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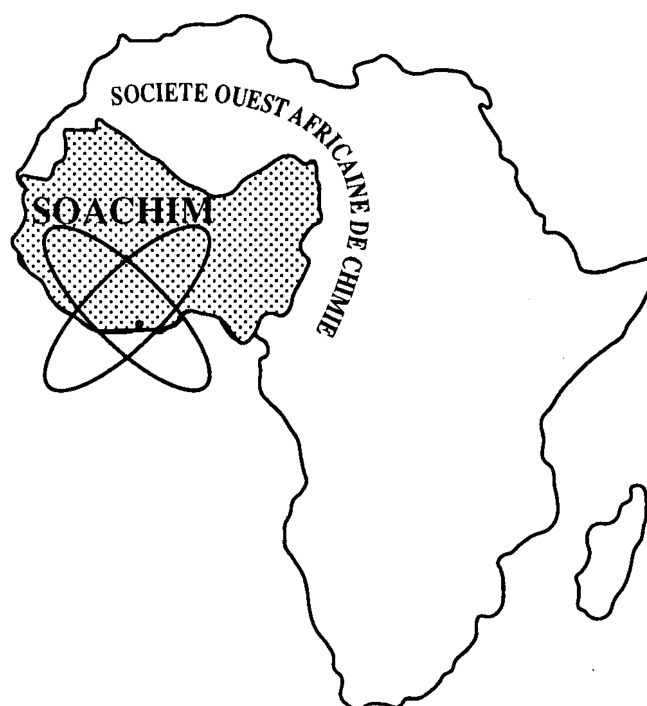

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Antiradical and anticancer activities of extracts of *Euphorbia hirta* L. (Euphorbiaceae) from Burkina Faso

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Abstract: *Euphorbia hirta* L. is a medicinal plant traditionally used around the world for the treatment of various pathologies. Studies reported in the literature showed that this plant species has numerous pharmacological properties, including antioxidant and antiproliferative properties which could be due to the presence of phenolic compounds. This study aimed to evaluate the anti-free radical and anticancer properties of *Euphorbia hirta* L. extracts on U937 and K562 cancer cell lines. The antiradical activity was realized by using the DPPH method and the antiproliferative effect was evaluated using the trypan blue method. Apoptotic activity was measured by Hoechst and Mito Tracker Red staining methods. The obtained results showed that ethyl acetate (AcOEt) and butanol (BuOH) extracts had the highest significant antiradical activities with IC_{50} values of 3.92 ± 0.01 µg/mL and 7.48 ± 0.07 µg/mL than the reference compound Trolox (IC_{50} = 3.81 ± 0.04 µg/mL), Cytotoxicity results revealed that acetate extract possesses very remarkable antiproliferative and apoptotic effects with IC_{50} values of 16.60 ± 4.40 µg/mL and 29.70 ± 2.10 µg/mL on U937 cancer cells. These results demonstrated that ethyl acetate extract of *Euphorbia hirta* L. *is* a promising candidate for the formulation of anticancer phytomedicines.

Keywords: Medicinal plant*;* DPPH; antioxidant; cytotoxicity; cancer; flavonoids

Activités antiradicalaire et anticancéreuse des extraits de *Euphorbia hirta* L. (Euphorbiacé) du Burkina Faso

Résumé : *Euphorbia hirta* est une plante médicinale utilisée traditionnellement dans beaucoup de contrées pour le traitement de diverses pathologies. Il est rapporté dans la littérature que l'espèce végétale possède de nombreuses propriétés pharmacologiques telles que antioxydantes et antiprolifératives. Ces activités biologiques sont imputables aux composés phénoliques, particulièrement aux flavonoïdes. Cette étude a porté sur l'évaluation des propriétés antiradicalaire et anticancéreuse sur deux lignées cellulaires cancéreuses (U937 et K562) des extraits de *Euphorbia hirta.* Pour ce faire, l'activité antiradicalaire a été évaluée par la méthode DPPH. L'effet antiprolifératif a été évalué par la méthode de trypan bleu. L'activité apoptotique a été mesurée par deux méthodes complémentaires telles que les colorants Hoechst et Mito Tracker Red. Comparativement au Trolox (IC_{50} = 3,81 ± 0,04 µg/mL), les extraits à l'acétate d'éthyle (AcOEt) et au butanol (BuOH) ont montré une activité antiradicalaire hautement significative avec des IC_{50} de 3,92 ± 0,01 µg/mL et 7,48 ± 0,07 respectivement. Les résultats de la cytotoxicité ont révélé que AcOEt possède des effets antiprolifératif et apoptotique très remarquables avec des IC_{50} de 16,60 ± 4,40 µg/mL et 29,70 ± 2,10 µg/mL respectivement sur la lignée U937. Ces résultats démontrent que le fraction acétate de *Euphorbia hirta* L. serait donc un candidat potentiel dans la formulation de phytomédicaments anticancéreux.

