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ULPC-MS/MS phenolic quantification and *in vitro* anticancer potential of *Gmelina arborea* Roxb. (Verbenaceae)

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ABSTRACT

Phenolic secondary metabolites are recognized as excellent antioxidants and are subject of numerous scientific studies. The present study was aimed firstly, to quantify the phenolic constituents contained in the hydroacetic crude extract obtained from *Gmelina arborea* leaves by UPLC-MS/MS and secondly, to investigate *in vitro* its anticancer potential against 6 cancerous cell lines. The quantitative analysis revealed a significant abundance in flavonoids (75.50%) and a presence of hydroxycinnamic acid derivatives (24.49%). The results of cytotoxicity survey clearly indicate that the hydroacetic crude extract shows a good anticancer activity against C6 glioma cells.

Keywords: *Gmelina arborea*, phenolic constituents, UPLC-MS/MS, anticancer activity.

INTRODUCTION

The cancer is a disease characterized by an anarchical proliferation of abnormal cells capable to invade and to destroy healthy cells of the organism. In 2012, it was estimated to 14.1 millions, the number of new cases of cancer and to 8,2 millions, the number of death caused by this disease [1]. In general, the use of African medicinal plants in the folkloric treatment of the cancer and those of Côte d'Ivoire notably, is insufficiently documented. Côte d'Ivoire presents an excellent biodiversity flora to which is added a rich traditional pharmacopeia. Among its plants with medicinal properties which compose it, *Gmelina arborea* finds its place. Indeed, *G. arborea* is widely used traditionally in the treatment of diarrhea, diabetes, hypertension, malaria and others diseases [2, 3]. It is a fast growing tree reaching 30 m in height with simple green leaves, oval and opposite [4]. This plant species contains antioxidant phenolic compounds [5, 6]. The effects of its flavonoids on the antioxidant activity and the erythrocytes osmotic stability were studied [7]. The results of this survey are reported here, like a contribution in the research of new anticancer agents from plant origin.

MATERIALS AND METHODS

Preparation of hydroacetic crude extract (HACE)

G. arborea leaves were collected at the Nangui Abrogoua University in Abidjan and authenticated at the National Floristic Center (CNF), in the Félix Houphouët-Boigny University (Abidjan/Côte d'Ivoire) by the eminent Professor Laurent AKÉ-ASSI in June 2010. A voucher specimen has been deposited at the LCBOSEN for future reference. The leaves were cleaned, dried in an air conditioned room and reduced in powder to obtain the crude extract. HACE was obtained from plant powder (5 g) previously treated by petroleum ether (50 ml) (Sigma-Aldrich), macerated in acetone-water mixture (100 ml, 70: 30 v/v) and left under magnetic agitation during 24 h. The filtrate was lyophilized for the phenolic quantification and the cytotoxicity survey.

PHENOLIC QUANTIFICATION

The lyophilisate (1 mg) was dissolved in methanol-water mixture (1 ml, 50: 50 v/v) by a vortex. The mixture was filtered with a filter (0.2 µm porosity) then diluted to the 1/10th for injection. LC-MS analysis was performed on a Waters Acquity UPLC chain coupled to a mass spectrometer (Bruker Amazon X) by UPLC-ESI-IT-MSn. A C18 column Waters Acquity BEH 150 × 1 mm reverse phase particles (1.7 µm diameter) was used at 35°C. The mobile phase was constituted of a binary system of solvents A (H₂O/HCO₂H, 1%) and B (CH₃OH/HCO₂H, 1%). The column flow rate was set at 0.08 ml/min with a gradient program as shown in Table 1. The injection volume was 0.5 µl.

Table 1: Gradient mode during 43 min

| Time (min) | 0 | 10 | 12 | 25 | 30 | 35 | 38 | 43 |
|------------|----|----|----|----|----|----|----|----|
| % A | 98 | 70 | 70 | 25 | 10 | 10 | 98 | 98 |
| % B | 2 | 30 | 30 | 75 | 90 | 90 | 2 | 2 |

UV-visible spectra were recorded from 250 to 600 nm with a pitch of 2 nm. The phenolic compounds contents were calculated from the integration of the surfaces of the molecular peaks at wavelength of maximum absorbance of each compound in the UV; and an external calibration with the corresponding standard or a neighboring molecule. Quantification was made while referring to gallic, ellagic, caffeic, protocatechuic acids, quercetin-3-O-glucoside, and epicatechin (Sigma-Aldrich). The contents were expressed as equivalent to reference molecule for the molecular family.

