

Extraction, Purification, Anticancer Activity Studies of Triacanthine Isolated from *Bridelia duvigneaudii* J. Léonard (Phyllanthaceae)

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Abstract

Objective: The main objective of this study was to isolate alkaloids from *Bridelia duvigneaudii* leaves and to evaluate their cytotoxic activity on prostate and leukemia cancer cells in order to discover potential natural anticancer products. **Methodology:** Alkaloids were extracted by organic solvents from the basified aqueous phase containing plant extracts and then purified by open column chromatography. The compounds were characterized by direct interpretation of their spectral (IR, 1D NMR, 2D NMR and MS) data and by comparison of their physico-chemical data with the literature. Antiproliferative assays were performed on two cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) methodology. **Results:** A total of 1.018 g of alkaloids was obtained from 2528 g of *B. duvigneaudii* leaves (0.04% of plant material). The chromatographic purification process gave three purified products, which were positive to Dragendorff's reagent: BRD4 (269 mg, 0.011%, R_f 0.3), BRD5 (165 mg, 0.006%, R_f 0.2) and BRD10 (584 mg, 0.023%, R_f 0.22). These three products exhibited cytotoxic activity against prostate cancer LAPC-4 cells with IC₅₀ values of 17.5 ± 0.50 µg/mL for BRD10, 19.2 ± 0.41 µg/mL for BRD5 and 22.3 ± 0.04 µg/mL for BRD4 and against leukemia HL-60 cells with IC₅₀ values ranging between 22.5 ± 0.17 and 24.3 ± 0.20 µg/mL while the positive control (6.5 µg/mL RM-581)

had got an IC_{50} value of 0.33 ± 1.52 $\mu\text{g/mL}$ on LAPC-4 cells and 1.45 ± 1.52 $\mu\text{g/mL}$ on HL-60 cells. The anticancer activity was highest at 100 $\mu\text{g/mL}$ of each purified product and was decreased significantly ($p < 0.05$) from 100 to 0.1 $\mu\text{g/mL}$. The structure elucidation of BRD4 by IR, MS and NMR allowed the identification of triacanthine, a purine-derived alkaloid of 203 Da and the empirical formula $C_{10}H_{13}N_5$ carrying a dimethylallyl group in position N3. **Conclusion:** This study showed that triacanthine is an alkaloid of *B. duvigneaudii* possessing an antiproliferative activity on LAPC-4 and HL-60 cancer cell lines.

Keywords

Bridelia duvigneaudii, Phyllanthaceae, Alkaloids, Triacanthine, Prostate Cancer, Leukemia

1. Introduction

Cancer has become a major public health issue worldwide, in both developed and developing countries. Indeed, biomedical statistics prove that cancer is one of the leading causes of death worldwide. Thus, from 2008 to 2018, the incidence of this pathology increased by 33.7% (12 million in 2008 and 18.1 million in 2018) and its mortality increased by 28% (7.5 million in 2008 and 9.6 million in 2018). Cancer mortality could increase by 50% from now to 2030, according to World Health Organization (WHO) projections. By that time, nearly 70% of new cancer cases could occur in developing countries [1]–[3].

Faced with this emerging scourge in the world, and especially in low-income countries as DR Congo, its fight comes down to early diagnosis, the risk factors avoidance, the installation of diagnostic and modern treatment infrastructures, and the research of new active compounds that could be accessible to all people regardless of their living standard. Given the side effects of anticancer drugs on the market, in particular their cytotoxicity on blood cells [4], the need for new active molecules with new mechanisms of action and having fewer side effects becomes a necessity. Therefore, researchers were focused on medicinal plants, which are always an important source of new therapeutic chemicals [5]. Indeed, the biological screening of substances from ethnobotanical knowledge currently remains a credible alternative in this quest and natural products from plants have attracted the attention of researchers for their potential as preventive and curative cancer agents. Plant derivatives are also considered inhibitors of different phases of cancer cell development [6]. Currently, about 60% of anticancer drugs are isolated from plants and about 3000 plants harvested around the world are recognized as having anticancer properties [7]. Natural anticancer drugs belong to the group of secondary metabolites including alkaloids, flavonoids, terpenoids, lignans, limonoids, quinones, saponins, stilbenoids and xanthenes [8] [9]. Besides, alkaloids are the most important natural products due to their diverse biological properties, such

as cytotoxicity and structural diversity [10].

Bridelia duvigneaudii J. Leonard (*Bridelia katangensis* J. Leonard) is used in traditional medicine in Haut-Katanga, Southern Province of DR Congo. As examples, the stems bark or root decoction is taken orally to treat diarrheal diseases, lower abdominal pain in women, dysmenorrhea, headache and gonorrhea. It can also be used to wash wounds or instilled to treat eye pain. The decoction is also used in gargle while roots and/or leaves powder is used to treat tooth decay. Stem or root bark powder is used to treat hemorrhoids [11]. Phytochemical analysis showed that *B. duvigneaudii* contains alkaloids, anthocyanins, quinones, saponins, steroids, tannins and terpenoids. The aqueous methanolic crude extracts and total alkaloids of this species showed antimitotic activity on *Sorghum* sp seeds [12]-[16].

