and seven known triterpenes (Gade et al., 2021). In that study, euphol was detected for the first time from Loranthaceae family plant and have exhibited good cytotoxic effect on C6, U87MG and PC-3 cancer cells. Another study also showed that euphol has anticancer activity on a large panel of cancer cell lines (Silva et al., 2018). Despite the well documented anticancer activity of euphol, its mechanism of action in cancer cells is unknown, especially in C6, U87 and PC-3 cancer cells. In our continue study, we want to investigate the mechanism of action of euphol on signaling pathways, particularly on the MAP Kinase and PI3K signaling pathway in glioblastoma and prostate cancer cells.

Materials and Methods

General experimental procedures

Euphol was analyzed using spectroscopic methods Proton (1H) and carbone (13C) data. Nuclear Magnetic Resonance (NMR) spectroscopy spectra were recorded in CDCl3 on an Avance 600 Bruker. The chemical shift are expressed in part per million (ppm) relative to TMS ($\delta = 0$) and the coupling constant in Hertz (Hz). NMR multiplicities are reported using: s = singlet, d = doublet and t = triplet. Colum chromatography was performed on silica gel (Merck, 230–400 Mesh) and thin layer chromatography (TLC) on precoated silica gel plates Merck (TCL Silica gel 60 F254).

Plant material

The leaves of Tapinanthus sp. (Loranthaceae) were collected during the month of May 2017 at Padarmé village located in Bibémi subdivision, North Region of Cameroon. The geographical coordinates of the place of collection of the plant are: Latitude 9.5304° or 9° 31′ 49.4″ North, Longitude 14.0617° or 14°3′ 42.1″ Est. Tapinanthus sp. is an unlisted plant species from the collection of plants listed at the National Herbarium of Cameroon (NHC) and no specimen in the herbarium had been found to match with this plant species. It's a hemi parasitic plant harvested on Combretum glutinosum, the host plant. This later have been identified at the National Herbarium of Cameroon under voucher number: 38091 HNC.

Extraction and isolation

The air-dried powder leaves of Tapinanthus sp. (1.30 Kg) was extracted by maceration in 7 L of methanol during 48 h with intermittent stirring. The obtained solution was filtered, followed by removing of solvent under reduced pressure using a rotary evaporator. The procedure of the extraction was repeated three times to afford 55 g of methanol crude extract. A quantity of the extract (26 g) was separated using glass column chromatography (h 1,000 mm, Ø 50 mm) on silica gel and eluted with hexane-ethyl acetate (100:0 → 0:100 %), then ethyl acetate-methanol (100:0 → 0:100 %) gradient system used as mobile phases. The 100 mL fractions each were collected from the column chromatography and a total of 195 fractions were obtained and grouped into four series (A-D) according to their TLC profiles. All series (A-D) were presented in the form of a trail regards to their TLC profile and were not of interest. The isolated compounds crystallized in some obtained fractions. Then, compounds were filtered on a filter paper and washed with the solvent or system of solvent in which the compound crystallized. The chromatographed extract led to the isolation of seven compounds such as tapinantoside, β-amyrine, a mixture of β-sitosterol and stigmasterol, ursolic acid, oleanolic acid, daucosterol and euphol which we investigate in this work (Gade et al., 2021).

Cell line and cell culture

The U87 human glioblastoma cell line and the C6 rat glioblastoma cell line were cultured in F25 flask (Thermo Ficher Scientific, Inc.) containing DMEM 1g/L glucose with GlutaMAXTM (Gibco) and sodium pyruvate (Invitrogen), supplemented with 10% of fetal calf serum and 100 U/mL penicillin and 100 g/mL streptomycin (Gibco). The PC-3 prostate cancer cell line was maintained in the same conditions except the use of DMEM medium with 4.5 g/L glucose. All the cells were incubated at 37°C in a controlled humidified atmosphere 95 % air, with 5 % CO_2. Upon reaching near-confluency, the cells were passaged using 0,25 % Trypsin-EDTA (Invitrogen).

XTT cytotoxicity assay

Cell viability was determined using the Cell Proliferation Kit II (XTT) (Promega) containing (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2Htetrazolium-5-carboxyanilide inner salt (XTT labeling reagent) and N-methyl dibenzopyrazine methyl sulfate (PMS, electron-coupling reagent). The metabolically active cells convert XTT into an orange formazan dye. Cells were plated on to 96-well plates at a cell density of 5×10^3 cells/well in 100 µL medium for U87 cells, 3×10^3 cells/well in 100 µL medium for C6 cells and 4×10^3 cells/well in 100 µL medium for PC-3 cells. After a 24h incubation, the medium was replaced with fresh medium containing different concentrations of euphol. Control cells were incubated in the presence of DMSO used to dissolve the compound. The final concentration of DMSO in the medium was less than 0.5 %. After 72h of treatment, cell viability was determined by adding 50 µL of XTT labeling reagent mixture solution in each

well and incubating the plate for 4h. The absorbance of formazan formed was measured at 490 nm using a spectrophotometric microplate reader. Results were expressed as percentages of cellular viability and the control was considered as the 100 value.

