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Dianzitoukoulou Louis Donald

University Marien Ngouabi,
Brazzaville, CONGO; Receptors,
Regulations, and Tumor Cells,
University of Poitiers, Bat B36,
TSA 51106, 86073 Poitiers Cedex
09, France, Europe.

Muller Jean-Marc

Université de Poitiers, Bat B36,
TSA 51 106, 86073 Poitiers
Cedex 09, France, Europe.

Gombert Jean-Marc

CHU de Poitiers, 2 Rue de la
Milétrie, 86021 Poitiers Cedex
France, Europe.

Ouamba Jean-Maurille

University Marien Ngouabi,
Brazzaville, CONGO; Receptors,
Regulations, and Tumor Cells,
University of Poitiers, Bat B36,
TSA 51106, 86073 Poitiers Cedex
09, France, Europe.

Séité Paule

Université de Poitiers, Bat B36,
TSA 51 106, 86073 Poitiers
Cedex 09, France, Europe.

Vannier Brigitte

Université de Poitiers, Bat B36,
TSA 51 106, 86073 Poitiers
Cedex 09, France, Europe.

Correspondence

Vannier Brigitte

Université de Poitiers, Bat B36,
TSA 51 106, 86073 Poitiers
Cedex 09, France, Europe.

Cytotoxicity and mode of action of *Klainedoxa gabonensis* Pierre ex Engl. on U87 Glioblastoma cancer cell line

Dianzitoukoulou Matsima Louis Donald, Muller Jean-Marc, Gombert Jean-Marc, Ouamba Jean-Maurille, Séité Paule and Vannier Brigitte

Abstract

Cytotoxic effects of an aqueous extract of a plant used in traditional Congolese medicine *Klainedoxa gabonensis* Pierre ex Engl. (KG) were studied on the aggressive U87 glioblastoma cell line. An aqueous extract (E2) was prepared from barks of KG and tested at different concentrations using an MTT assay (IC₅₀ of 65 µg/ml). The E2 KG extract exerts a cytotoxic effect on the U87 glioblastoma cell line, through the negative regulation of cell proliferation pathways: P-PDK1 and P-Akt are both down regulated upon treatment by E2. KG could also play a role in autophagic processes by converting LC3-I in LC3-II. Both electron and confocal microscopy support the implication of E2 in autophagy. The effect on E2 on other cell lines will be studied in the future. This study brings results supporting the effects of *Klainedoxa gabonensis* Pierre ex Engl., in the treatment of cancers in traditional Congolese medicine.

Keywords: Plant extract, Antiproliferative, *Klainedoxa gabonensis* Pierre ex Engl., Autophagy, Glioblastoma, Mwandza

Abbreviations: E2: aqueous KG extract; 7AAD: 7- Amino-Actinomycin D; AV: Annexin V; DMEM: Dubelco Modified Eagle Medium; HPLC: High Pressure Liquid Chromatography; KG: *Klainedoxa gabonensis* Pierre ex Engl.; TFA: TriFluoroacetic Acid

1. Introduction

Klainedoxa gabonensis Pierre ex Engl. is a plant (*Irvingiaceae*) growing in several countries in Africa. It is used in traditional medicine in Congo for different diseases as Mwandza (skin disease), bacterial infections or cancers (Burkill, 1994) [5]. In this work, we decided to test the cytotoxic properties of an aqueous extract of KG barks and assess its cytotoxicity on a glioblastoma cancer cell line, U87 (ATCC-HTB14), a current model used in our laboratory and one of the very aggressive form of brain tumors.

To mimic as close as possible the conditions used by traditional healers, we decided to work with aqueous extracts rather than organic fractions. The use of water also lowers the risk of solvent dependent cytotoxicity. The National Cancer Institute (Monks *et al.* 1991) [9] recommends the use of concentrations lower than 250 µg/ml to observe significant results. Thus, a 100 µg/ml threshold concentration was used to evaluate the cytotoxicity of different plants on the U87 cell line, leading to select *Klainedoxa gabonensis* Pierre ex Engl. on the basis of IC₅₀ value. We next studied its properties and the cellular and molecular mechanisms involved in its potential antitumor activity.