Thus, the main objective of this study was to isolate *B. duvigneaudii* alkaloids and to evaluate in vitro their cytotoxic effect on prostate and leukemia cancer cells in order to discover natural anticancer products.

2. Experimental Procedures

2.1. General Experimental Procedures

We have carried out Infrared spectra on a Perkin branded Spectrum One device (Norwalk, CT, USA) using KBr pellets at a concentration of 4% by mass of the product. The mass spectrometry was conducted on Micromass Autospec 3F Mass Spectrometer using 70 eV by electronic impact for molecular ionization and fragmentation. The NMR spectra were recorded at 19°C on a Bruker Avance 400 device with a frequency of 400.13 MHz for the ¹H nucleus and 100.61 MHz for the ¹³C nucleus in solutions of chloroform and methanol deuterated containing tetramethylsilane trace. The chemical shifts were reported in ppm using the tetramethylsilane signal as a reference and the coupling constants are expressed in Hz. The complete elucidation of the molecular structure was based on ¹H-NMR, ¹³C-NMR, DEPT-135 experiments and the 2-D experiments: COSY, NOESY, HSQC and HMBC. 1D- and 2D-NMR spectra were recorded at 400 MHz for ¹H and 100.6 MHz for ¹³C on a Bruker AM-400 NMR spectrometer (Billerica, MA, USA). Silica gel (200 - 300 mesh, Merck) was used for column chromatography. The 96-well microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, USA), and a microplate reader (Molecular Devices, Sunnyvale, CA, USA) were used for cell proliferation assays.

2.2. Plant Material

Bridelia duvigneaudii was collected from December 2010 to January 2011 by Kimsimba Kibuye, a botanist technician in the Department of Geography of the Faculty of Science at University of Lubumbashi. The voucher specimens were stored at the Herbarium of the Faculty of Science (Voucher number KM4223). After drying in the sun the samples, at the Faculty of Sciences laboratory, were crushed in a metal mortar, sieved and stored in plastic bags at room temperature.

2.3. Preparation of Dragendorff Reagent

We have dissolved 0.85 g of bismuth nitrate (Merck) in 10 mL of glacial acetic acid (Merck) and 40 mL of distilled water to yield solution A. We have also dissolved 8 g of potassium iodide (Merck) in 20 mL of distilled water to generate solution B. 5 mL of solution A and 5 mL of solution B were transferred into a 100 mL flask. We added 20 mL of acetic acid to the mixture and diluted it with distilled water to the mark [13].

2.4. Extraction and Isolation

Dry leaves of *B. duvigneaudii* (2.528 kg) were macerated with petroleum ether at room temperature for 24 h and this operation was performed twice. The dry marc (solid residue) mixed with Na_2CO_3 (1 kg) is moistened with water (1 L). The basified residue was kept for 48 h and then extracted with CHCl_3 for 24 h. The filtration was evaporated under vacuum to dryness with a rotavapor (BiBBy RE) and the dry residue was acidified with 0.5 M citric acid (Merck). The aqueous phase was basified with 20% Na_2CO_3 to reach the pH 9 and extracted three times with CHCl_3 . After decantation, phase separation and evaporation of the organic phase, 6512 mg of total alkaloids (0.26% from dry leaves) were obtained. The total alkaloidal extract (6.512 g), chromatographed on open silica gel column 60 F₂₅₄, was successively eluted with EtOAc:MeOH system (100:0, 90:10, 80:20, 70:30, 60:40, 0:100). The different fractions were subjected to thin-layer chromatography (TLC) using the EtOAc:MeOH:Isopropylamine (IPA) system (9:1:0.05) and the plates were revealed with Dragendorff's reagent. Fractions (2040 mg, 0.081% of dry material) with similar chromatograms (Positive to Dragendorff's reagent) were chromatographed again on silica gel column eluting with EtOAc: MeOH (100:0, 90:10, 0:100) to afford 11 fractions, of which three fractions were positive to Dragendorff's reagent. After evaporation under vacuum on a rotary evaporator at 30 °C - 40 °C, this process yielded 3 positive Dragendorff fractions: BRD4 (269 mg, 0.011%), BRD5 (165 mg, 0.006%) and BRD10 (584 mg, 0.023%). The three obtained fractions (BRD4, BRD5 and BRD10) were then analyzed by NMR, but only BRD4 was found pure enough for a full characterization by FTIR, MS and NMR (^1H , ^{13}C , DEPT, COSY, NOESY, HSQC and HMBC).