Total protein extraction

U87, C6 and PC-3 cells were seeded in Petri dishes and allowed to grow till 90 % confluence. Then, the culture medium was removed, and the cells were treated with medium containing euphol at 20 and 40 µg/mL for 1h, 6h and 24h. Control cells were incubated with 0.5 % of DMSO solution. After treatment, the culture medium was removed and cells were washed twice with cold PBS. Cells were scrapped into ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA pH 8, 0.5 % CHAPS and glycerol 10 %) supplemented with protease and phosphatase inhibitors (Pierce 88669 40X). After 30 min on ice, lysates were centrifuged for 20 min at 13,000g at 4°C. The supernatants were collected and stored at - 80°C. Protein concentration was determined using the DC Protein Assay (Bio-Rad).

Western immunoblot analysis

Proteins (20 µg) were resolved in 10% SDS–PAGE and transferred on PVDF (polyvynilidene fluoride) membranes. Membranes were blocked using 5% nonfat milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (Sigma–Aldrich) (TBST) for 1h at room temperature and then incubated overnight at 4°C with the following primary antibodies diluted in blocking solution: phospho-Akt (Ser473) (1:1,000) (Cell Signaling), phospho-p44/42 MAPK (Erk1/2) (1:1,000) (Cell Signaling), Akt (pan) (1:1,000) (Cell Signaling) and p44/42 MAPK (Erk1/2) (1:1,000) (Cell Signaling). After 3 washes with TBST for 10 minutes each, membranes were incubated for 1h at room temperature with goat anti-rabbit (1:20,000) (Sigma-Aldrich) or goat anti-mouse secondary antibodies (1:20,000) (Calbiochem) conjugated to horseradish peroxidase in blocking solution. After 3 washes as before, chemiluminescent signals were generated using Luminata forte (Millipore) and images were captured with PXi system (Syngene).

Assay of caspase-3/7 activity

The Apo-ONE® Homogeneous Caspase-3/7 kits assay (Promega) was used to determine the enzymatic activity of caspases in cells treated with euphol for 6 and 24h according to the manufacturer's instruction. The assay is based on the cleavage of non-fluorescent caspase substrate by caspase-3/7 that creates a fluorescent product. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample. The intensity of the emitted fluorescence was measured at 485 nm using a spectrophotometric microplate reader (Mithras, Berthold).

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). IC_{50} values are calculated from the sigmoidal nonlinear regression curve. Statistical comparisons were performed with the Friedman test using GraphPad Prism. The statistical significance was set at $P < 0.05$.

Results

Structural elucidation of euphol

Euphol (Figure 1) was isolated in our previous study (Gade et al., 2021) as a white powder soluble in methanol. Its structure has been identified using spectroscopic data and by comparison with literature data (Lin et al., 2000). The molecular formula of euphol, $C_{30}H_{50}O$ was determined from its ESI-MS spectrum which pointed out in positive mode the pseudomolecular ion peak [M+3Na]3+ at m/z 495 with six degrees of unsaturation, corresponding to the molecular weight 426. It responded positively to the Liebermann-Bürchard test, characteristic of triterpene skeleton. Euphol was detected for the first time in Loranthaceae family plant. Its NMR data from proton spectrum and carbon spectrum are presented in the following text.