2. Materials and Methods

2.1 Plant material and extraction: Barks of KG were collected in the region of the Cuvette and in Brazzaville, Congo in March 2012. Plant samples were identified by botanists of the C.E.R.VE (Center of Studies and Research on Plant Resources, Brazzaville, Congo). Aqueous extracts were prepared with 30g of barks in 300ml of distilled water, boiled for 15 min. After filtration, extracts were lyophilized and conserved at 4 °C. The KG aqueous extract is called E2.

2.2 HPLC Analysis: Qualitative HPLC analysis was performed on a Waters™ device equipped with a photodiode array detector (PDA) 996 and a 626 pump. The chromatography was performed on a C18 column (150mm x 4.60mm x 5 µm, Phenomenex). The elution was effected with a gradient from solvent A (H₂O, 0.05% TFA) to solvent B (Acetonitrile, 0.05% TFA) and a 1ml/min flow rate.

The gradient shape was: 0-15% acetonitrile in 2 min, then 15-60% acetonitrile in 15 min, then 60-100% acetonitrile in 2 min. PDA Millennium software was used for data analysis. The 3D (optical density /wavelength/elution time) spectrum of each component eluted from the column was compared with those of a bank of standard 3D spectra of various phenolic plant compounds previously recorded in the HPLC device.

2.3 Cell culture

U87-MG cells (ATCC-HTB14) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100µg/ml) (Lonza, Walkersville, Inc) at 37 °C and 5% CO₂.

2.4 MTT assay

The MTT assay is a colorimetric assay measuring cellular metabolic activity via NAD (P) H-dependent cellular oxidoreductase enzymes that reflect cell viability. Cells were seeded at the concentration of 1000 cells/well on 96 wells plates. The plant extract was added for 24 H at different concentrations (1µg/ml to 1000µg/ml). MTT assay was then performed as described in (Berridge *et al.*, 2005) [4].

SDS PAGE/ Western blot: Cell extracts were prepared from control or E2 treated cells. Cells pellets were suspended in RIPA buffer supplemented by proteases inhibitors. Protein concentration was determined by BCA Protein Assay according manufacturer's recommendations (SIGMA). Samples were loaded on SDS-PAGE. After the electrophoresis, samples were transferred on PVDF (Andrique *et al.*, 2012) [1]. PVDF Membranes were then saturated in PBS BSA 1% and incubated overnight with antibodies according to manufacturer's recommendations.

2.5 Antibodies

Anti-P-AKT(Thr308), is a rabbit polyclonal from Cell Signaling Technologies, (#2965S); anti-AKT is a rabbit from Cell Signaling Technologies (#4691S); Anti- Phospho-PDK1 (Ser241) is a rabbit polyclonal from Cell Signaling Technologies Antibody (#3061) and Anti-LC3 is a rabbit polyclonal from Cell Signaling Technologies LC3A/B Antibody (#4108).

2.6 Apoptosis analysis by cytometry

U87-MG cells were seeded (1.10⁶ cells) in a Petri dish in 10 ml of DMEM+FCS 10%. After 24H, cells were treated by E2 (50 or 100µg/ml) for 24H. The next day, cells were labeled with the Apoptosis Detection Kit with 7-AAD/ annexin V (Biollegend, #640934) and analyzed by cytometry.

2.7 Cell cycle analysis by cytometry

U87-MG cells were seeded (1.10⁶ cells) in a petri dish in 10 ml of DMEM+FCS 10%. After 24H of incubation, cells were treated by E2 (50 or 100µg/ml) for 24H. Cells were trypsinized and stained with propidium iodide (BD Bioscience, 556547) and analyzed by cytometry.