2.5. Spectral Data for BRD4

BRD4 was obtained as an amorphous reddish solid. IR (KBr) ν_{max} (cm^{-1}): 3209, 3078, 2962, 1690, 1589, 1551 - 1412, 1358, 879, 779. ESI-MS: 203 (27%, M^+), 188 (73%, $\text{M}^+ - ^\circ\text{CH}_3$), 135 (100%), 108 (49%), 81 (7%), 69 (23%) and 53 (9%). CI-MS: 204 (100%, $(\text{M} + \text{H})^+$), 188 (22%), 135 (20%). HREIMS: calculated mass for $\text{C}_{10}\text{H}_{14}\text{N}_5$ ($\text{M} + \text{H}$) was 204.1124 Da; found 204.1243 Da. ^1H -NMR: δ (ppm, CDCl_3 , 400 MHz): 1.75 (s, 3H, CH_3 -13), 1.90 (s, 3H, CH_3 -14), 4.98 (d, 6.6 Hz; 2H, CH_2 -10), 5.45 (t, 6.5 Hz, 1H, CH-11), 8.08 (s, 1H, CH-8), 8.13 (s, 1H, CH-2). ^1H -NMR: δ (ppm, Acetone- d_6 , 400 MHz): 1.76 (s, 3H, CH_3 -13), 1.90 (s, 3H, CH_3 -14), 5.05 (d, 6.6 Hz; 2H, CH_2 -10), 5.55 (t, 6.5 Hz, 1H, CH-11), 7.94 (s, 1H, CH-8), 8.34 (s,

¹H, CH-2). ¹H-NMR: δ (ppm, CD₃OD, 400 MHz): 1.80 (s, 3H, CH₃-13), 1.88 (s, 3H, CH₃-14), 5.01 (d, 6.6 Hz; 2H, CH₂-10), 5.50 (t, 6.5 Hz, 1H, CH-11), 8.04 (s, 1H, CH-8), 8.33 (s, 1H, CH-2). ¹³C-NMR: δ (ppm, CDCl₃/CD₃OD, 400 MHz): 18.2 (C13), 25.7 (C14), 47.5 (C10), 116.2 (C11), 120.4 (C5), 141.5 (C12), 142.0 (C2), 150.1 (C4), 153.1 (C8), 154.6 (C6). ¹³C-NMR: δ (ppm, Acetone-*d*₆, 400 MHz): 18.2 (C13), 21.2 (C14), 48.1 (C10), 118.6 (C11), 118.7 (C5), 140.4 (C12), 145.0 (C2), 150.6 (C4), 151.4 (C8), 156.0 (C6). ¹³C-NMR: δ (ppm, CD₃OD, 400 MHz): 18.2 (C13), 21.1 (C14), 48.7 (C10), 118.2 (C11), 119.0 (C5), 141.6 (C12), 145.8 (C2), 150.4 (C4), 151.4 (C8), 156.4 (C6).

2.6. Antiproliferative Activity on Cancer Cells

The antiproliferative assay was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Cell Titer 96 Aqueous, Promega, Nepean, ON, Canada), which allowed us to measure the number of viable cells. Prostate cancer LAPC-4 cells were kindly provided by Robert E. Reiter from the University of California (Los Angeles, CA, USA) while leukemia HL-60 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). In brief, triplicate cultures of 1×10^4 cells in a total of 100 μ L of medium (RPMI-1640, Sigma, Saint Louis, MO, USA) in 96-well microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, USA) were incubated at 37 °C under 5% CO₂ humidified atmosphere. Compounds were tested at different concentrations (0.1, 1, 10 and 100 μ g/mL) and the aminosteroid RM-581 [12]–[16] was used as a positive control. They were first solubilized in DMSO and diluted at multiple concentrations with culture medium, added to each well and incubated for 3 days. Control cells were treated with the medium and dimethyl sulfoxide (DMSO) only (final DMSO concentration < 0.5%). Following each treatment, MTS (20 μ L) was added to each well and incubated for 4 h. MTS is converted to water-soluble colored formazan by dehydrogenase enzymes present in metabolically active cells. The plates were subsequently analyzed at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results are expressed as cell proliferation and the control (basal proliferation of culture medium and DMSO) is fixed at 100%.

2.7. Statistical Analysis of the Data

The statistical analysis of the data was done using the GraphPad Prism version 6 software. In fact, for the univariate analysis, the results are expressed in the form of numbers, percentages, and mean $\pm$ standard deviation. For multivariate analysis, ANOVA One Way was used to compare several variables. The Tukey test was applied with a 95% significance level ($p < 0.05$). The concentration inhibiting 50% of cell proliferation (IC₅₀) was calculated using the graphical method.

3. Results and Discussion

3.1. Extraction and Purification of Alkaloids

The alkaloid extraction from 2528 g of *B. duvigneaudii* leaves yielded 6512 mg of

total alkaloids, which accounted for 0.257% of the plant material used. The alkaloid extract was purified using column chromatography on silica gel (60F₂₅₄ silica from Merck). The purification was carried out utilizing a gradient of chloroform (CHCl₃) and methanol (MeOH) from 100:0 to 0:100 as eluent, and we obtained fractions F8 to F12 that were positive to Dragendorff's reagent. The mixture (F8 to F12) was subjected to an additional silica gel column chromatography with EtOAc:MeOH (100:0, 90:10, 0:100) as eluent. After silica gel column chromatography, the obtained fractions were subjected to TLC in the solvent system EtOAc:MeOH:IPA (8:2:0.05), and three fractions were positive to Dragendorff's reagent (BRD4: 269 mg, 13.21%, Rf 0.3), (BRD5: 165 mg, 8.10%, Rf 0.2), and (BRD10: 584 mg, 28.68%, Rf 0.22) (**Table 1**).