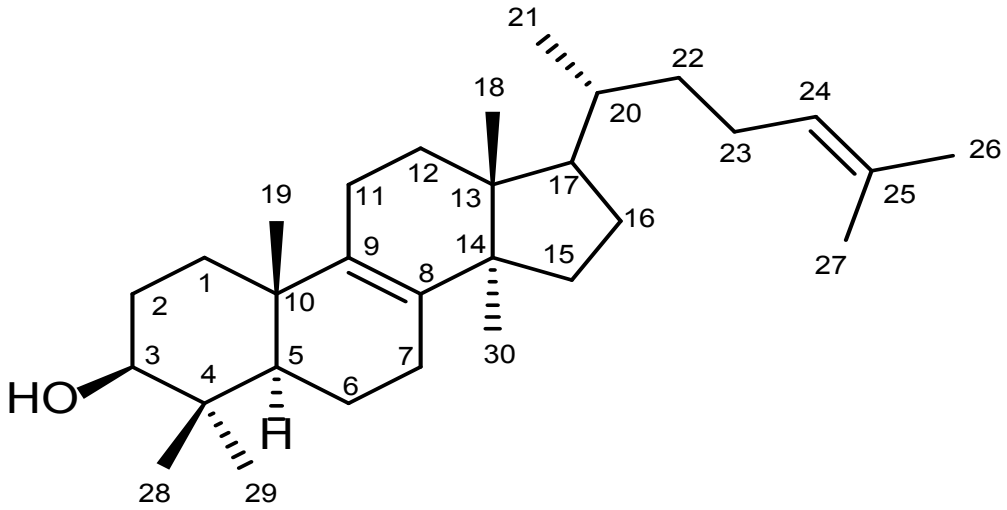

Figure 1. Structure of Euphol

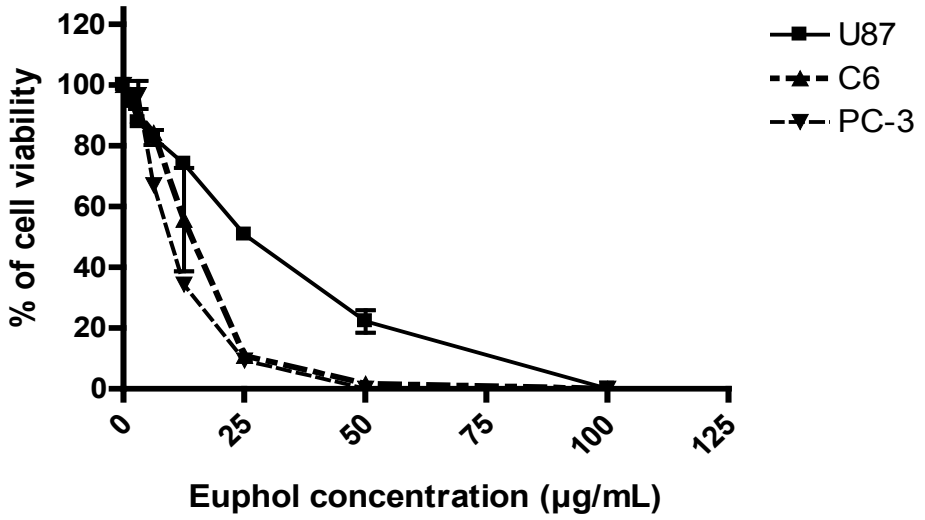

Figure 2. Effect of Euphol on the Cell Viability of U87, C6 and PC-3 Cancer Cells. Cell viability was measured after 72 h of treatment with euphol by an XTT assay. The results are expressed as the mean percentage ± SEM of three independent experiments. Control cells were treated in the presence of DMSO (at a final concentration of 0.5 %) and considered as 100 % viability. Euphol inhibited cell viability in a dose-dependent manner after 72 h of exposure.

Euphol NMR data

White powder, ESI-MS m/z 495 [M+3Na]3+. 13C NMR (150 MHz, CDCl3): δ 35.2 (C-1), 27.6 (C-2), 79.0 (C-3), 38.9 (C-4), 50.9 (C-5), 18.9 (C-6), 27.9 (C-7), 133.5 (C-8), 134.0 (C-9), 37.2 (C-10), 21.5 (C-11), 28.1 (C-12), 44.1 (C-13), 50.0 (C-14), 30.8 (C-15), 29.7 (C-16), 49.6 (C-17), 15.5 (C-18), 20.1 (C-19), 35.8 (C-20), 18.9 (C-21), 35.4 (C-22), 24.7 (C-23), 125.2 (C-24), 130.8 (C-25), 17.6 (C-26), 25.7 (C-27), 24.4 (C-28), 28.0 (C-29) and 15.6 (C-30). 1H NMR (600 MHz, CDCl3): δ 3.23 (dd, J = 4.6, 11.8 Hz, H-3), 0.80 (s, H-18), 0.95 (s, H-19), 0.87 (d, J = 6.1 Hz, H-21), 5.09 (t, J = 7.2 Hz, H-24), 1.68 (s, H-26), 1.61 (s, H-27), 0.87 (s, H-28), 1.00 (s, H-29) and 0.75 (s, H-30).