2.8 Transmission Electronic Microscopy

After E2 treatment at 100µg/ml for 24H, cells were harvested by trypsinization and washed with PBS and fixed for 1H with a solution of glutaraldehyde 3%, Cacodylate 0.1M, pH 7.4. After a PBS wash, cells were post-fixed with OSO4 1% for 45 min. Dehydration of the sample was made by successive baths of alcoholic solutions. Cells were then scrapped and resuspended in Ethanol 100%. A pellet of cells obtained by centrifugation (1000rpm 10 min) was then

included in an Epon solution (Electron Microscopy Sciences). 0.1 mm sections of cells were prepared with an ultramicrotome UC6 (LEICA) and stained with uranyl acetate /lead citrate solution. Observations were made on a JEOL1010 transmission electron microscope at 80 KV.

2.9 Fluorescent Microscopy

Goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 (1:500, Invitrogen) and DRAQ5 for nuclear labelling (1:500, Cell Signaling Technologies) were incubated 1H at room temperature. Cells were examined with a spectral confocal FV-1000 station installed on an inverted microscope IX-81 (Olympus) with Uplan Sapo x60 oil, 1.2 NA, objective lens. Multiple fluorescence signals were acquired sequentially to avoid cross-talk between image channels. Fluorophores were excited with the 488 nm line of an argon laser (Alexa fluor488) and 633 line of an He Ne laser (DRAQ5). The emitted fluorescence were detected through spectral detection channels between 500–530 nm for green fluorescence and through a 650 nm long pass filter for far red.

3. Results

3.1 Cytotoxicity of E2 aqueous extract on the U87 Cell line

An aqueous extract has been prepared as mentioned in the material and methods section. This extract was then tested on U87 cells for 24H. An MTT assay was performed to determine the IC₅₀. Results shown in figure 1 indicate that an IC₅₀ (50% of dead cells) of 62.5 µg/ml is obtained after 24H of treatment. Other cell lines have been tested as U251 cell line (data not shown). Interestingly, an IC₅₀ of 250µg/ml has been obtained on the latter indicating that U250 and U87 cell line differs in their response to the E2 extract probably because of gene and/or protein different expression patterns.

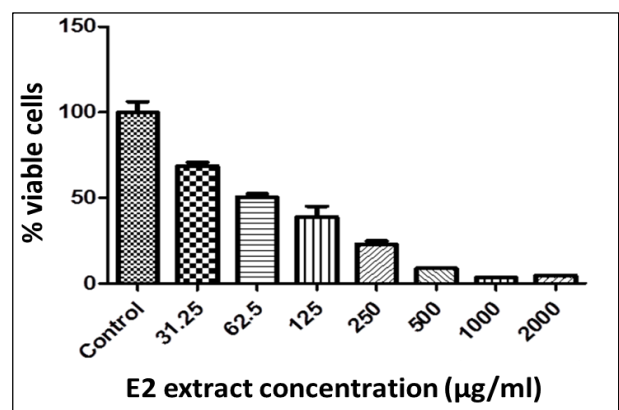


Fig 1: Effect of E2 on cell viability of U87 glioblastoma cell line.

The aqueous extract E2 has been incubated with U87 glioblastoma cell line for 72H in DMEM FCS 10%. An MTT assay has been performed. The viability of untreated cells was regarded as 100%. Results shown are the means ± SD.

3.2 Analysis of apoptosis by cytometry

A 7AAD/AV labeling was performed on the U87 cell line in order to determine the type of cell death occurring in cells after an E2 treatment for 24H. 7AAD (7-amino-actinomycin D) is a marker of nonviable cells in cytometry (O'Brien, *et al.*, 1995) [11] while annexinV is a protein having a strong affinity for phosphatidylserine, used to detect apoptotic cells (Vermees *et al.*, 1995) [16]. The results of the cytometry analysis are presented in Figure 3.