Table 1. Characteristics of fractions obtained from extract 1 (the mixture of fractions 8 - 12).

Fraction	Code	Revelation with Dragendorff's reagent	Rf	Weight (g)	Yield (%)
1	BRD1	-		0.321	15.77
2	BRD2	-		0.044	2.16
3	BRD3	-		0.122	5.99
4	BRD4	+	0.3	0.269	13.21
5	BRD5	+	0.2	0.165	8.10
6	BRD6	-		0.022	1.08
7	BRD7	-		0.054	2.65
8	BRD8	-		0.100	4.91
9	BRD9	-		0.210	10.31
10	BRD10	+	0.22	0.584	28.68
11	BRD11	-		0.145	7.12

From the total alkaloidal extract purification works, three positive fractions to Dragendorff's reagent were obtained. These three fractions gave 1.018 g (0.04%) of alkaloids from 2528 g of plant material (dry leaves). In fact, the purification of *B. duvigneaudii* allowed obtaining three alkaloidal fractions, which could be considered as three purified alkaloids: BRD4 (269 mg, 0.011%, Rf 0.3 and positive to Dragendorff's reagent), BRD5 (165 mg, 0.006%, Rf 0.2 and positive to Dragendorff's reagent) and BRD10 (584 mg, 0.023%, Rf 0.22 and positive to Dragendorff's reagent). The three purified products were first evaluated as anticancer agents and secondly characterized by spectral methods such as Infrared (IR), MS and NMR to determine their structures.

3.2. Anticancer Activity Assessment

We report herein the results of our ongoing research into natural products. In fact, the three purified alkaloids (BRD4, BRD5 and BRD10) were tested for cytotoxicity on LAPC-4 prostate cancer cells and HL-60 leukemia cells. For comparison

purposes, a blank test was performed upon RM-581-102 (named RM-581), an aminosteroid known for its efficiency against prostate cancer cells spreading as well as LAPC-4 cells [12]–[16], was used as a positive control (Table 2, Table 3).

Table 2. Evaluation of the anticancer activity of purified fractions on prostate cancer LAPC-4 cells.

Fraction	Cell growth (%)				IC ₅₀ (μg/mL)
	100 μg/mL	10 μg/mL	1 μg/mL	0.1 μg/mL	
Control	100.0 ± 1.7 ^a	100.0 ± 1.7 ^a	100.0 ± 1.7 ^a	100.0 ± 1.7 ^a	
BRD4	1.7 ± 0.04 ^d	100.5 ± 2.2 ^a	97.5 ± 2.1 ^a	98.5 ± 4.9 ^a	22.3
BRD5	3.6 ± 0.4 ^{cd}	90.2 ± 2.0 ^b	101.9 ± 3.8 ^a	103.8 ± 1.7 ^a	19.2
BRD10	1.5 ± 0.5 ^d	89.7 ± 3.1 ^b	102.4 ± 3.2 ^a	97.5 ± 4.9 ^a	17.5
RM-581	6.5 μg/mL	3.2 μg/mL	0.6 μg/mL	0.06 μg/mL	0.33
	11.4 ± 1.5 ^b	16.7 ± 1.2 ^c	47.9 ± 1.5 ^b	70.3 ± 2.0 ^b	

Values in the same column with different exponents (a, b, c and d) show significant differences ($p < 0.05$, Tukey test results).

The cytotoxicity study showed that the three extracts were cytotoxic on LAPC-4 cells (Table 2). In fact, at 100 μg/mL the extracts fully inhibited the cell proliferation, namely 1.5% cell growth for BRD10, 1.7% for BRD4 and 3.6% for BRD5, thus representing 98.4%, 98.3% and 96.4% of cell growth inhibition, respectively. However, the positive control RM-581 had the best anticancer activity among the fractions tested. At 6.5 μg/mL it inhibited the cell growth to 11.4% (88.6% of inhibition). The statistical analysis ($p < 0.05$) carried out at 100 μg/mL showed that there are three groups of results for which the averages had any significant difference. This is the control group, the positive control group and the BRD10, BRD4 and BRD5 groups. At 10 μg/mL, statistical tests also showed that there are three groups of results without any significant difference: the control and BRD4 groups, the BRD5 and BRD10 groups and the positive control group. Besides, at 1 and 0.1 μg/mL, the statistical tests ($p < 0.05$) showed that there is no significant difference between the results obtained with the extracts and those of the control. However, at 10 μg/mL, the extracts BRD5 (9.8% inhibition) and BRD10 (10.3% inhibition) showed a very low activity while the BRD4 extract was inactive at this concentration. By further reducing the concentration of the extracts from 10 to 0.1 μg/mL, the cytotoxicity of all the extracts was canceled. In contrast, the positive control (RM-581) remained active from 0.06 to 6.5 μg/mL with the cell growth inhibition ranging from 29.7% to 88.6%, respectively.