Euphol decreases viability of U87, C6 and PC-3 cancer cells

The effect of euphol on cell viability was assessed on the glioblastoma cell lines, U87 and C6, and the prostate cancer cell line, PC3. Temozolomide and docetaxel were used as reference drugs. The results are reported in (Table 1). All tested cells were sensitive to euphol treatment that induced a dose-dependent decrease (Figure 2) in the number of viable cells. PC-3 cells showed the highest sensitivity to euphol treatment with an IC_{50} value equal to 21.33 µM, while IC_{50} values were 59.97 and 38.84 µM for U87 and C6, respectively. Among the tested glioblastoma cells, the C6 cells line was more sensitive than U87 MG cells to euphol treatment. Euphol was found to be more active than temozolomide and less active than docetaxel, both used as reference drugs.

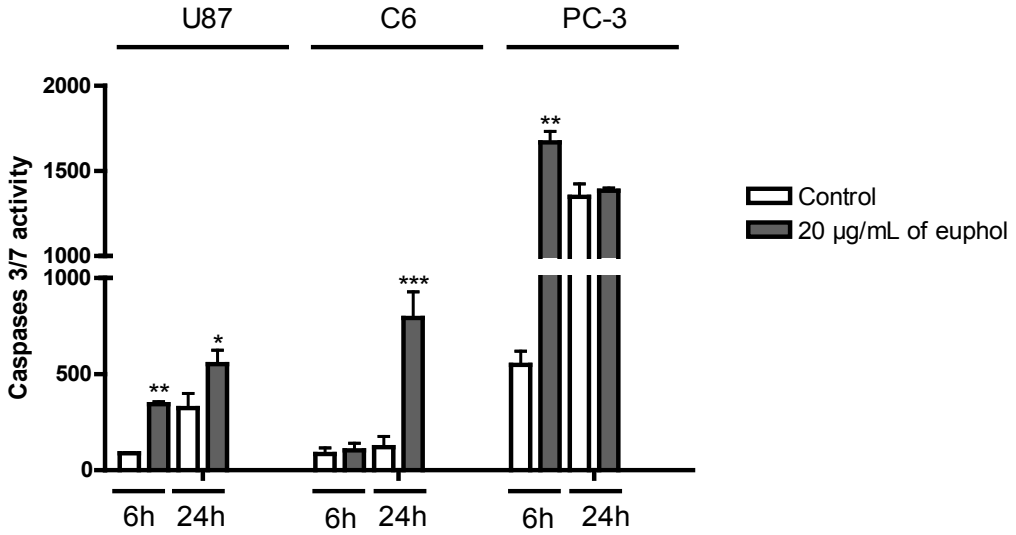

Figure 3. Effect of Euphol on Caspase-3/7 Activity in U87, C6 and PC-3 Cells. Cells were treated with 20 µg/mL of euphol for 6h and 24h. Results were obtained from two independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