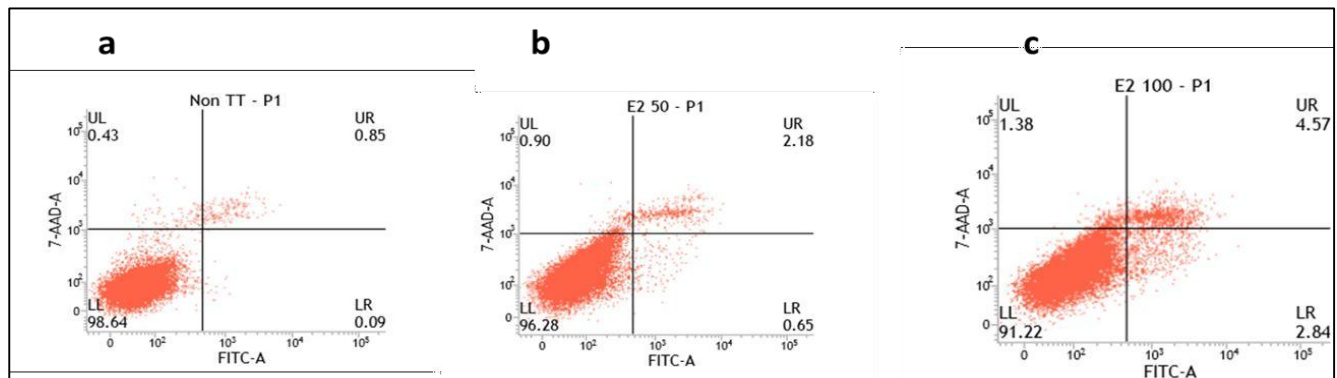


Fig 2: Flow cytometry analysis of U87 cell line after E2 treatment for 24H.

Cells were labelled with 7 AAD (dead cells detection) and annexinV (apoptotic cells detection) and analyzed by flow cytometry. (a) Untreated cells; (b) E2 (50 μ g/ml) treated cells and (c) E2 (100 μ g/ml) treated cells.

The assay shows that treated cells are spread in 3 sub-populations: AV-/7AAD-, live cells (LL panel); (AV+/7AAD-), cells in early apoptosis (LR panel); (AV+/7AAD+) and cells in late apoptosis (UR panel).

Results (Figure2) show that very few cells are in apoptosis after the treatment with E2 at different concentrations: 0.65% apoptotic cells are observed for the 50 μ g/ml E2

concentration, 2.84% for the 100 μ g/ml concentration. Thus, apoptosis is not significantly detected in E2 treated cells. While apoptosis is easily detected by cytometry, other types of cell death require other methods as western blots or electron microscopy. We first tried to characterize the cell death phenomenon by electron microscopy.

3.3 Electron microscopy

Cells were prepared as described in the material and methods section. 0.1 mm sections were stained and observed with an electron microscope. Micrographs are presented in figure 3.

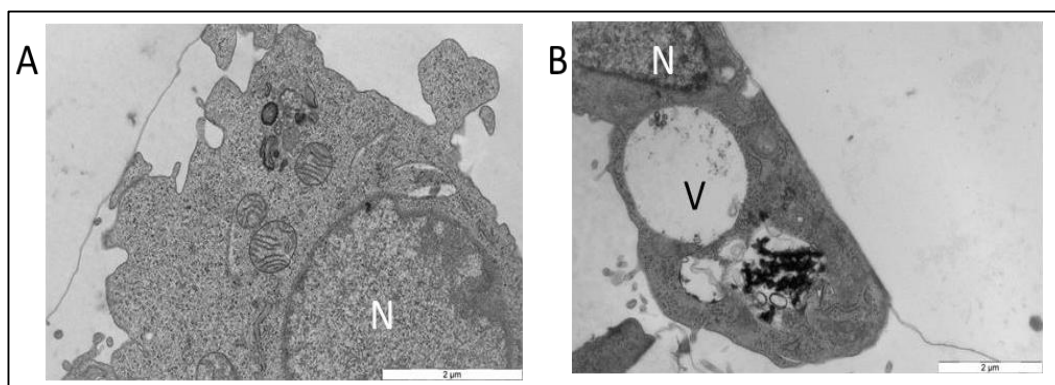


Fig 3: Electron microscopy on U87 cell line after E2 (100 μ g/ml) treatment for 24H.