CELL CULTURES

Cancerous cells were cultured in «Eagle» culture medium modified by Dulbecco (DMEM) (Sigma-Aldrich) supplemented with fetal calf serum (FCS) (10%), 2 mM L-glutamine and Penicillin Streptomycin (1%) at 37°C in a humidified atmosphere with 5% CO₂. Breast cancer stem (MDA-MB 231), skin cancer stem (melanoma and MDA-MB 435 B16F10), colon cancer stem (Caco-2) and brain cancer stem (glioma cells SNB75 and C6) were used to study the cytotoxicity activity of HACE. They were purchased from American Type Culture Collection (ATCC).

IN VITRO CYTOTOXICITY STUDY

Cell survival was assessed by colorimetric MTT (3 bromide (4,5-dimethylthiazol-2-yl)-2,5-diphenyl) test (Sigma-Aldrich) [8]. Cells were cultured in 100 µl environment and incubated for 24 hours. After obtaining an adherent cell layer, the environment was decanted and replaced with the extract at concentrations ranging from 0.125 to 2 mg/ml. After 72 hours, the wells were emptied. The cells were washed with PBS and incubated with 100 µl of culture containing MTT (2 mg/ml) for 4 hours at 37°C and 5% CO₂. Color intensity produced indicates the relative number of living cells, determined at 570 nm using a micro plate reader (Multiscan Labosystems). The viability percentage (VP) was calculated with the equation.

$$VP = (\text{Abs sample} / \text{Abs control}) \times 100.$$

STATISTICAL ANALYSIS

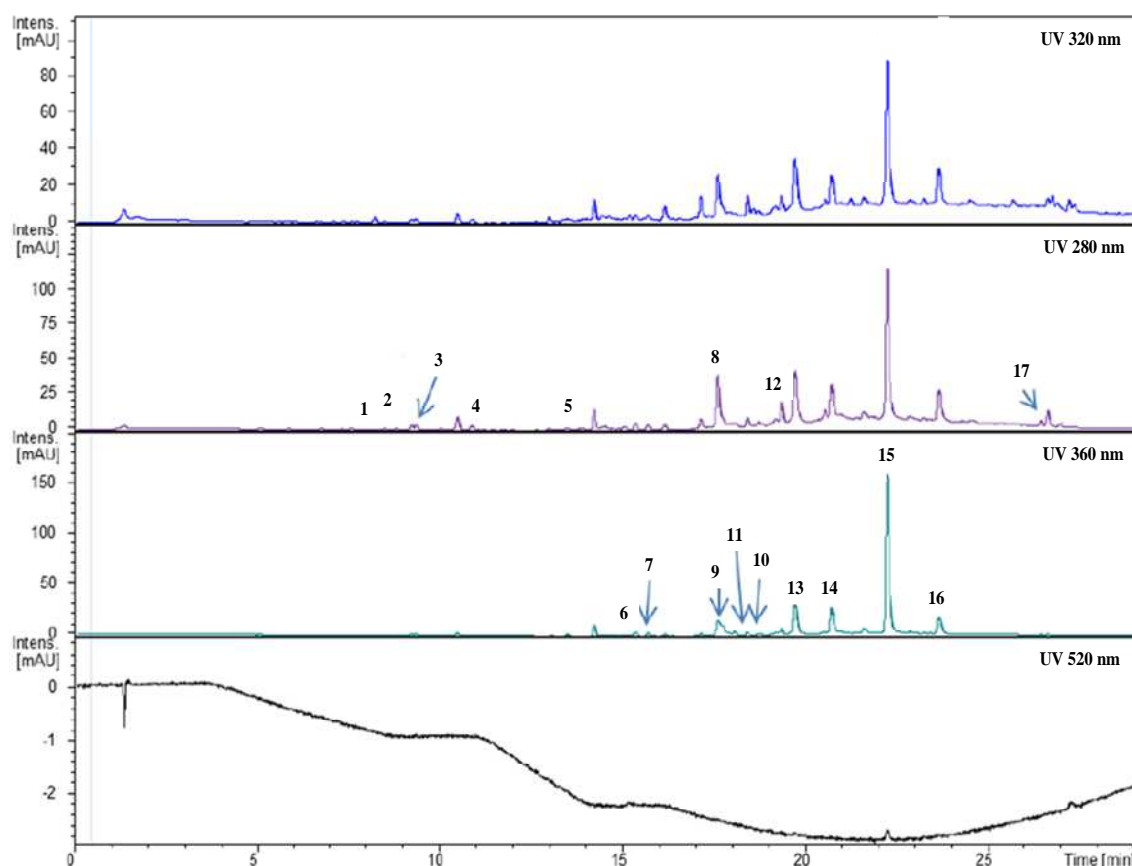
The *in vivo* data was analyzed by One-Way Analysis of Variance (ANOVA) followed by Turkey-Kramer multiple comparison tests, and $p < 0.05$ was considered significant [9]. Statistical analysis was implemented using SAS version 9.1. [10].