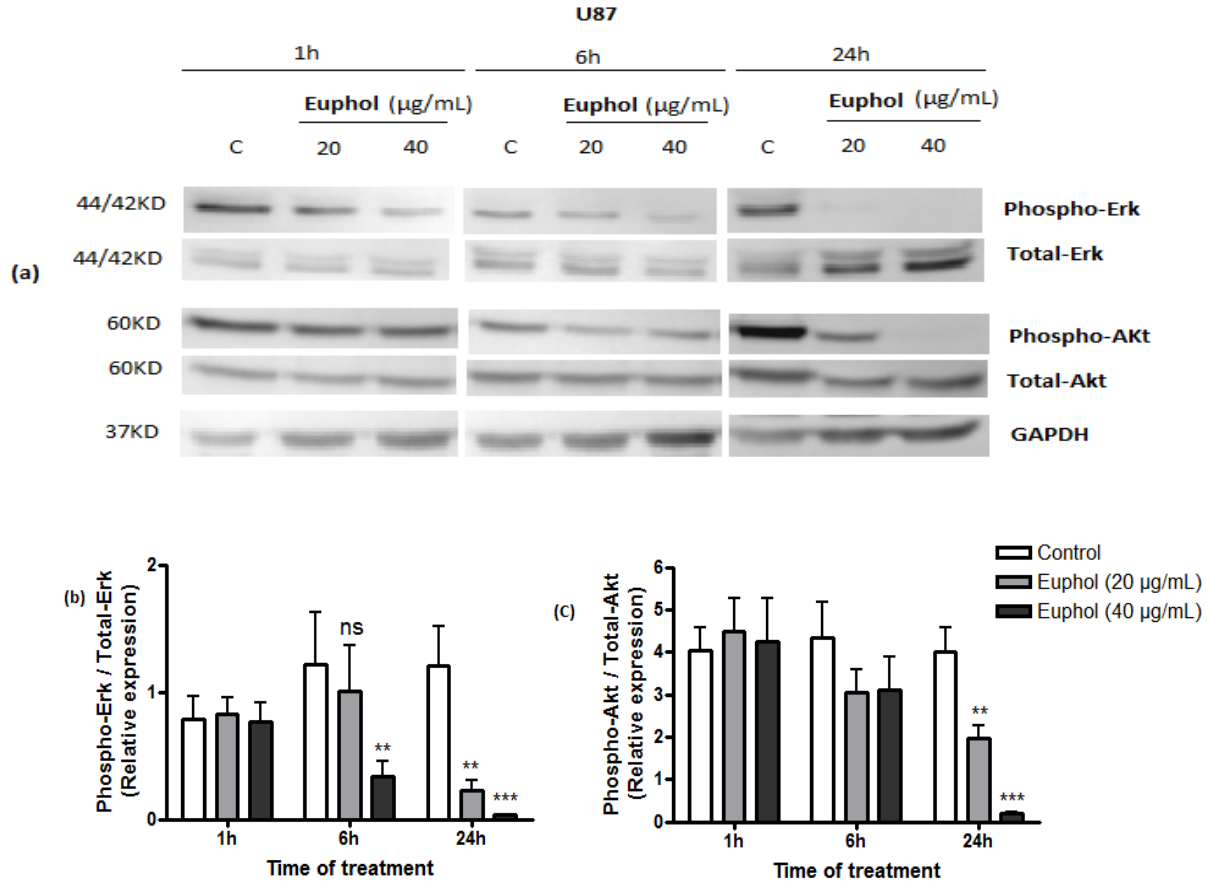

Figure 4. Western Immunoblot Analyses of the Expression of Erk and Akt in Euphol-Treated U87 Cells. Representative experiment (a) and quantification of active phospho-Erk / total Erk (b) and active phospho-Akt / total Akt (c). Cells were treated for 1h, 6h and 24h (c: control cells). GAPDH was used as loading control. Results were obtained from three independent experiments performed in duplicate. ** $p < 0.01$ and *** $p < 0.001$.

Euphol increase caspase-3/7 activity in U87, C6 and PC-3 cells: induction of apoptosis

To determine whether the decreased cell viability induced by euphol in U87, C6 and PC-3 cells is correlated with apoptosis, cells were treated with 20 µg/mL of euphol concentration and exposed to the Apo-ONE® Homogeneous Caspase-3/7 reagent. Caspases-3/7 belong to the family of cysteine proteases and are a prominent cell death marker and a point of convergence as effector enzymes in the caspase cascade activation that typically occur during apoptosis (Martin and Pognonec, 2010 ; Julien and Wells, 2017). The results of this test are presented in (Figure 3). This figure indicates that the treatment of U87, C6 and PC-3 cells with euphol induces a significant increase in caspase-3/7 activity compared with untreated control cells. In the U87 cells, a significant increase in caspase-3/7 activity was observed after 6 h ($p < 0.01$) and 24h ($p < 0.05$) of treatment. For ¬C6 cells, we rather observed a significant ($p < 0.001$) increase in caspase-3/7 activity occurring after 24h of treatment. However, in the PC-3 cells, the elevated caspase-3/7 activity was observed after 6h, but no activity of those enzymes was detected after 24h treatment. These results demonstrate the cytotoxic effet of euphol in U87, C6 and PC-3 cells.

Table 1. Results of the Cytotoxicity Effect of Euphol

Samples	U87	C6	PC-3
		IC_{50} (ÂµM)	
Euphol	59.97	38.84	21.33
Temozolomide	172.00	144.32	NT
Docetaxel	NT	NT	2.05

NT, non tested

Effect of euphol on Erk1/2 and Akt protein expression in U87, C6 and PC-3 cells

We next evaluated the effect of euphol on Erk 1/2 and Akt protein expression, their total and phosphorylated active forms, in glioblastoma and prostate cancer cells. Erk 1/2 and Akt proteins are the main component respectively of MAP Kinase and PI3K signaling pathways involved in tumoral progression. Cells were treated at various time points with 20 and 40 µg/mL of euphol for C6 and U87 cells, and 15 and 30 µg/mL for PC-3 cells, for 1h, 6h and 24h. Proteins were analyzed by Western immunoblotting (Figure 4). The results indicated that the treatment of U87 cells with euphol significantly ($P < 0.05$) affected MAP Kinase and PI3K signaling pathways in a dose- and time-dependent manner. Euphol strongly reduced Erk1/2 (ratio phosphor-Erk/ total-Erk) and Akt (ratio phospho-Akt/ total Akt) activity in U87 cells particularly after 24h treatment compared to control cells. The decrease in the level of Erk and Akt protein activity in U87 cells induced by euphol