0.1mm sections were prepared and analyzed by electron microscopy. N: Nucleus; V: vacuole. Figure 3. A shows a normal cytoplasm, nucleus and mitochondria while Figure 3.B is a representative micrograph of what has been observed in E2 treated cells: A vacuolization of the cytoplasm indicates the potential presence of autophagy. Bar: 2 μ m In A, non-treated cells have a dense cytoplasm without any vacuoles and normally appearing mitochondria. In contrast, treated cells (Figure 3.B) contain large cytoplasmic vacuoles comparable to autophagosomes, suggesting that E2 might induce autophagy in U87 cells. To further investigate this hypothesis, western blotting analysis was carried out to detect LC3II, the conjugated form of LC3I, the Microtubule-associated protein 1A/1B-light chain 3 protein, recruited to autophagosomal membranes during autophagy (Tanida *et al*, 2008) [14].

3.4 Expression and conversion of LC3 I in LC3 II in U87 cells after E2 treatment

A reliable method for monitoring autophagy and autophagy-related processes, including autophagic cell death is to highlight the conversion of the cytosolic form of LC3 (LC3-I) to LC3 phosphatidylethanolamine conjugate (LC3-II) by western blotting (Tanida *et al*, 2008, Behrends, C., *et al*.

2010) [14]. U87 cells were treated with increasing concentrations of E2 ranging from 50 to 200 μ g/ml for 24H before protein extracts were analyzed by western blotting using LC3 antibodies. Results are presented in Figure 4.

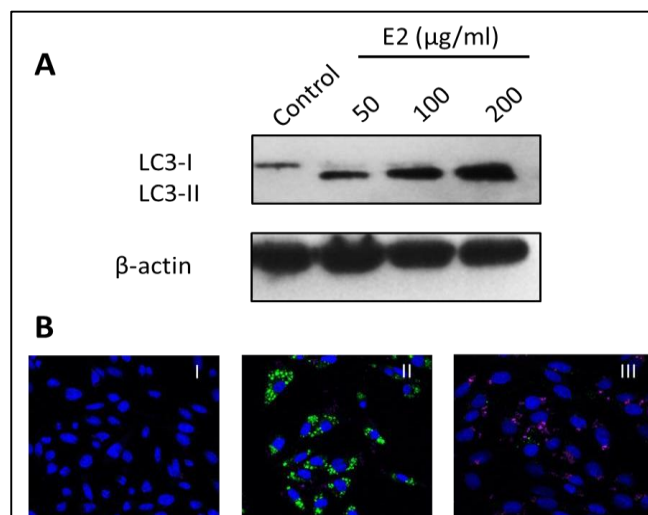


Fig 4: LC3 Detection in E2 treated cells. A: western blot analysis of LC3 expression in E2 treated U87 cells.

U87 cells were treated for 24H with different concentrations of E2 (50, 100 or 200 µg/ml). Protein extracts were prepared from the treated of control cells and analyzed by SDS-PAGE and western blot using an LC3 antibody. In control, only the LC3-I form is observed. LC3-II is detected at the E2 concentrations from 50 to 200µg/ml for 24H in a dose-dependent manner. B: LC3 labeling in U87 cells after E2 treatment. U87 cells were treated with E2 (50µg/ml) for 24H. Panel I shows U87 cells in control conditions. In panel II, Chloroquine, an autophagy inhibitor provokes an accumulation of LC3 in structure resembling autophagosomes. In panel III (E2 treated cells), a red fluorescence is observed in cell compartments.