RESULTS AND DISCUSSION

PHENOLIC QUANTIFICATION

Chromatograms (Figure 1) show 17 extractable phenolic compounds detected^o: hydroxycinnamic acid derivatives, flavones and flavonols.

Figure 1: Chromatograms of HACE at 320, 280, 360 and 520 nm



The extractable phenolic compounds contents obtained are summarized in Table 2.

Table 2: Phenolic quantification results from *Gmelina arborea*

| Pics | RT (min) | λ (nm) | intégration | Area | Compound identified | Molar mass | Molecular formula | Content (mg/g of powder) |
|--------------|----------|----------------|-------------|--------|---------------------|------------|----------------------|--------------------------|
| 1 | 8 | 320 | | 1.85 | HCAD* | 338 | $C_{16}H_{18}O_8$ | 0.07 |
| 2 | 8.5 | 320 | | 6.13 | HCAD* | 338 | $C_{16}H_{18}O_8$ | 0.22 |
| 3 | 9.3 | 320 | | 17.95 | HCAD* | 488 | $C_{25}H_{28}O_{10}$ | 0.58 |
| 4 | 10.9 | 320 | | 22.75 | HCAD* | 354 | $C_{16}H_{18}O_9$ | 0.54 |
| 5 | 13.5 | 320 | | 9.29 | HCAD* | 326 | $C_{15}H_{18}O_8$ | 0.20 |
| 6 | 15.3 | 360 | | 21.86 | Flavone | 564 | $C_{26}H_{28}O_{14}$ | 0.59 |
| 7 | 15.7 | 360 | | 19.77 | Flavone | 564 | $C_{26}H_{28}O_{14}$ | 0.54 |
| 8 | 17.6 | 320 | | 209.86 | HCAD* | 548 | $C_{27}H_{32}O_{12}$ | 7.67 |
| 9 | 17.9 | 320 | | 57.69 | HCAD* | 624 | $C_{29}H_{36}O_{15}$ | 1.73 |
| 10 | 18 | 360 | | 20.53 | Flavonol | 448 | $C_{21}H_{20}O_{11}$ | 0.44 |
| 11 | 18.4 | 360 | | 20.66 | Flavonol | 464 | $C_{21}H_{20}O_{12}$ | 0.46 |
| 12 | 19.3 | 320 | | 82.00 | HCAD* | 494 | $C_{22}H_{22}O_{13}$ | 2.70 |
| 13 | 20 | 360 | | 185.76 | Flavone | 448 | $C_{21}H_{20}O_{11}$ | 7.42 |
| 14 | 21 | 360 | | 133.79 | Flavone | 448 | $C_{21}H_{20}O_{11}$ | 5.24 |
| 15 | 22.5 | 360 | | 889.57 | Flavone | 286/572 | $C_{15}H_{10}O_6$ | 25.28 |
| 16 | 23.9 | 360 | | 95.33 | Flavone | 270 | $C_{15}H_{10}O_5$ | 3.61 |
| 17 | 26.4 | 320 | | 17.67 | HCAD* | 356 | $C_{16}H_{20}O_9$ | 0.43 |
| Total | | | | | | | | 57.72 |

