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Original Article

Cytotoxic Activity of *Annona Muricata* L, *Momordica Charantia* L. and *Launaea Taraxacifolia* Willd. from Benin: A Flow Cytometric Approach

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ABSTRACT

Background: Cancer is rising worldwide, causing approximately 9.7 million deaths in 2022. Treatments are costly, have significant side effects, and are sometimes unavailable in Benin. Medicinal plants offer promising avenues for new therapeutic agents. **Objective:** This study uses flow cytometry method to evaluate the cytotoxic activity of *Annona muricata* (Annonaceae), *Momordica charantia* (Cucurbitaceae) and *Launaea taraxacifolia* (Asteraceae), three plants from the Beninese pharmacopoeia known for their anti-cancer properties. **Materials and Methods:** The leaves of *A. muricata*, *L. taraxacifolia* and whole plants of *M. charantia* were collected in southern Benin. Extracts were prepared by aqueous decoction and sequential extraction with solvents of increasing polarity (dichloromethane, ethyl acetate, methanol). The phytochemical profiles of the extracts were determined using TLC and tube tests. The cytotoxicity of the extracts was evaluated on THP-1 cancer cells and PBMC healthy cells. After treatment with the extracts, cell viability was measured by flow cytometry after staining with Thiazole Orange and Hoechst 33258. **Results:** The dichloromethane extracts *of A. muricata* and *M. charantia*, and the ethyl acetate extract of *M. charantia* and *L. taraxacifolia* showed significant cytotoxic effects on THP-1 cells. Their half-maximal inhibitory concentrations (IC_{50}) were 139.6 µg/mL, 72.89 µg/mL, 81.88 µg/mL and 106.3 µg/mL, respectively. These extracts also demonstrated good selectivity toward normal cells. Phytochemical screening revealed the presence of alkaloids, coumarins, flavonoids, anthracene glycosides and triterpenes in the active extracts. **Conclusion:** This study demonstrated the cytotoxic potential of three medicinal plants from the Beninese pharmacopoeia which may serve to develop further new anticancer therapies.
KEYWORDS: Benin, cancer, cytotoxicity, plant extracts, selectivity, THP-1 cells.

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INTRODUCTION

Cancer is a disease that has been found in humans for thousands of years, with traces of tumors discovered in human skeletal dating back to 3,400 BC[1]. Cancer is a major public health problem worldwide due to its epidemiological characteristics and the difficulties associated with its management, particularly in developing countries.

Epidemiologically, cancer is one of the leading causes of death worldwide. According to the World Health Organization, cancer alone caused 9.7 million deaths in 2022[2]. In addition, 20 million new cases of cancer were recorded in the same year, and this number is expected to increase by 77% by 2050[2]. On the African continent, approximately 1.2 million new cases of cancer were recorded in 2022, with more than 760,000 deaths[3]. In Benin especially, approximately 7,500 new cases were recorded in 2022, resulting in more than 5,000 deaths[3]. These figures are likely to be underestimated due to several factors specific to our countries, such as limited access to healthcare facilities. Cancers therefore represent a heavy burden for healthcare systems, not only because of its epidemiological characteristics, but also because of the difficulties associated with its treatment.

Cancer treatment requires multiple therapeutic resources such as surgery, radiotherapy, hormone therapy, immunotherapy, targeted therapies, and chemotherapy. However, these have several limitations, such as limited accessibility, particularly in developing countries, significant side effects, and the possibility of treatment failure, particularly due to cancer-cells resistance to treatments[4,5]. This situation therefore highlights the need for further research to develop new effective and accessible anti-cancer agents in our countries. In this regard, traditional medicine, particularly medicinal plants, represents a promising area for exploration. Many of the anticancer molecules currently in use are derived from medicinal plants such as vinca alkaloids and taxanes[6]. In Benin, a study conducted by Tohinnou Houeze et *al.* (2019) identified 112 species traditionally used in the treatment of cancer[7]. Among the most frequently cited species are *Annona muricata L.*, *Momordica charantia L.* and *Launaea taraxacifolia* Willd. However, despite their strong therapeutic potential, the traditional uses of these medicinal plants often lack reliable scientific data to prove their efficacy and safety. Furthermore, most studies assessing their cytotoxic activity rely on colorimetric assays, which may lack precision. The aim of this study was therefore to evaluate the cytotoxic activity of *Annona muricata L.*, *Momordica charantia L.* and *Launaea taraxacifolia* Willd, three plants from the Beninese pharmacopoeia, through a flow cytometry approach.