Mots clés : *Plante médicinale ; DPPH• ;* antioxydant ; cytotoxicité ; cancer ; flavonoïdes

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1. Introduction

According to the World Health Organization, cancer is the second leading cause of death in many countries, with approximately 8.8 million deaths in 2015 and 9.6 million in 2018 [1, 2]. The number of new cancer cases worldwide is rising rapidly, from 12.7 million in 2008 to 14.1 million in 2012, and 18.1 million in 2018 [2]. Statistics indicate that this number could rise to 19.3 million by 2025. In Burkina Faso, 12,045 new cancer cases were recorded in 2020, with 8,695 deaths [3]. The development of cancer is associated with several factors, including oxidative stress. Indeed, numerous epidemiological studies have demonstrated that oxidative stress plays a role in the pathogenesis of various chronic non-communicable diseases, including cancer [4, 5]. Free radicals are potential carcinogens as they facilitate mutagenesis, tumor promotion, and the spread of cancer [6]. However, previous studies have shown that the incidence of leukemia and lymphoma could be considerably reduced by a diet rich in bioactive phytochemicals that influence cancer development [7]. In this regard, the identification of natural bioactive compounds that suppress proliferation and/or induce apoptosis could be a useful complementary strategy for controlling the development and progression of cancer. Currently, in addition to conventional anti-tumor drugs used in chemotherapy, there is growing interest in plant-derived bioactive compounds that could play a role in cancer treatment. The critical role of selectivity in cancer chemotherapy is highlighted by the pharmacological screening of natural molecules that avoid cytotoxicity against normal cells [7]. Evidence from in vitro and in vivo studies have shown that dietary consumption in antioxidants is associated with a lower risk of cardiovascular disease and cancer [8, 9]. *Euphorbia hirta L.* is an herbaceous plant found in very sunny environments. It grows annually on dry land and in wetter areas. Raja et al. and Patil et al. showed in 2011 that *Euphorbia hirta* L. has antiproliferative properties on Hep2 and EL-4 cancer cells [10, 11]. However, a few studies have been done on the antiproliferative and apoptotic activities of the polyphenols contained in the leafy stems of this species [7, 12-15]. The objective of this study is to investigate the cytotoxic effects of phenolic compound-enriched extracts of *Euphorbia hirta* on two human cancer cell lines (U937 and K562).

2. Materials and Methods

2.1. Plant Material and Extraction

The whole plant excluding the roots of *Euphorbia hirta* L. was collected in February 2014 in Ouagadougou, Burkina Faso, at GPS coordinates 12°23'33.6"N; 001°32'20.8"W. The plant was identified by the late Professor Jeanne Millogo, a botanist at the Laboratory of Biology and Vegetable Ecology at Joseph KI-ZERBO University. The leafy stems were washed and dried at room temperature in a ventilated area. The dry material was then ground into a fine powder using an electric grinder (IRSAT, Ouagadougou, Burkina Faso). Finally, extraction was carried out according to a protocol described in the laboratory [16]**,** with minor modifications. A total of four (04) extracts were prepared: DCM (dichloromethane fraction)**;** AcOEt (ethyl acetate fraction); BuOH (butanol fraction), and H_2O (residual aqueous fraction).