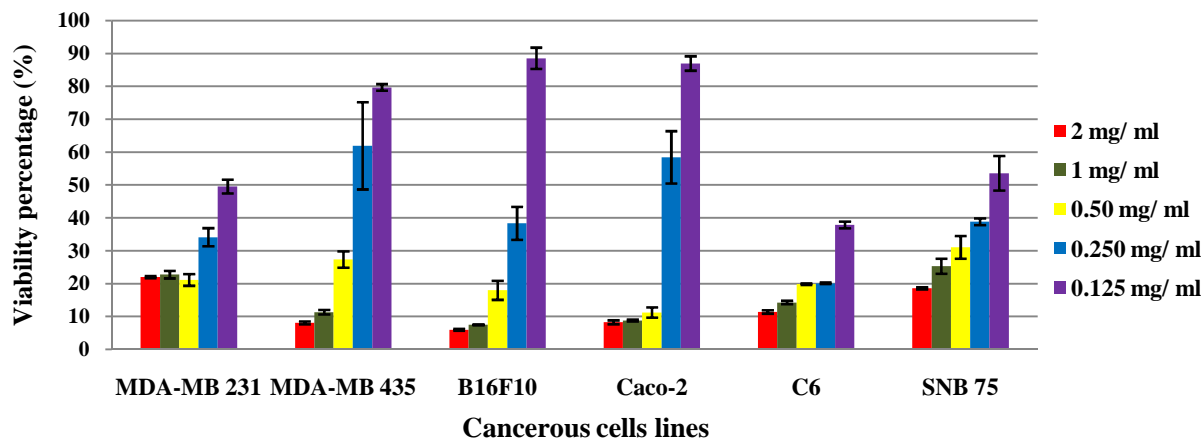
HCAD *: hydroxycinnamic acid derivative

Hydroxycinnamic acid derivatives contents are 14.14 mg/g (24.49%) whereas those of flavonoids are 43.58 mg/g (75.50%) (42.68 mg/g in flavones and 0.9 mg/g in flavonols). We note *G. arborea* leaves richness in flavonoids, which according to a recent study, exhibited a good antioxidant power but also a low hemolytic power [7].

IN VITRO CYTOTOXICITY STUDY

The Figure 2 shows the viability percentage (VP) of cancerous strains treated by HACE during 72 hours, with different concentrations ranging from 0.125 to 2 mg/ml. Generally a gradual decrease in cancerous cells is noticed with however, significant differences between the VP. A good cytotoxic activity against C6 (VP < 40%) and MDA-MB 231 (VP < 50%) emerges.

Figure 2: Cancerous strains viability percentage



The results of statistical analysis (Table 3) showed that HACE exhibits a modest cytotoxic effect against cancerous strains, notably against C6 (average VP of about 20.696%). However, for concentrations ranging from 0.25 to 2 mg/ml, the VP are all less than 50% (Table 4).