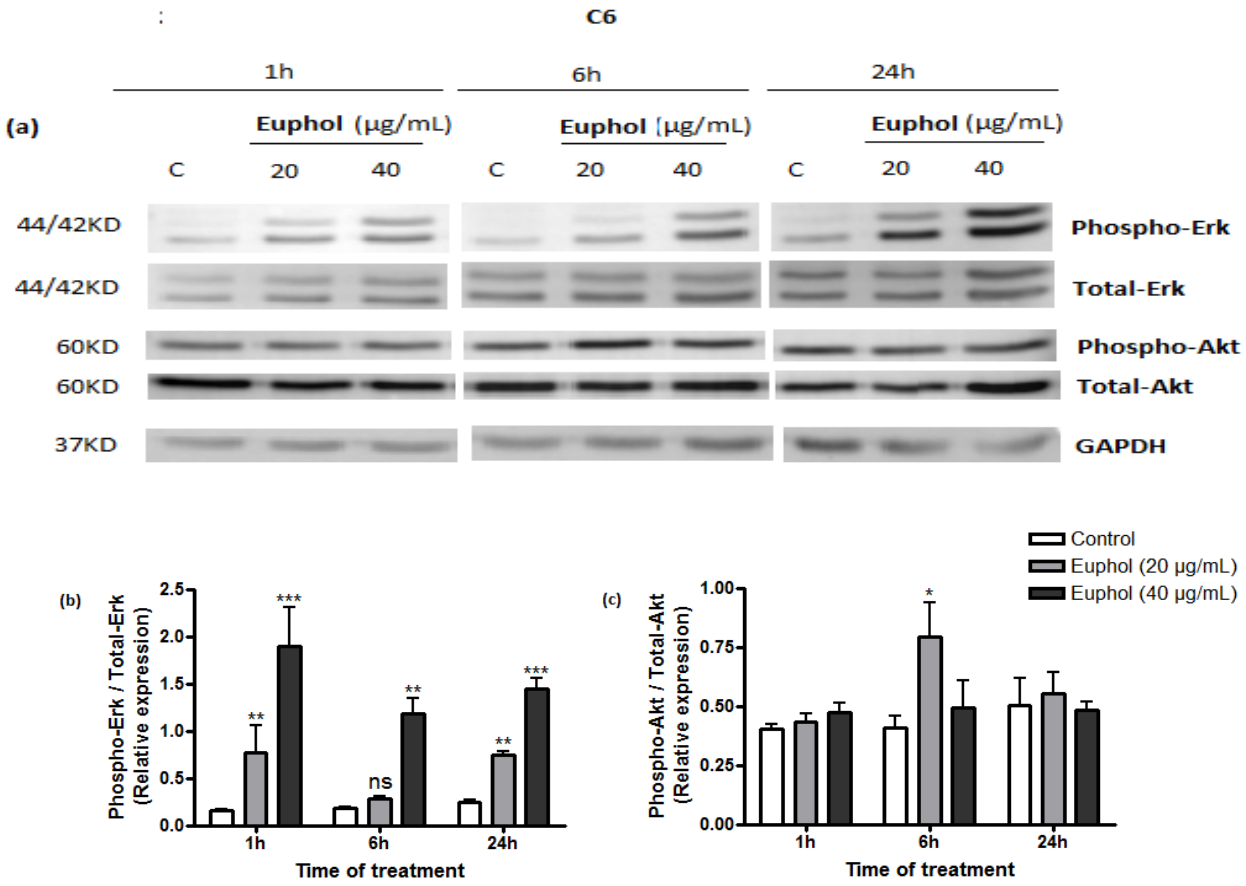

Figure 5. Western Immunoblot Analyses of the Expression of Erk and Akt in Euphol-Treated C6 Cells. Representative experiment (a) and quantification of active phospho-Erk / total Erk (b) and active phospho-Akt / total Akt (c). Cells were treated for 1h, 6h and 24h (c: control cells). GAPDH was used as loading control. Results were obtained from three independent experiments performed in duplicate. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

is then correlated with apoptosis induced in that cells by the same compound (Figure 3).

In C6 glioblastoma cells, euphol induced a reverse effect compared with those in U87 cells. Treatment of C6 cells with euphol significantly ($P < 0.05$) pointed out a persistent and strong activation of Erk1/2 at all-time treatment (1h, 6h and 24h), (Figure 5a) and transient activation of Akt just at 6h (Figure 5b). This result is correlated with the high cytotoxic effect induced in that cells by euphol (Figure 2 and Figure 3).