The three purified extracts were also tested on HL-60 cells and statistical analysis was performed as well (Table 3). They were only active on HL-60 cells at 100 μg/mL. Indeed, at this concentration, the purified alkaloids showed a strong inhibitory effect on cell growth ranging between 97.0% and 98.4%. As for the tests carried out on LAPC-4 cells, it was observed that the product used as the positive control showed the best activity than the purified alkaloids studied on HL-60 cells.

Indeed, cell growth of $12.0\% \pm 1.0\%$ was observed for RM-581 at $6.5 \mu\text{g/mL}$, which represents 88.0% of cell growth inhibition. By decreasing the concentration from 10 to $0.1 \mu\text{g/mL}$, the purified alkaloids become inactive on HL-60 cells. However, the inhibitory activity of positive control decreased. The statistical analysis ($p < 0.05$) showed that there was no significant difference in the activity of all extracts at $100 \mu\text{g/mL}$. In addition, at $10 \mu\text{g/mL}$, BRD4 and BRD5 showed significant differences compared to BRD10, which showed higher cell growth compared to the other extracts. Finally, from 1 to $0.1 \mu\text{g/mL}$, the BRD4 and BRD5 extracts did not show any significant differences in activity compared to the control.

Table 3. Evaluation of the anticancer activity of purified fractions on leukemia HL-60 cells.

Fraction	Cell growth (%)				IC ₅₀ ($\mu\text{g/mL}$)
	100 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	0.1 $\mu\text{g/mL}$	
Control	100.0 ± 6.4^a	100.0 ± 6.4^c	100.0 ± 6.4^{ab}	100.0 ± 6.4^{ab}	
BRD4	1.6 ± 0.2^c	120.4 ± 2.7^b	98.6 ± 1.9^{ab}	94.5 ± 2.8^{bc}	22.5
BRD5	2.9 ± 0.3^c	111.9 ± 4.1^b	98.8 ± 1.5^{ab}	95.8 ± 2.4^{abc}	24.0
BRD10	3.0 ± 0.2^c	142.3 ± 1.7^a	108.2 ± 10.2^a	105.9 ± 3.7^a	24.3
RM-581	6.5 $\mu\text{g/mL}$	3.2 $\mu\text{g/mL}$	0.6 $\mu\text{g/mL}$	0.06 $\mu\text{g/mL}$	1.2
	12.0 ± 1.0^b	20.6 ± 5.0^e	89.5 ± 4.0^b	87.8 ± 3.4^c	

Values in the same column with different exponents (a, b, c and d) show significant differences ($p < 0.05$, Tukey test results).

The IC₅₀ values determined by the graphical method showed that the purified alkaloids were toxic to the cells tested [17]. In fact, on LAPC-4 cells, the purified fractions are active with an IC₅₀ of $17.5 \mu\text{g/mL}$ for BRD10, $19.2 \mu\text{g/mL}$ for BRD5 and $22.3 \mu\text{g/mL}$ for BRD4. Besides, on HL-60 cells, they are active with IC₅₀ values ranging between 22.5 and $24.3 \mu\text{g/mL}$. However, the purified fractions were less toxic than the reference product RM-581, which had an IC₅₀ of $0.33 \mu\text{g/mL}$ on LAPC-4 cells and $1.15 \mu\text{g/mL}$ on HL-60 cells.

The evaluation of the anticancer activity of *B. duvigneaudii* alkaloids on LAPC-4 and HL-60 cancer cells confirmed the results obtained during the antimitotic activity evaluation of total alkaloids obtained from *Sorghum sp* seeds [12]–[16]. Indeed, the first study showed that the total alkaloids extracted from *B. duvigneaudii* were extremely toxic on *Sorghum sp* seeds with an inhibiting concentration of 50% of seed germination (IG50) ranging between 3.18 - 3.66 mg/mL . Similarly, the second study conducted by the interaction of purified alkaloids fraction of *B. duvigneaudii* on two types of cancer cell lines (LAPC-4 and HL-60) at $100 \mu\text{g/mL}$ showed that these alkaloidal fractions were toxic on these cancer cells with the IC₅₀ ranging respectively between $17.5 - 22.3 \mu\text{g/mL}$ on LAPC-4 cells and $22.5 - 24.3 \mu\text{g/mL}$ on HL-60 cells. These results show that seeds in germination can be used in the antiproliferative preliminary test [16]. The antiproliferative activity observed for the three alkaloid fractions encouraged us to elucidate the structure

of BRD4, BRD5 and BRD10. However, since the preliminary results of NMR analysis showed that only BRD4 was completely pure, we only fully characterized this fraction.