2.2. Biological Material

Acute myeloid leukemia U937 and chronic myelogenous leukemia K562 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Cells were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS; Sigma-Aldrich, Borneum, Belgium) and 1% (v/v) antibiotic/antimycotic (penicillin, streptomycin, and amphotericin; BioWhittaker, Verviers, Belgium) at 37°C and 5% CO_2. Cells were routinely checked for mycoplasma contamination. All experiments were conducted on cells in exponential growth, in a culture medium containing 10% (v/v) FCS.

2.3. Evaluation of antiradical activity

The DPPH• free radical was used to assess the antiradical potential of the different extracts. In this study, the antiradical activity of all *Euphorbia hirta* L. extracts were evaluated by measuring the optical densities of the DPPH• radical after it was exposed to free radical scavenging substances. For that, 50 µL sample of each extract, prepared in methanol at different concentrations, was mixed with 200 µL of the DPPH• radical. After incubation in the dark for 10 minutes, the absorbance of the reaction mixture was measured at 517 nm using a UV-visible spectrophotometer (SPECTROTOstar NANO, BMG LABTECH, Ortenberg, Germany). Trolox was used as the standard. The positive control contained 50 µL of methanol with DPPH• and the blank contained methanol only. IC_{50} values, measured three times and expressed in µg/mL, were determined using the equations of the calibration curves for each sample. IC_{50} is the value of the extract concentration that would trap 50% of the free radicals contained in the

reaction mixture. More this value is lower and more the antiradical activity of the extract is better.

2.4. Evaluation of anticancer Activity

2.4.1. Antiproliferative Effect

The antiproliferative effect was assessed using the trypan blue exclusion test to determine the number of viable cells in the suspension culture medium [17]. Cancer cell lines U937 and K562 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic and incubated at 37°C and 5% CO_2. Cells were allowed to grow exponentially 24 hours before treatment. For each treatment, cells were seeded in *6-well* plates at 200,000 cells/mL; 2 mL/well [18]. Different concentrations of the crude extract prepared in DMSO were added to each well, while the negative control received the same volume of DMSO. In all experiments in this study, the volume of DMSO used did not exceed 0.25% to avoid significant toxicity of the solvent on the cells tested. At regular time intervals of 24 h, 48 h, and 72 h, 20 µL of the suspended cells were added to 20 µL of trypan blue [17, 18]. 20 µL of the mixture was placed on a slide for reading by Cedex XS. The number of cells surviving of each extract and the percentage of viability are provided by computer-controlled software.

2.4.2. Apoptotic effect

The induction of apoptosis by the different extracts was assessed using two different methods: Hoechst staining and Mito Tracker Red (MTR) staining to measure mitochondrial membrane potential (MMP) [18, 19]. Hoechst 33342 (Molecular Probes, Invitrogen) is a cell-permeant dye that binds to chromatin and allows the monitoring of nuclear morphology leading to the quantification of the number of cells with fragmented and/or condensed nuclei. Cell-permeant Mito Tracker Red (MTR; Invitrogen, Molecular Probes) accumulates in the mitochondria depending on the mitochondrial membrane potential. Living cells accumulate MTR dye in mitochondria, resulting to an increase of fluorescence intensity, while apoptotic cells exhibit reduced fluorescence intensity.

After exposure to the different extracts for 24 h and 48 h, 1 mL of suspended cells was incubated for 15 min at 37°C and 5% CO_2 with a precise volume of Hoechst 33342 solution at 1 µg/mL. At the end of incubation, the percentage of dead cells was estimated as the ratio of the number of cells with apoptotic fragmented nuclear morphology to the total number of cells in at least 3 random fields of 300 cells, according to equation 1, using a fluorescence microscope (Olympus, Hamburg, Germany).

$$\% \text{ Cells death} = \frac{\text{Number of cells with fragmented/condensed nuclei}}{\text{Total number of cells}} \text{x}100 \qquad (1)$$