As expected, in non-treated cells, an inhibitor of autophagy, only the soluble cytoplasmic LC3-I (18kDa) form was detected, showing the absence of autophagy in U87 native cells (Figure 4.A). In contrast, in cells treated with 50 to 200 µg/ml E2 concentrations, LC3-II (16kDa) form, a feature of autophagy process, is detected in a dose-dependent manner. These results strongly suggest that autophagy occurs during E2 treatment and that the vacuoles observed by electron microscopy are likely to be autophagosomes.

To visualize the localization of LC3 in E2 treated cells, we used the same LC3 antibody as in western blot for fluorescent microscopy. In control cells, we could expect a signal corresponding to the LC3-I basal level observed in western blot. LC3 labeling is probably too diffused to be seen (Figure 4.B, panel I). It could also be explained by the weak affinity of the anti-LC3 antibody for LC3-I, the major form present in absence of autophagy. In cells treated with chloroquine, an inhibitor of autophagy, LC3-II is accumulated in cells resulting in a strong green signal located in endosomes (Figure 4.B, panel II). In E2 treated cells, (Figure 4.B, panel III), the signal observed is red indicating that it could be located in acid compartments such as autophagolysosomes. Such artefacts of the fluorescence detection have already been described (Barth *et al.*, 2010) [3]. Both western blot and fluorescent microscopy results suggest that the E2 cytotoxic effect involves autophagy in the U87 cell line.

3.5 Regulation of expression and /or phosphorylation of proteins of the PI3K/Akt signaling pathway

The PI3K/Akt signaling pathway plays a major role in the proliferation and its deregulation is often documented in cancer cells (Zhang *et al.*, 2010) [17]. Thus, it was interesting to investigate the expression and regulation of different proteins of this pathway after an E2 treatment. Protein extracts of E2 treated cells (50, 100 or 200µg/ml) were prepared. After electrophoresis and western blots, membranes were incubated with several antibodies against proteins implicated in the PI3K/Akt pathway. LY294002, an inhibitor of the PI3K/Akt pathway, was used as control. Results are shown in Figure 5.

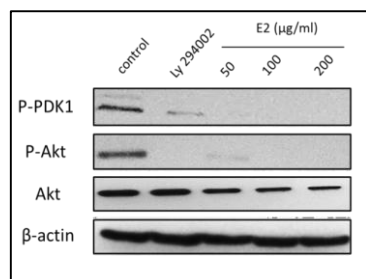


Fig 5: PI3K/Akt signaling pathway protein expression and regulation after E2 treatment

U87 cells were treated at different concentrations of E2 (50, 100 and 200 µg/ml). Ly294002 was used as an inhibitor of PI3K. Protein extracts were analyzed by SDS-PAGE and western blot using P-PDK1 (Ser241), P-Akt (Ser473) and β actin antibodies.

Both P-PDK1 and P-Akt are down-regulated upon treatment of U87 cells with E2. Akt itself seems to be also mildly down-regulated. Actin was used as gel loading control. The down regulation of these three proteins indicates that E2 contains molecules repressing the PI3K/Akt pathway by inhibiting the phosphorylation of both P-PDK1 and P-Akt. Thus, the inhibition of proliferation by E2 shown through the MTT assay (Figure 1) is confirmed by this experiment.

3.6 Cell cycle analysis after E2 treatment

The E2 extract contains several molecules and it might act at different levels of the cell proliferation. In order to see the effect of E2 on the cell cycle, we analyzed propidium iodide labeled cells by cytometry on one hand. On the other hand, cyclin D1 expression was analyzed by SDS-PAGE and western blot, with a cyclin D1 antibody. Results are shown in Figure 6.