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MATERIALS AND METHODS

Collection and extraction

The plant material used in our study consisted of leaves from *Annona muricata* L. (Annonaceae) and *Launaea taraxacifolia* Willd. (Asteraceae), as well as whole plants of *Momordica charantia* L. (Cucurbitaceae). The three species were harvested during the daytime in June 2023 in Abomey, a town in southern Benin, with the following GPS coordinates:

- *Annona muricata* L.: 77° E (7°10'55" N 1°59'6" E);

-*Momordica charantia* L.: 303° NW (7°8'3" N 1°59'23" E);

- *Launaea taraxacifolia* Willd.: 26° NE (7°11.2" N 1°59.5" E).

The plant species were identified by the National Herbarium of Benin, and a specimen of each was registered under the respective numbers YH 1023/HNB, YH 1025/HNB and YH 1024/HNB. After harvesting, the samples collected were washed and dried at room temperature of 20-25°C for 8 days and then powdered. Four extracts were prepared for each species. First, an aqueous decoction was obtained by boiling 100 g of powdered plant material in 1000 mL of water. Secondly, a sequential extraction was carried out by successively macerating the powdered plant material in solvents of increasing polarity, namely dichloromethane, ethyl acetate and methanol, at 10% (w/v). The extracts obtained were then filtered four times, concentrated in a rotary evaporator (VWR, HB 10 SO93), and evaporated to dryness in an oven at 40°C. The dry extracts were then stored at +4°C away from light until use.

Cell culture

THP-1 cells were used as cancer cells. The THP-1 cell line offers several advantages, including easy availability, favorable culture characteristics (e.g., good doubling time, up to 25 passages), and a homogeneous genetic background, which minimizes variability and thereby enhances reproducibility[8]. They were cultured in a culture medium consisting of RPMI 1640 (Thermo Fisher Scientific ®) minimum medium supplemented with 10% fetal bovine serum (FCS) Thermo Fisher Scientific ®, 1% penicillin + streptomycin (Pen/Strep 1X) and L-glutamine at a final concentration of 2 mM. The incubator temperature was maintained at 37°C in an atmosphere containing 5% CO_2. To ensure optimal growth, the cell density in the flasks was maintained within a range of $0.1x10^6$ to $1x10^6$ cells/mL. A subculture was performed each time the cell concentration exceeded the upper limit of this range.

Cell viability tests

Cell viability assays were conducted using flow cytometry (BD FACSCanto ®) according to the method described by Grimberg *et al.*, with optimizations for THP-1 cell line[9]. The cells were labelled with two fluorochromes, Hoechst 33258 (Sigma Aldrich®) at 1 µg/mL and Thiazole Orange at 0.1 µg/mL. Hoechst 33258 passes through the membrane of living and dead cells and binds to DNA, while Thiazole Orange (Sigma Aldrich®) binds to both DNA and RNA[10]. Because RNA degrades rapidly in dead cells, Thiazole Orange fluoresces strongly in live cells and weakly in dead cells, thus enabling differentiation. To prepare the final test solutions, each extract was dissolved in a mixture of culture medium and DMSO to obtain a stock solution. Each stock solution was then gradually diluted to obtain concentrations of 12.5, 25, 50, 100, 250 and 500 µg/mL for each extract. The final DMSO concentration did not exceed 0.5%. THP-1 cells in active proliferation phase were seeded in 96-well plates at 10^5 cells per well. After centrifugation, the culture medium was removed and replaced with fresh medium containing the extract at the desired concentration. Plates were incubated for 48 hours at 37°C in 5% CO_2. Initially, only the 250 µg/mL and 500 µg/mL concentrations were tested to identify the most active extracts. The most active extracts were then further tested across the full range of concentrations (12.5–500 µg/mL). Cisplatin (Sigma Aldrich®) was used as a positive control and 0.5% DMSO as a negative control. At the end of the incubation period, cells were rinsed with PBS (Thermo Fisher Scientific ®), stained for 30 minutes with the HO/TO solution, and analyzed by flow cytometry. This allowed determination of the IC_{50} values for each extract and for cisplatin (CIS). A plant extract was considered to have significant cytotoxic activity if its IC_{50} was ≤ 100 µg/mL[11,12].