The application of the MTR staining method follows the following procedure: After a regular treatment (24 h and 48 h), 500 µL of treated cells were incubated for 15 min at 37°C, 5% CO_2 with MTR (100 nM). At the end of incubation, the percentage of cells showing loss of mitochondrial membrane potential was estimated according to equation 2, using a FACS Caliber (Becton Dickinson, San José, California) set at 488 nm (emission: 585 nm; FL3-H: 625 nm). A total of 10,000 events were recorded using Cell Quest software (Becton Dickinson, San José, California), and further analysis was performed using FlowJo 10 software (Tree Star Inc., Ashland, OR, USA).

$$\% \text{ Cells death} = \frac{\text{Number of cells with MMP loss}}{\text{Total number of cells}} \text{x}100 \qquad (2)$$

3. Results and discussion

3.1. Antiradical Activity

The antiradical properties of the different fractions of *Euphorbia hirta* L. were evaluated by monitoring the behavior of $DPPH^{\bullet}$ radicals in the presence of each extract at different concentrations. The equations of the regression curves obtained were used to determine IC_{50}. The various IC_{50} obtained values are shown in **Table I**.

Table I shows that the best antiradical activities are found in the AcOEt and BuOH fractions, as shown by their lower IC_{50} values. The acetate fraction is the most active with a IC_{50} of 3.92 ± 0.01 µg/mL which is closely equal to the value of Trolox (IC_{50} = 3.81 ± 0.04 µg/mL). The antiradical properties of these extracts could be due to the presence of flavonoids which are able to inhibit the enzymes responsible for the production of reactive oxygen species (ROS) or to trap free radicals [20, 21]. This result is in accordance with that reported by Karanga et *al.,* 2017, who showed that *Euphorbia hirta L.* fractions are rich in phenolic compounds particularly flavonoids [16].

Due to the lowest IC_{50} value of AcOEt and BuOH extracts of *Euphorbia hirta L.*, further investigation on their anticancer properties has been conducted.

3.2. Anticancer Activity

3.2.1. Cytotoxic screening of different extracts

The anticancer properties of the two *Euphorbia hirta* L. extracts were assessed based on their ability to induce cell death and inhibit cell proliferation. To do this, the antiproliferative and apoptotic effects of these extracts at a concentration of 50 µg/mL were

studied on the two cancer cell lines, U937 and K562. The obtained results on the antiproliferative effect are shown in Figure 1.

Figure 1 shows the result of the antiproliferative tests of the AcOET and BuOH extracts on U937 and K562 cells after 24h, 48h, and 72h treatment times. One can see in Figure 1 that after 24 hours, the number of U937 cells treated with the AcOEt extract and the negative (DMSO) and positive (VP16) control wells indicates that the extract was able to block any attempt at cell proliferation completely. Overall, we found that the well containing AcOEt had a lower number (< 200,000 cells/mL) of cells seeded at t = 0 h after 24 h treatment. This result shows that the extract was able to prevent the cells from multiplication. The considerable reduction in the number of cells initially present in the well-treated with AcOEt shows that, in addition to its antiproliferative effect, it also has an apoptotic effect. In the same order as observed in the anti-radical test, the BuOH fraction showed a weak effect than AcOET one on the antiproliferative activities but inhibited significantly the proliferation of U937 cancer cells. Furthermore, in the wells treated with AcOEt, the number of K562 cells initially introduced decreased considerably, showing that the extract has an antiproliferative effect on the K562 cell line. However for the BuOH extract, the number of cells in the well treated with this extract and in the negative control well were similar, as can be seen from the proliferation curves for BuOH and the negative control (DMSO), which grew in the same order. This result shows that this extract has a very moderate antiproliferative effect on K562 cells. Subsequently, the apoptotic effects of the two *Euphorbia hirta* L. extract on the same cancer cell lines were assessed by quantifying the percentage of cells showing apoptotic nuclear fragmentation and confirmed by analysis of the loss of mitochondrial membrane potential (MMP) (Figure 2).