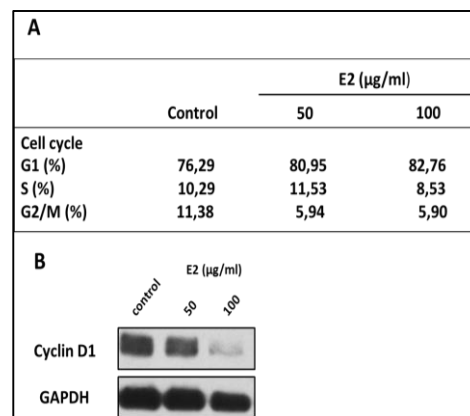


Fig 6: Cell cycle analysis and cyclin D1 expression in E2 treated cells

A: Cells treated with or without E2 (50µg/ml or 100µg/ml) for 24h were labeled with propidium iodide and analyzed by cytometry. B: Western blot analysis of cyclin D1 expression. Cells were treated with E2 at two concentrations (50 or 100µg/ml) for 24 H. Protein extracts were prepared and analyzed by SDS-PAGE 8%. GAPDH was used as a gel loading control in absence of treatment, 76.29% of U87 cells are in phase G1, 10.29% in phase S and 11.38% are in the G2/M phase (Figure 6.A). After E2 treatment (50 or 100 µg/ml) for 24H, the number of cells in the G1 phase seems to increase with the E2 concentration used. The number of cells in the G2 phase decreases from 11.38% to 5.9%. These results seem to indicate that E2 provokes an increasing number of cells blocked in the G1 phase. These data are in accordance with the fact that E2 blocks proliferation. In addition, cyclin D1, a key protein in the cell cycle regulation is down regulated by E2 (Figure 6.B).

4. Discussion

Klainedoxa Gabonensis is a plant widely used in traditional medicine in Congo and other African countries for several diseases ranging from diarrhea, sterility, and dermatitis to several types of cancers (Nkanwen *et al.*, 2013) [10]. The aim of this study was to document the effects of an aqueous extract of KG using the glioblastoma U87 cell line used as a model of cancer cell line. A previous study has shown that a

methanolic extract of KG has anti-bacterial activity on several bacteria. So far, all the studies have described various properties of KG organic extracts but none actually focused on the aqueous extract properties even though it corresponds to the popular and traditional uses by the population and traditional healers. Our study has determined for the first time the IC₅₀ of 65 µg/ml of the aqueous extract (E2) on the glioblastoma U87 cell line. Previous studies have characterized the types of molecules present in KG organic extracts. A new lanostane triterpenoid, 2-hydroxy-24-methylenelanostan-1,8-dien-3-one named klainedoxalano-tenone with one new steroid, 6-O-acyl-β-d-glucosyl-β-sitosterol named klainedoxasterol together with ten known compounds including six triterpenoids, two steroids and two tannins were isolated from the stem bark of KG (Nkanwen *et al.*, 2013) [10]. Moreover, Methyl gallate has been found to have anti-cancer activities on C6 and U373 glioma cells (Lee *et al.* 2013) [8]. Other studies have also documented the effects of acid gallic and quercetin on several cancer cell lines (Pallauf *et al.*, 2013; Aoki *et al.*, 2007; Psahoulia *et al.*, 2007) [12, 2, 13]. An HPLC analysis and fractionation of E2 will be carried out in the future to determine what molecule is responsible for the effects described in this work.

We also characterized the type of cell death induced by E2 in U87 cell line at cellular and molecular levels. Cytometry experiments showed that apoptosis, a phenomenon often described in the effect of anti-cancer drugs, is not involved (Hickman, 1992; Kanzawa *et al.* 2004) [6, 7]. Instead, E2 seems to provoke autophagy: a strong vacuolization of the cytoplasm is observed by electron microscopy and the conversion of LC3-I in LC3-II, a signature of autophagosomes formation, is also present in E2 treated U87 cells. Furthermore, a down-regulation of P-PDK1, P-Akt and Akt, three proteins of the PI3K/Akt pathway, has been observed on U87 cell line indicating that E2 blocks the cell proliferation.

E2, also acts on the cyclin D1 protein expression as shown by the western blot experiments.

Further work will aim to characterize the effects of E2 on other cancer cell lines to determine the action spectrum of E2. Other studies will also be necessary to characterize the specificity of E2 on cancer versus normal cells.

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