3.3. Identification of the Active Molecule of the BRD4 Fraction

3.3.1. Infrared Spectral Data of BRD4

The BRD4 infrared spectrum showed an antisymmetric elongation vibration band at 3209 cm^{-1} assigned to the primary amine function $\nu\text{ NH}_2$. The intense band at 1690 cm^{-1} , which is associated with the in-plane deformation vibration, confirmed this assignment and the 779 cm^{-1} bands associated with the out-of-plane deformation vibration of δNH_2 . For the different types of CH bonds, spectral data revealed the $\nu\text{ CH}$ band at 2962 cm^{-1} , attributed to the aliphatic CH absorption, the 3078 cm^{-1} band of aromatic $\nu\text{ CH}$ and the 1358 cm^{-1} band assigned to the $\nu\text{ CH}$ of the methyl group. There are also bands around 1589 cm^{-1} assigned to the imine function $\nu\text{ (C=N)}$. Besides, four bands appearing between $1412 - 1551\text{ cm}^{-1}$ are those of elongation vibrations of the bond $\nu\text{ (C=C)}$. A similarity between the FTIR spectrum of BRD4 and the FTIR spectrum of adenine was noted (**Table 4**).

Table 4. Comparison of FTIR data between Adenine and Compound BRD4.

Observed function	Wavenumber (cm^{-1})	
	Adenine*	BRD4
$\nu\text{ (NH}_2\text{)}$	3286	3209
$\delta\text{ (NH)}$	1668	1690
$\nu\text{ (CH}_2\text{)}$	2972	2962
$\nu\text{ (C = C)}$	1502	1551
$\nu\text{ (C = N)}$	1601	1589

*Masoud *et al.* [17] [18].

Thus, on the FTIR spectrum of BRD4, the antisymmetric elongation vibration $\nu\text{ (NH}_2\text{)}$ appears at 3209 cm^{-1} while it appears around 3286 cm^{-1} in the adenine spectrum. Moreover, the deformation bands $\delta\text{ (NH}_2\text{)}$ appear at 1690 cm^{-1} for BRD4 and 1668 cm^{-1} for adenine [17] [18]. The $\nu\text{ (CH}_2\text{)}$ vibration is located at 2972 cm^{-1} for adenine but is displaced to 2962 cm^{-1} for BRD4. Two absorption bands are observed around 1502 and 1601 cm^{-1} on the spectrum of adenine, they respectively correspond to the vibration of elongation bands of the C=C and C=N bonds [17]-[20]. These two bands appear respectively at 1551 and 1589 cm^{-1} on the BRD4 spectrum. The overlap of the IR spectral data of BRD4 and those of adenine highlights the existence of structural similarity for these two substances.

3.3.2. MS Spectral Data of BRD4

The electronic impact (EI) mass spectrum showed a peak of 203 Da at the highest molecular mass with an intensity of 27%; it was the molecular ion. To confirm

that the ion observed at the highest molecular mass is not a fragment, another spectrum has been obtained by chemical ionization (CI). Then, the CI-MS spectrum showed that the molecular ion (M^+) was also the base peak (100%) at m/z 204 Da. This mass corresponds to that of the molecular ion $(M + H)^+$, which confirms that the molecule studied has the molecular mass of 203 Da. Its HREIMS revealed a $(M + H)^+$ peak at m/z 204.1243 (calc. 204.1244), consistent with a molecular formula of $C_{10}H_{13}N_5$ with seven degrees of unsaturation.

The analysis of MS fragments revealed 7 characteristic peaks (EI mass spectrum). The peak of 203 Da is the molecular ion $C_{10}H_{13}N_5^+$ (M^+) and the peak of 188 Da reflects the loss of a methyl group, which gave the ion $C_9H_{10}N_5^+$ $(M-CH_3)^+$. Moreover, the fragment represented by the peak of 135 Da ($C_5H_5N_5^+$) demonstrates the existence of an aromatic system following the loss of the 69 Da fragment on the molecular ion [19]. The peaks of 108 and 81 Da correspond respectively to the fragment ions $C_4H_4N_4^+$ and $C_3H_3N_3^+$ obtained as the result of a loss of neutral fragments HCN and $H_2C_2N_2$ on the 135 Da fragment. The 69 Da (23%) fragment represents the isopent-2-enyl cation. Finally, the 53 Da fragment represents the $C_2HN_2^+$ ion and/or the $C_3H_3N^+$ ion. The $C_2HN_2^+$ fragment is more likely because it results from a loss of a neutral fragment whereas $C_3H_3N^+$ requires a molecular rearrangement during its metastable state [21]-[27].

3.3.3. 1H -NMR Spectral Data of BRD4

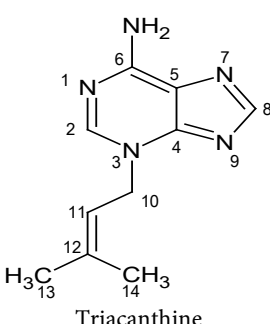
The 1H -NMR spectrum acquired in $CDCl_3$ indicates 7 characteristic signals grouped in 3 zones. The first zone of aliphatic protons comprises two singlets between 1.75 and 1.90 depending on the deuterated solvent used, which indicate the methyl protons of a dimethylallyl group. In the second zone, it was observed a doublet at 4.98 ppm ($J = 6.5$ Hz) and a triplet at 5.45 ppm ($J = 6.6$ Hz), which are the signals of the two methylene protons coupled with a methine proton. The broad singlet at 6.3 ppm indicates the protons attached to the nitrogen atom, which, in turn, is also attached to the aromatic ring. Finally, the third zone at about 8 ppm contains two characteristic singlet signals at 8.08 and 8.13 ppm for the protons of an aromatic nucleus.