Figure 2 shows that, regardless of the method used, more than 80% of U937 cells incubated with AcOEt extract at 50 µg/mL died after 48 h of treatment. However, for K562 cells, the results obtained showed that AcOET also exhibited cytotoxicity, but to a lesser extent than U937. The percentage of cell death was around 40% at 50 µg/mL after 48 h, showing that U937 cells were more sensitive to treatment with AcOEt than K562 cells. As for BuOH, the percentages of dead U937 and K562 cells were similar to those obtained with the negative control (DMSO), proving that BuOH had no significant apoptotic effects on the two cancer cell lines. Furthermore, it was found that after 24 hours of treatment with etoposide (VP16) at a concentration of 30 µM, 100% of the U937 cells initially introduced into the well had died. However, K562 cells were less sensitive to VP16 toxicity, as shown by the low percentages of dead cells (around 40%) and the longer treatment time (48 h). This result agrees with those of the extracts and confirms the relatively low sensitivity of the K562 line to treatment compared to U937 with the different extracts.

In summary, cytotoxic screening revealed that AcOEt had very remarkable antiproliferative and apoptotic effects on the U937 cancer cell line compared with the VP16 positive and DMSO negative controls. BuOH showed no remarkable cytotoxic effects on either cancer cell line. Subsequent cytotoxic analyses were therefore focused on the AcOEt extract on the U937 cancer cell line.

3.2.2. Dose-dependent antiproliferative effects of AcOEt extract

The antiproliferative activity of AcOEt extract was assessed at different concentrations on acute myeloid cells (U937) by the trypan blue exclusion assay at regular time intervals of 24 h, 48 h, and 72 h. The results obtained are shown in Figure 3.

Concerning the dose-dependent antiproliferative effect of the extract on lymphoma, the results presented in Figure 3 show that the U937 cancer cell line is sensitive to treatment with AcOEt, which showed a remarkable effect on cell proliferation. Compared with the negative control (0 µg/mL), the extract significantly blocked the proliferation of U937 cells in a dose- and time-dependent. Indeed, after treatment at different concentrations for 72 h, the number of viable cells in the wells treated with the high concentrations (50 µg/mL) did not increase, but rather the opposite, characterized by decreasing curves that tended towards zero after 72 h.

Table I: Antiradical activities of different fractions of *Euphorbia hirta* L. and Trolox

IC_{50} (µg/mL)				
***E. hirta L.* fractions**				**Trolox**
DCM	**AcOEt**	**BuOH**	**Residual H_2O**	
22.83 ± 0.41	3.92 ± 0.01	7.48 ± 0.07	27.59 ± 0.28	3.81 ± 0.04

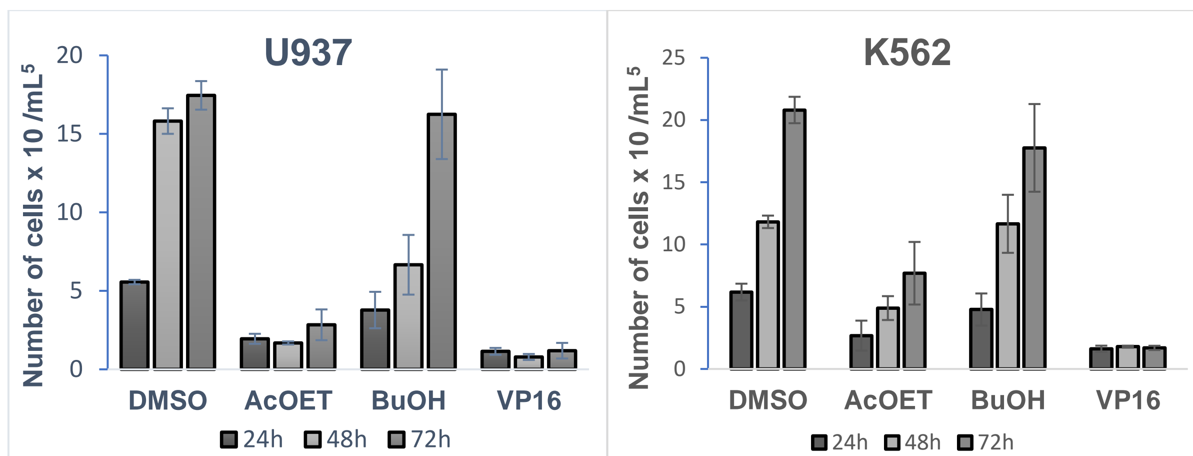

Figure 1: Antiproliferative effects of different extracts of *Euphorbia hirta* L. at 50 µg/mL and DMSO (used as negative control) on U937 and K562 cells after 24 h, 48 h, and 72 h. AcOEt: Ethyl acetate extract from leafy stems; BuOH: Butanol extract from leafy stems; VP16: etoposide (used as positive control).