Selectivity test

The selectivity test was performed on peripheral blood mononuclear cells (PBMCs) isolated from venous blood through the Ficoll-Hypaque method described by Böyum *et al*[13]. Once isolated, PBMCs were washed twice in PBS to remove platelets, and then resuspended in culture medium as described above. PBMCs were cultivated in 96-well plates and exposed to the same concentrations of extracts as used for THP-1 cells. Plates were then incubated for 48 hours at 37°C with 5% CO_2. After incubation, cells were rinsed with PBS, stained with the HO/TO solution for 30 minutes, and analyzed by flow cytometry. This experiment allowed for the determination of the CC_{50} (concentration causing 50% reduction in PBMC viability) for each extract. The Selectivity Index (SI) was then calculated as follows: $SI = CC_{50} / IC_{50}$ [14]. An extract was considered selective when its SI was >1[15].

Phytochemical screening

The phytochemical screening of extracts from the three plants was performed according to the methods described by Wagner & Bladt, and Houghton & Raman[16,17]. The detection of alkaloids, coumarins, flavonoids, anthracene glycosides, cardiotonic glycosides, lignans, naphthoquinones, as well as bitter principles was carried out by thin-layer chromatography (TLC) following the procedures described by Wagner and Bladt. Tube tests were employed to identify tannins, saponins, and terpenoids/steroids.

Data analysis

All tests were performed in triplicate. Cytometry data were analyzed using FlowJo® (v10.10.0). The gating strategy involved selecting the target cell population (THP-1 or PBMCs), eliminating cell doublets to retain only single cells, and distinguishing live from dead cells (figure 1). At least 10,000 events were acquired per sample.

The processed data were further analyzed using GraphPad Prism® (v10.2.3). One-way ANOVA followed by Tukey's post-hoc test was used to evaluate statistically significant differences among groups, including comparisons between each sample and the control. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Viability tests

Preliminary viability assays showed that four of the twelve tested extracts exhibited a statistically significant cytotoxic activity against THP-1 cells compared to the negative control. These were the dichloromethane extracts of *Annona muricata* L. (AMFD) and *Momordica charantia* L. (MCFD), and the ethyl acetate extracts of *Momordica charantia* (MCFA) and *Launaea taraxacifolia* Willd. (LTFA). At 250 and 500 µg/mL, AMFD reduced cell viability to 6.0% and 8.2% respectively (Figure 2A); MCFD to 28.8% and 1.1% (Figure 2 B); MCFA to 22.7% and 3.1% (Figure 2 B); and LTFA to 18.6% and 3.7% (Figure 2 C). Negative controls maintained viability above 90%, while cisplatin reduced it to near 0%. These results led to the selection of AMFD, MCFD, MCFA, and LTFA for further analysis.