3.3.4. ^{13}C -NMR Spectral Data of BRD4

The ^{13}C -NMR spectrum indicated the presence of 10 peaks that means the structure studied has 10 carbon atoms, which corroborates the MS data. Besides, the analysis of the DEPT-135 spectrum indicated only 6 peaks on this spectrum. Indeed, this spectrum showed two methyl signals at 18.2 and 21.1 ppm. The signals appearing at 145.8 and 151.4 ppm were those of the aromatic methines and a signal at 118.2 ppm is a non-aromatic ethylenic methine attached to the dimethylallyl group. Moreover, the peak appearing at 48.7 ppm is attributed to methylene. Finally, the signals appearing at 156.4, 150.4, 141.6 and 119.0 ppm on the ^{13}C -NMR spectrum, and which are absent on the DEPT-135 spectrum, are those of quaternary carbons, two of them being located at the 2-ring junction of the aromatic system [21]. To support the different NMR correlations and couplings, two-

dimensional NMR was used, including COSY, NOESY, HSQC and HMBC experiments. Different correlations obtained by 2D-NMR and mentioned in **Table 5** suggest the structure of triacanthine.

Table 5. Chemical Shifts and COSY, NOESY, HSQC and HMBC correlations of BDR4 in CD₃OD.

Structure	Assignment	$\delta^{13}\text{C}$ (ppm)	$\delta^1\text{H}$ (ppm)	COSY	NOESY	HSQC	HMBC
 Triacanthine	CH-2	145.8	8.33	H-2/H-2	H-2/H-10 H-2/H-11	H-2/C-2	H-2/C-4, C-6, C-10
	C-4	150.4	-	-	-	-	-
	C-5	119.0	-	-	-	-	-
	C-6	156.4	-	-	-	-	-
	CH-8	151.4	8.04	H-8/H-8	-	H-8/C-8	H-8/C-4, C-5
	CH ₂ -10	48.7	5.01	H-10/H-11	H-10/H-2 H-10/H-14	H-10/C-10	H-10/C-2, C-4, C-11, C-12
	CH-11	118.2	5.50	H-11/H-10	H-11/H-2 H-11/H-13	H-11/C-11	H-11/C-10, C-12, C-13, C-14
	C-12	141.6	-	-	-	-	-
	CH ₃ -13	18.2	1.80	H-13/H-13	H-13/H-11	H-13/C-13	H-13/C-11, C-12, C-14
	CH ₃ -14	21.1	1.88	H-14/H-14	H-14/H-10	H-14/C-14	H-14/ C-11, C-12, C-13

The spin systems were determined by the COSY and NOESY spectra. On the NOESY spectrum, the H-14 protons were correlated to the H-10 protons, which in turn were correlated to the H-11 proton on the COSY spectrum. The correlations obtained by NOESY between H-11 and H-2 and H-14 to H-10 prove that the H-11 proton was in the trans-position concerning the C-14 carbon. This implies that there is a double bond between two carbons on the side chain attached to the aromatic system. Moreover, the HSQC spectrum allowed the identification of protons correlating with carbon (H-C correlation). Protons H-11 (5.50 ppm), H-10 (5.01 ppm) and H-2 (8.33 ppm) were carried by carbon C-11 (118.2 ppm), carbon C-10 (48.7) and carbon C-2 (145.8 ppm), respectively. HMBC correlations allow the positioning of different groups. Thus, the correlations between H-13 and C-11, C-12 and C-14 as well as between H-14 and C-11, C-12 and C-13 were detected. Other key correlations were also detected with H-2 (C-4, C-6 and C-10), H-8 (C-4 and C-5), H-10 (C-2, C-4, C-11 and C-12) and H-11 (C-10, C-12, C-13 and C-14). All these correlations allow us to know the junction point of the side chain with the N-3 of the aromatic system.

Correlations shown on the HSQC spectrum identified two methyl groups (18.2 and 21.1 ppm), one methine (116.2 ppm) and the DEPT spectrum showed methylene (48.7 ppm) in the allylic position assigned to C-10. Moreover, the correlations

obtained by COSY and NOESY showed that there is a double bond between two carbons on the side chain attached to the aromatic system. So, the C₅H₇ fragment is the isopentyl, which allows the linking of all the information obtained by all the techniques used [21]-[27].

3.3.5. Comparison of BRD4 and Triacanthine Spectral Data

For comparison, the ¹H-NMR and ¹³C-NMR spectral characteristics of BRD4 and triacanthine are summarized in **Table 6**.

Table 6. ¹H- and ¹³C-NMR spectral data (δ in ppm) of BRD4 and triacanthine for comparison.