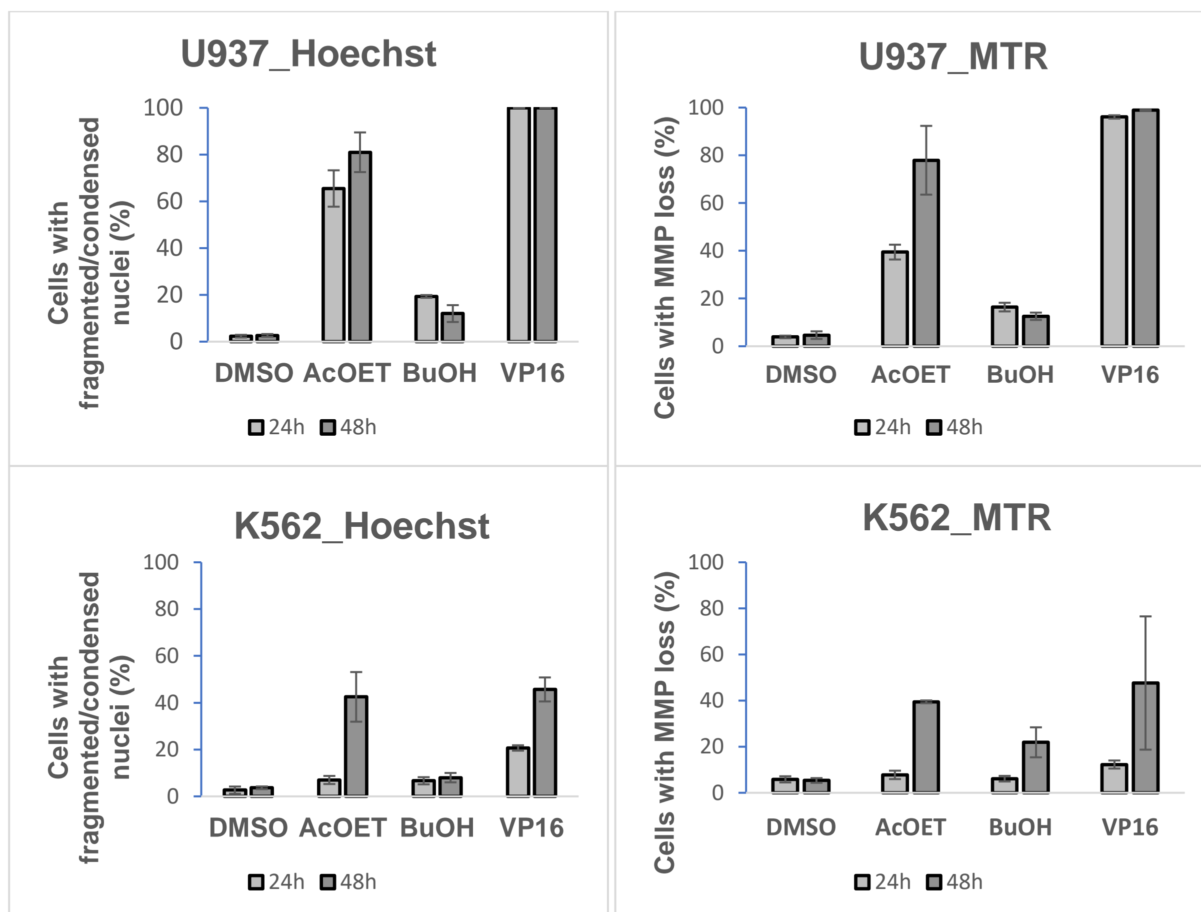

Figure 2: Apoptotic effects of different extracts at 50 µg/mL and DMSO (used as negative control) on U937 and K562 cells by Hoechst and Mito Tracker Red staining analysis for 24 h and 48 h. AcOEt: Ethyl acetate extract from leafy stems; BuOH: Butanol extract from leafy stems; VP16: etoposide (used as positive control).