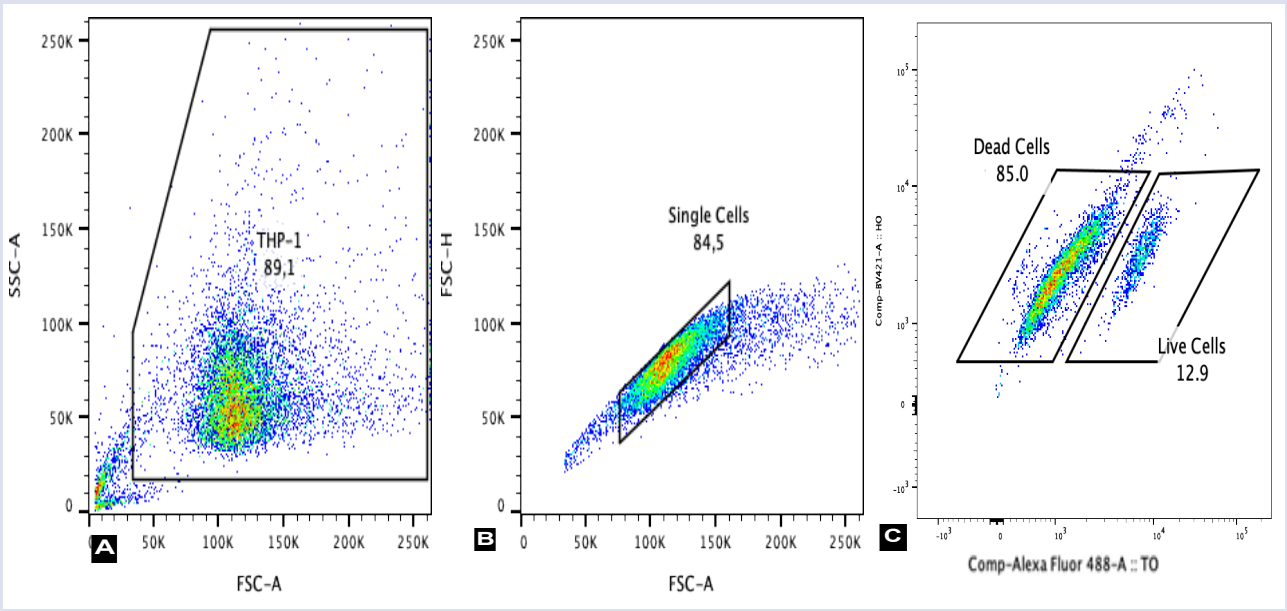

Figure 1. Gating strategy for flow cytometry data analysis.

A: Selection of target cells. B: Doublet exclusion. C: Identification of live and dead cells.