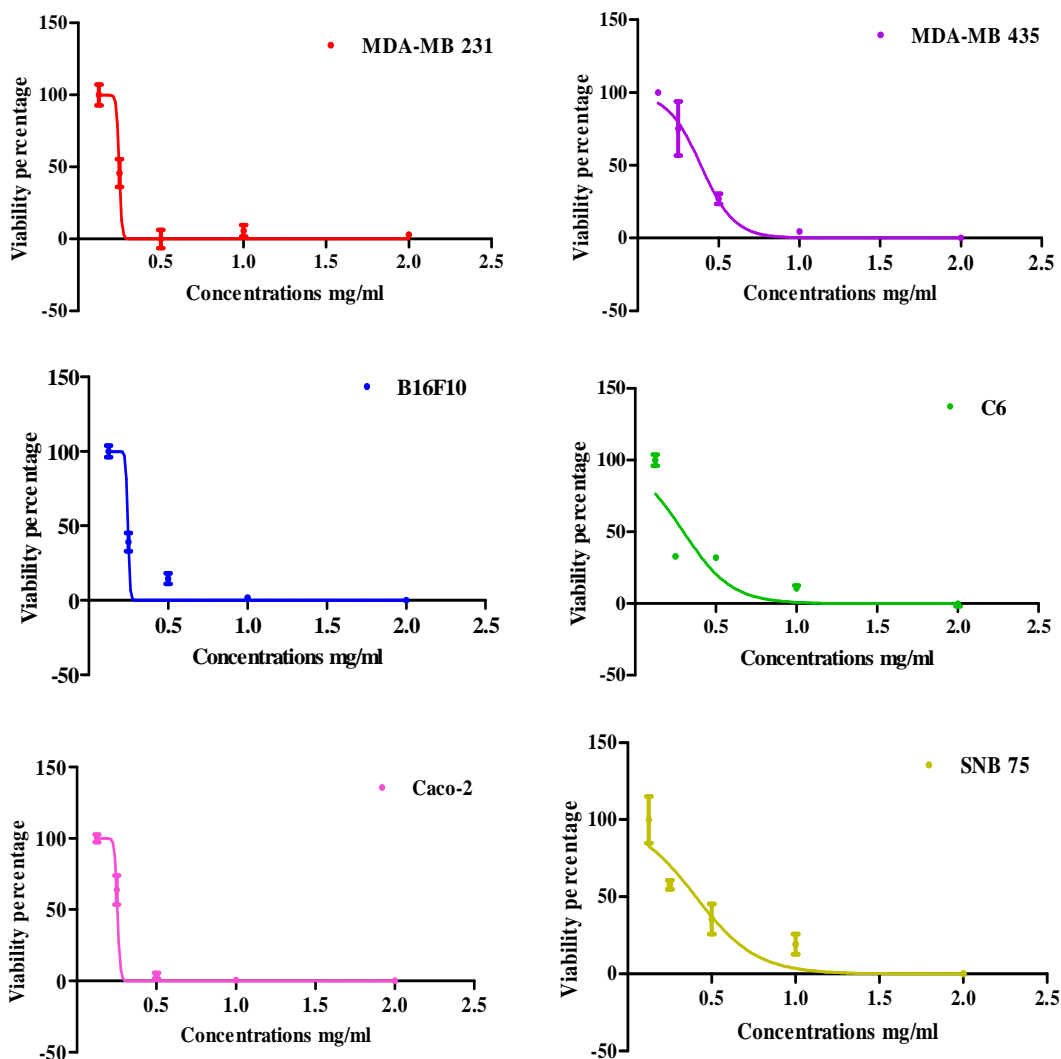


Figure 3: Anticancer activity of HACE

Table 3: Cytotoxic effect of HACE against 6 cancerous strains

| Cancer stem | N | Means ± Standard deviation | F | P |
|-------------|----|------------------------------|------|--------|
| MDA-MB 231 | 15 | 29.898 ± 11.391 ^a | 3.70 | 0.0046 |
| MDA-MB 435 | 15 | 37.654 ± 29.833 ^a | | |
| B16F10 | 15 | 31.649 ± 31.880 ^a | | |
| Caco-2 | 15 | 34.719 ± 33.578 ^a | | |
| C6 | 15 | 20.696 ± 9.537 ^b | | |
| SNB75 | 15 | 33.452 ± 12.742 ^a | | |

(*) Means followed by the same letter were not significantly different

Table 4: Effect of HACE on C6 concentrations

| Concentrations | N | Means ± Standard deviation | F | P |
|----------------|----|------------------------------|--------|----------|
| 0.125 | 18 | 66.016 ± 20.513 ^a | 955.65 | < 0.0001 |
| 0.25 | 18 | 41.946 ± 15.793 ^b | | |
| 0.5 | 18 | 21.425 ± 0.223 ^c | | |
| 1 | 18 | 14.974 ± 7.037 ^{cd} | | |
| 2 | 18 | 12.362 ± 6.074 ^d | | |

(*) Means followed by the same letter were not significantly different

All IC₅₀ values determined (< 1 mg/ml) (Figure 3) are given in Table 5.

Table 5 : IC₅₀ values

| Cancer stem | MDA-MB 231 | MDA-MB 435 | B16F10 | Caco-2 | C6 | SNB75 |
|--------------------------|------------|------------|--------|--------|-------|-------|
| IC ₅₀ (mg/ml) | 0.246 | 0.379 | 0.246 | 0.250 | 0.304 | 0.404 |

The results of this work show a correlation between HACE anticancer activity and its phenolic composition. In fact, studies reported that flavonoids are caused cellular apoptosis [11, 12]. Others have clinically demonstrated their anticancer effect against different types of cancer (breast, lung, prostate). Their regular use protects against the risk of developing gastric cancer [13, 14].

CONCLUSION

The quantification of extractable phenolic components of *Gmelina arborea* leaves was performed by ULPC-MS/MS from its hydroacetic crude extract, which has demonstrated its modest *in vitro* effective anticancer activity against 6 cancerous strains. Through this contributive survey, we show that the use of the plants in traditional therapy can offer positive answers in the research of new medicines.

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