This decrease is proof that the number of cells initially introduced into the wells had decreased; therefore, the extract not only prevented the cells from multiplication but also induced apoptotic cell death. For lower concentrations (≤ 30 µg/mL), the curves in the treated wells began to increase but remained below those of the negative control. This result shows that the extract partially inhibits cell proliferation. The relative position of the different curves in the treated wells compared with the negative control (curves below that of the DMSO) shows that AcOEt inhibits the growth of U937 cells.

In summary, these results show that the antiproliferative activity of the extract is linked to both the dose administered and the treatment time.

3.2.3. Dose-dependent apoptotic effects of AcOEt extract

Concerning the apoptotic effect of the different extracts on U937 cells, the results presented in Figure 4 show that the percentages of apoptotic cells following incubation for 24 h and 48 h at increasing concentrations of extracts are significantly different compared with the negative control (DMSO).

Figure 4 shows that as the extract concentration increases, the percentage of dead cells also increases, regardless of the method used. This percentage reached approximately 85% for a concentration of 50 µg/mL of the extract. These results agree with those obtained previously. The analysis therefore shows that AcOEt significantly induces apoptosis in U937 cells in a dose-dependent compared to the negative control (0 µg/mL). The percentages of dead cells after 24 h were lower than those obtained after 48 h of exposure to the toxic substance. The optimal treatment time for the cells could therefore be set at 48 hours. To support the overall cytotoxic results, the IC_{50} values of the antiproliferative and apoptotic potential of the AcOEt extract against U937 cancer cells were calculated. The algebraic values are shown in Table II.

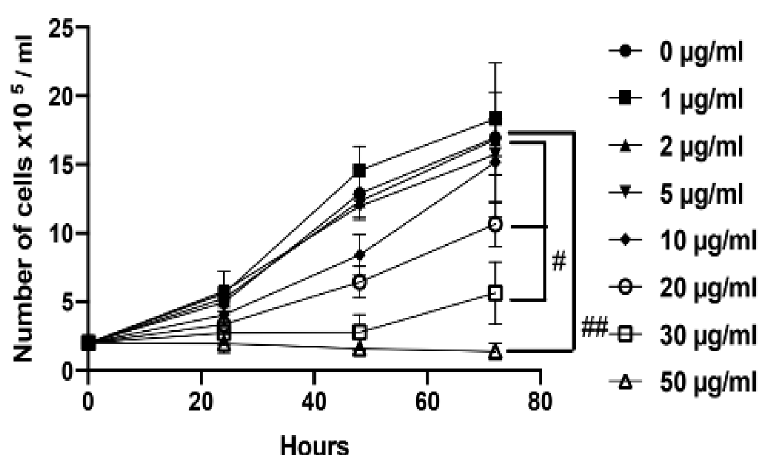

Figure 3: Antiproliferative effects of AcOEt at different concentrations and DMSO (used as negative control) on U937 cells after 24 h, 48 h, and 72 h.