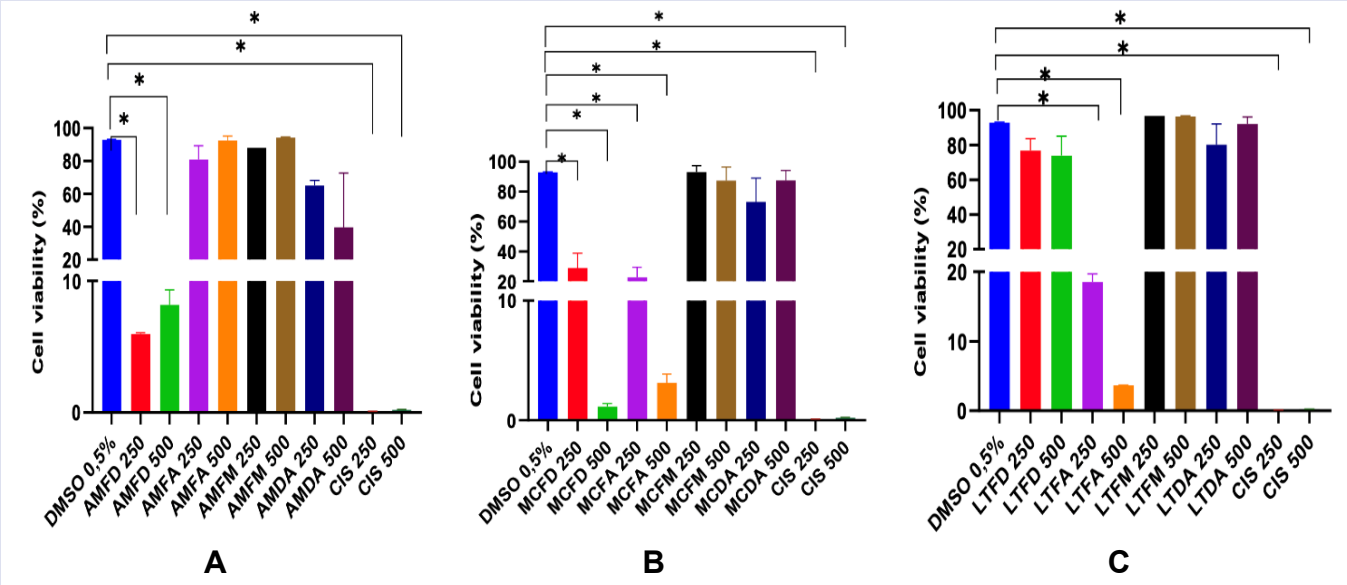

Figure 2. Preliminary viability test with extracts of **A**: *Annona muricata* L (AM). **B**: *Momordica charantia* L.(MC) and **C**: *Launaea taraxacifolia* Willd. (LT)

(FD: dichloromethane extract; FA: Ethyl acetate extract, FM: Methanol extract, DA: Aqueous decoction).

The results presented are the mean values ± SEM of three independent trials (** $p < 0.05$ represents a statistically significant difference compared to the negative control)*.

In subsequent tests, all four extracts exhibited dose-dependent cytotoxic effects on THP-1 cells.

The dichloromethane extract of leaves of *Annona muricata* L. (AMFD) had an IC_{50} of 139.6 ± 39.6 µg/mL (Figure 3A). This result is similar to those found by Hasan *et al.* (2022), who reported an IC_{50} of 155.34 µg/mL for an ethyl acetate extract of *Annona muricata* L. leaves on HeLa cells[18]. However, other studies have reported significantly lower IC_{50} values. For instance Hadisaputri *et al.* (2021), observed IC_{50} values of 2.86 and 3.08 µg/mL for the ethyl acetate and n-hexane extracts of *Annona muricata* L. leaves on MCF-7 cells[19]. Several factors could explain these differences, such as the location and time of collection of the plant materials or the type of cells used. Importantly, our results were obtained by flow cytometry, which provides a more accurate discrimination between viable and dead cells, compared to conventional metabolic assays such as MTT. While our IC_{50} values may appear higher, they reflect the actual proportion of dead cells rather