The PC-3 human prostate cancer cells displayed the highest cytotoxicity sensitivity to euphol compared to those of U87 and C6 glioblastoma cancer cells with the lowest IC50 value (9,09 µg/mL). However, euphol showed no visible effect on Erk1/2 protein activity (Figure 6a). Phosphorylation of Erk 1/2 was not observed in control or treated cells. A transient significative increase of phospho-Akt/Akt protein expression was observed after 6h of treatment with 30 µg/mL euphol (Figure 6b).

Discussion

This investigation shows that euphol (a tetracyclic triterpene alcohol) isolated for the first time from Loranthaceae family plant, exhibits an important cytotoxic effect on U87, C6 and PC-3 cancer cells, and also generally modulates significantly the relative activity of Erk1/2 and Akt protein expression from the MAP Kinase and PI3K signaling pathway respectively, in the same cells. These strong cytotoxic properties of euphol could be correlated with its significant effects generally observed in these cell lines on the relative activity of Erk and Akt proteins.

The cytotoxicity of euphol varies significantly from glioblastoma to prostate cancer. Previous studies described the decrease of cell viability induced by euphol on a large panel of human cancer cell lines including glioblastoma and prostate cancer (Silva et al., 2018). A similar result was obtained with these latter. However, the decrease of cell viability induced by euphol on C6 glioblastoma cells is reported here for the first time. These results demonstrate that euphol has a significant cytotoxic effect on glioblastoma and prostate cancer cells and could be of value for cancer therapy.

Euphol downregulates Erk and Akt protein expression in U87 cells. This effect was also observed in a previous study by our research group in 2021 (Gade et al., 2021). The data demonstrated that the methanol extract of Combretum fragrans F. Hoffm, a medicinal plant, inhibits Erk and Akt activity in U87 cells. It is worth noting that Erk and Akt play an important role in cell proliferation, differentiation and survival (Coffer et al., 1998 ; Manning and Toker, 2017 ; Cao et al., 2019). They are implicated in