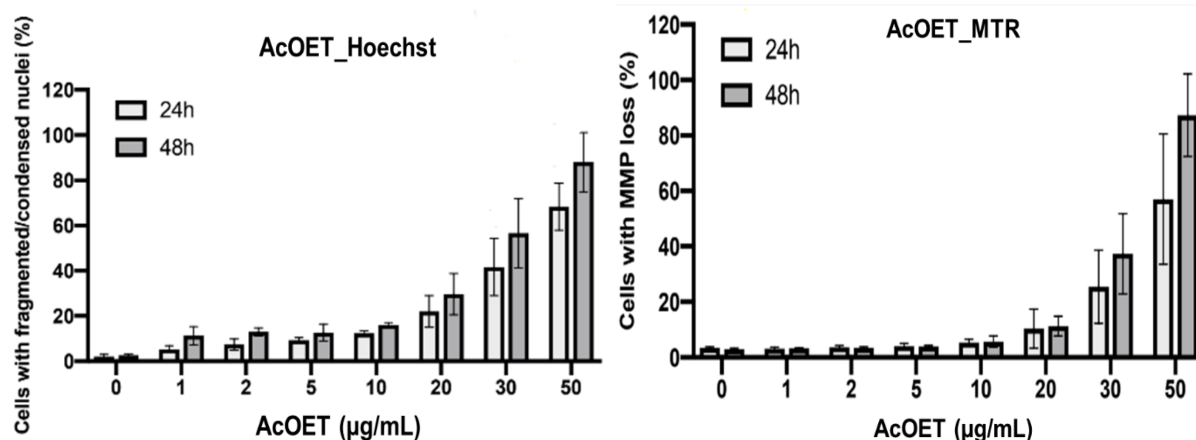

Figure 4: Apoptotic effects of AcOEt at different concentrations compared with DMSO (used as a negative control) on U937 cells using Hoechst and MitoTracker Red (MTR) staining methods for 24h and 48h.

Table II: IC_{50} (µg/mL) values of antiproliferative and apoptotic activities of AcOEt on cancer cell models U937

Investigated activities	Methods	Hours	AcOEt
			IC_{50} (µg/mL)
Antiproliferative effect	Trypan blue	24	20.3 ± 2.4
		48	16.6 ± 4.4
		72	24.5 ± 5.3
Apoptotic effect	Hoechst	24	35.4 ± 9.2
		48	29.7 ± 2.1
	Mito Tracker Red	24	38.4 ± 6.9
		48	33.9 ± 2.5

Analysis of the results in Table II reveals that AcOEt inhibits cancer cell proliferation very significantly, with a maximum inhibition rate that varies according to the concentration of the extract and the incubation time, which is set at 24 h, 48 h, and 72 h. The low values (≤ 50 µg/mL) of the IC_{50} show that the extract has a very remarkable antiproliferative activity against the U937 line. Furthermore, this activity was maximal at a concentration of 16.6 ± 4.4 µg/mL and after 48 h of treatment. This result therefore suggests that 48h is the optimal time for U937 cancer cells treatment with AcOEt. The study of the cytotoxic potential of AcOEt on lymphomas, carried out using the two complementary Hoechst and Mito Tracker Red staining methods, highlighted the high cytotoxicity of the extract, as shown by the low IC_{50} values (Table II). Regardless of the analytical method used, the extract was very active after 48 h of treatment, with an IC_{50} of 29.7 ± 2.1 µg/mL using the Hoechst staining method. The notable antiproliferative activity and apoptotic effect of the AcOEt extract of *Euphorbia hirta* L. could be justified by a synergy of action of certain compounds such as the flavonoids contained in the extracts highlighted in previous studies [16]. Indeed, studies carried out on plant extracts enriched with polyphenols and flavonoids in particular have highlighted the anti-cancer properties of these chemical groups. For example, Sawadogo et al. showed in 2011 that a flavonoid-enriched extract of *A. macrostachya* inhibited KB cancer cell proliferation by 95% at a concentration of 10 µg/mL [22]. The same author showed in 2015 that polymethoxyflavone, a flavonoid isolated from *L. ukambensis*, has relevant antiproliferative and pro-apoptotic effects against U937 cells [18]. Recent research has shown that pro-anthocyanidins (tannins) present in grapes induce apoptosis in U937, HL-60, and Jurkat [23].

4. Conclusion

The present study aimed to evaluate the antiradical and anti-cancer properties of the different extracts of *Euphorbia hirta* L. The ethyl acetate fraction ($IC_{50} = 3.92 \pm 0.01$ µg/mg) showed the best antiradical activity compared to Trolox (IC50 = 3.81 ± 0.04 µg/mg). The anti-cancer properties were evaluated on two cancerous cells that are U937 and K562. The results show that the ethyl acetate extract of the leafy stems of *Euphorbia hirta* L exhibits significant antiproliferative and apoptotic effects on the U937 cancer cell line. These results demonstrated that acetate fraction of *Euphorbia hirta* L. could therefore be used in the development of improved traditional medicines for the treatment of blood cancer.

5. Acknowledgments:

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