Assignment H/C	δ H (ppm)					δ C (ppm)				
	BRD4		Triacanthine			BRD4		Triacanthine		
	CDCl ₃	Acetone- <i>d</i> ₆	CD ₃ OD	CD ₃ OD *	CDP	CDCl ₃ /CD ₃ OD	Acetone- <i>d</i> ₆	CD ₃ OD	CD ₃ OD *	CDP
CH-2	8.13	8.34	8.33	8.24	8.64	142.0	145.0	145.8	144.8	148.4
C-4	-	-	-	-	-	150.1	150.6	150.4	156.7**	155.8
C-5	-	-	-	-	-	120.4	118.7	119.0	141.4***	120.5
C-6	-	-	-	-	-	154.6	156.0	156.4	150.7**	155.8
CH-8	8.08	7.94	8.04	7.80	8.70	153.1	151.6	151.4	153.2	149.1
CH ₂ -10	4.98	5.05	5.01	5.02	4.08	47.5	48.1	48.7	48.7	39.3
CH-11	5.45	5.55	5.50	5.53	5.33	116.2	118.6	118.2	118.3	121.3
C-12	-	-	-	-	-	141.5	140.4	141.6	121.2***	135.1
CH ₃ -13	1.75	1.76	1.80	1.76	1.70	18.2	18.2	18.2	18.2	18.3
CH ₃ -14	1.90	1.90	1.88	1.90	1.82	25.7	21.2	21.1	25.8	24.3

(*) Data from Boonyaratavej and Petsom [22]. (**) The C-4 and C-6 assignment seems inversed. (***). The C-5 and C-12 assignment seems inversed. CDP: ChemDraw Prediction.

The chemical shift of H-2 was 8.13 ppm in CDCl₃, 8.34 ppm in acetone-*d*₆ and 8.33 ppm in CD₃OD. Besides, H-8 is shifted to 8.08, 7.79 and 8.04 ppm in CDCl₃, acetone-*d*₆ and CD₃OD, respectively. These protons are significantly deshielded due to the aromatic nature of the purine ring. Aromatic proton signals are most often located in the relatively narrow region of 7.5 to 9.5 ppm [21]. This was found analogous to the chemical shifts of protons at 8.24 ppm (H-2) and at 7.80 ppm (H-8) acquired in the CD₃OD for triacanthine [22], but these chemical shifts were lower than the ChemDraw theoretical prediction of 8.64 ppm for H-2 and 8.70 ppm for H-8 in triacanthine. Except for H-10 protons (5.01, 5.02 and 4.08 ppm), a good similarity was also observed for experimental and predicted chemical shifts of other protons of BRD4 and triacanthine.

On the ¹³C-NMR spectrum, chemical shifts of carbon acquired in CDCl₃-CD₃OD, acetone-*d*₆ and CD₃OD also showed similarities. However, it was observed a difference in chemical shifts of C-4 (150.4 ppm), C-5 (119.0 ppm), C-6 (156.4 ppm) and C-12 (141.6 ppm) for BRD4 comparatively of the chemical shift of C-4 (156.7 ppm), C-5 (141.4 ppm), C-6 (150.7 ppm) and C-12 (121.2 ppm) for the same

carbons in triacanthine acquired in the CD₃OD for the two compounds. Indeed, it was observed that the C-4 and C-6 as well as C-5 and C-12 assignments seemed inverted. Thus, according to the similarities observed for chemical shifts of protons and carbons of BRD4 and triacanthine, it means that BRD4 from *B. duvigneaudii* spectral data are consistent with triacanthine spectral data, a purine-derived alkaloid. This alkaloid has already been isolated from *Holarrhena floribunda*, *H. mitis*, *H. congolensis*, *H. wulfsbergii* and *Gleditsia triacanthos* (Apocynaceae) and from *Childowia sanguinea* (Leguminosaceae) [21]-[27].

Triacanthine (6-amino-3-(α,α -dimethylallyl)adenine) is an adenine derivative with a dimethylallyl group in N3 position. It is the only natural substance known as an N-alkylation derivative of adenine at position 3 [21]-[27]. This study also showed that triacanthine has anticancer activity. Furthermore, various biological studies carried out on triacanthine showed that it had diverse biological activities [28]-[34].

4. Conclusion

In this study, we extracted and purified three alkaloid fractions (BRD4, BRD5 and BRD10) from *B. duvigneaudii* and only one fraction (BRD4) was sufficiently pure, and it has been identified as triacanthine (269 mg, 0.011%, R_f 0.3, C₁₀H₁₃N₅) for the first time with this species. These three fractions exhibited cytotoxic activity against prostate cancer LAPC-4 cells, with IC₅₀ values of 17.5 µg/mL for BRD10, 19.2 µg/mL for BRD5 and 22.3 µg/mL for BRD4, and against leukemia HL-60 cells, with IC₅₀ values ranging between 22.5 and 24.3 µg/mL. Our study is an added value for the advancement of science because the alkaloids that we have isolated are powerful against prostate cancer and leukemia and this has not been revealed in the literature. However, the triacanthine anticancer properties hostile to bladder cancer and colorectal cancer cells have already been published in the literature [28]-[34].

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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