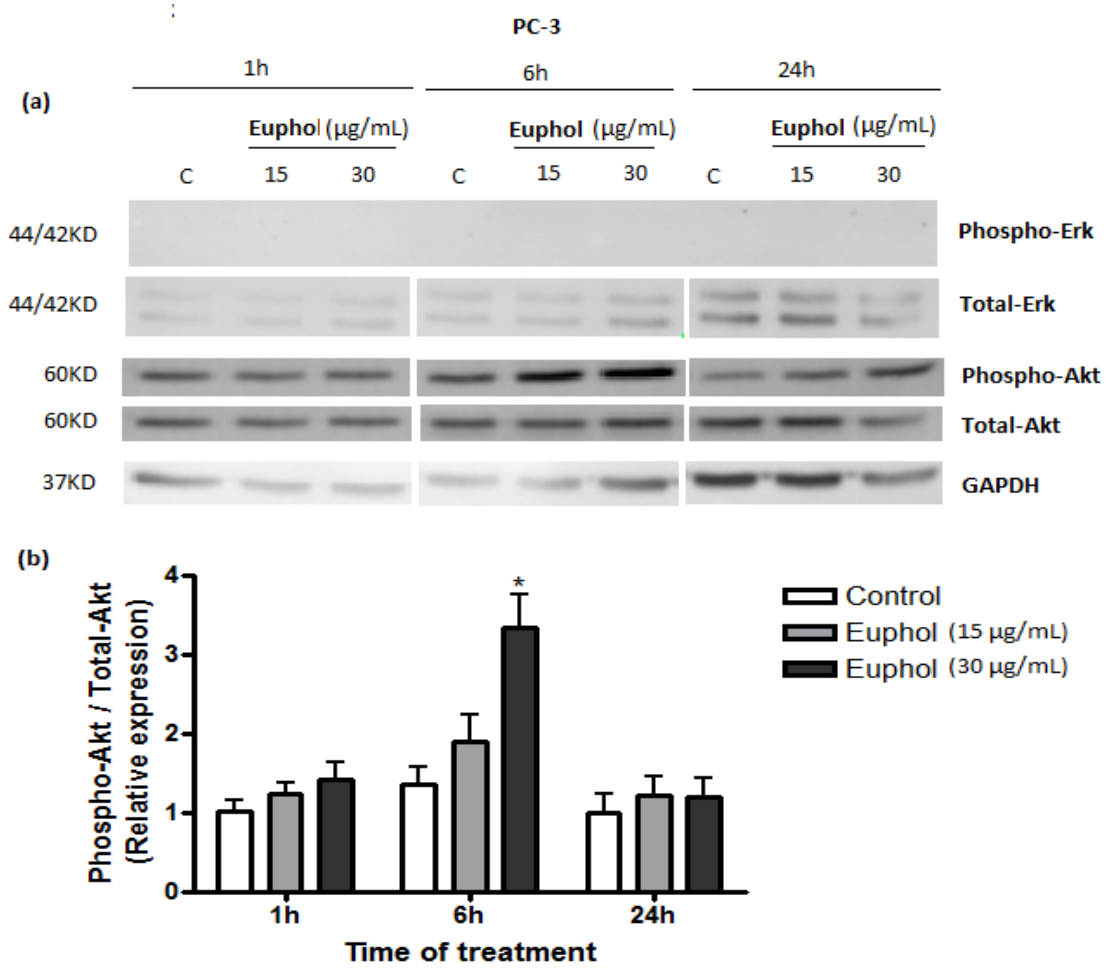

Figure 6. Western Immunoblot Analysis of the expression of Erk and Akt in euphol-treated PC-3 Cells. Representative experiment (a) and quantification of active phospho-Akt / total Akt (b). Cells were treated for 1h, 6h and 24h (c: control cells). GAPDH was used as loading control. Results were obtained from three independent experiments performed in duplicate. * $p < 0.05$

glioblastoma progression (Majewska and Szeliga, 2017), particularly the frequency of activated Akt increases with glioma progression (Zhao et al., 2017). Thus, the inhibition of Erk and Akt in U87 cells could induce cell death. This latter could be at the origin of the elevated capase-3/7 activity observed in that cell line in our experiments.

Euphol induced in C6 cells a strong and permanent activation of Erk at all-time treatment and also a cell death which was observed by the elevated caspase-3/7 activity. Other investigators also demonstrated that euphol could induce significant activation of Erk in gastric cancer cells (Lin et al., 2012). Prolonged ERK1/2 activation has been associated with cell growth arrest and cell death (Ballif and Blenis, 2001 ; Zhang et al., 2020). Although Erk1/2 is a pro-survival factor in the MAP kinase family and contributes to the regulation of cell proliferation and differentiation, under some circumstances, Erk1/2 can function in an apoptotic manner (Sugiura et al., 2021). Our results suggest that euphol-induced apoptosis in C6 glioblastoma cells may be triggered through long-term activation of Erk1/2 pathway.

In PC-3 prostate cancer cells, no effect was observed on Erk protein expression and activation when treated with euphol. As presented in (Figure 6), only a transient activation of Akt protein occurring at 6h was observed in these cells. Although the highest cytotoxic effect and cell death induced by euphol was observed in the PC-3 cell line, phospho-Erk levels could not be detected in control or euphol treated cells. This is corroborated by studies from other investigators that reported that metastatic PC3 and DU145 prostate cancer cells display relatively low levels of active phospho-Erk1/2 (Ballif and Blenis, 2001). In a previous study of our group, barely detectable levels of phospho-Erk were also reported in control PC3 cells but extracts from the plant Combretum fragrans F. Hoffm strongly activated Erk, a phenomenon that was correlated with induction of apoptosis (Gade et al., 2021) Further studies will thus be necessary to decipher the mechanism of action of euphol in PC-3 prostate cancer cells.

In this study we have demonstrated the cytotoxic effect of euphol on glioblastoma and prostate cancer cells and its regulation of Erk1/2 and Akt protein activity. Euphol-induced apoptosis in U87 cells was correlated with a significant downregulation of the relative activity of Erk1/2 and Akt signaling proteins, while in C6 cells the reverse effect was observed. The compound displayed the highest cytotoxic effect on PC-3 cells but had no effect on Erk1/2 activity and only transiently increased Akt activity. This suggests that euphol may acts on PC-3 cells through another signaling pathway. This study contributes to know

the mechanism of action of euphol in glioblastoma (U87 and C6) and prostate (PC-3) cancer cells and could be used as hits or leads for cancer drug discovery.

Author Contribution Statement

Conceptualization and Design: Isaac SilvÃ¨re Gade,Tagne Simo Richard, Corinne Chadeneau, Emmanuel Talla, Atchade, Nwabo Kamdje and Jean-Marc Muller. Collection and Assembly af data : Isaac SilvÃ¨re Gade, Paule Seite, Corinne Chadeneau, Brigitte Vannier, Sophie Laurent and CÃ©line Henoumont. Writing â•fi Original Draft and Editing : Isaac SilvÃ¨re Gade and Tagne Simo Richard. Proofreading And Language Editing: Sophie Laurent and CÃ©line Henoumont. Materials and Funding Acquisition: Armel H. Nwabo Kamdje, Tagne Simo Richard, Jean-Marc Muller. Supervision: Emmanuel Talla, Alex De ThÃ©odore Atchade, Jean-Marc Muller

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Ethical approal

Not applicable.

Conflict of interest

We the authors declare that we have not a conflict of interest.

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