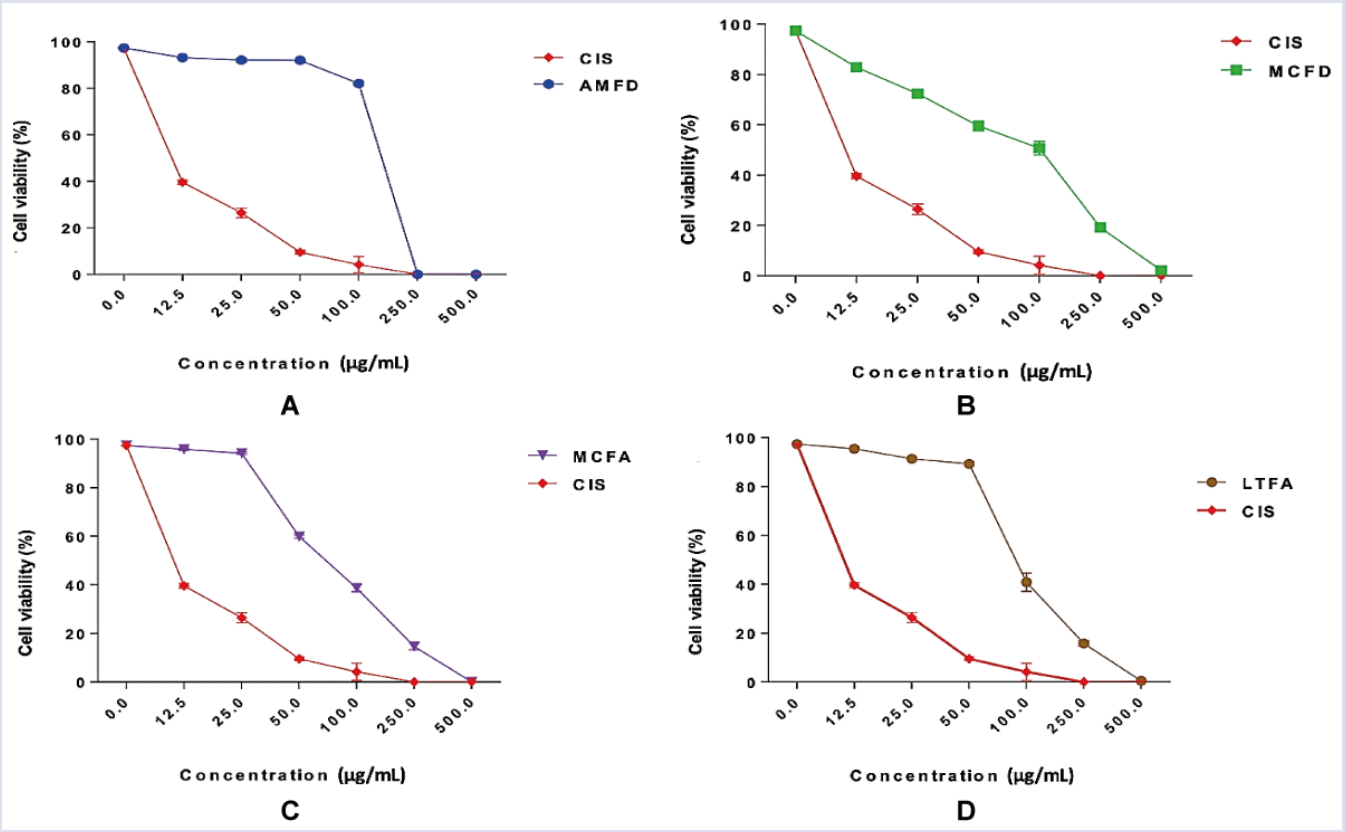

Figure 3. Cytotoxic activity profile of the extracts compared with cisplatin

A: dichloromethane extracts *of Annona muricata* L (AMFD).; **B**: dichloromethane extracts of *Momordica charantia* L. (MCFD); **C**: ethyl acetate extracts of *Momordica charantia* L.(MCFA) ; **D**: and the ethyl acetate extract of *Launaea taraxacifolia* Willd. (LTFA). Data are presented as means ± SEM of three independent trials.

than merely a reduction in metabolic activity, which can sometimes overestimate cytotoxicity. This overestimation may be due to several factors such as their dependence to cell metabolism, which can be altered without cell death, chemical interference by test compounds, and non-specific reduction of tetrazolium salts[20].

The dichloromethane and ethyl acetate extracts of the whole plant of *Momordica charantia* L. showed the highest activity against THP-1 cells, with IC_{50} values of 72.9 ± 7.0 µg/mL and 81.9 ± 12.6 µg/mL respectively. These results are similar to those reported by Jha *et al.* (2018), who obtained an IC_{50} of 102 µg/mL using a hydroethanolic extract of *Momordica charantia* L. fruit incorporated into silver nanoparticles[21]. The ethyl acetate extract of *Launaea taraxacifolia* Willd. showed an IC_{50} of 106.3 ± 20.8 µg/mL. This result differs from that reported by Thomford *et al.* in 2016, who obtained an IC_{50} of 2000 µg/mL for the aqueous extract of *Launaea taraxacifolia* Willd. leaves[22]. Such discrepancies may suggest that the active cytotoxic compounds present in *Launaea taraxacifolia* Willd. leaves are more likely to be soluble in organic solvents than in water. Figure 3 shows the cytotoxic activity profiles of each extract compared to cisplatin, which showed an IC_{50} of 7.92 ± 0.6 µg/mL.

In contrast to many previous studies that relied on colorimetric assays such as MTT for cytotoxicity assessment, the present work employed flow cytometry to evaluate cell viability. This methodological choice represents a significant improvement in accuracy, as flow cytometry allows direct quantification of live and dead cells based on membrane integrity and fluorescence staining, rather than relying on metabolic activity. The use of metabolic assays like MTT can sometimes lead to an overestimation of cytotoxicity, since several chemicals are capable of interfering with mitochondrial enzymes or altering the redox state of cells[23]. By adopting flow cytometric analysis, this study minimized such sources of bias and provided a more reliable estimation of the true cytotoxic potential of the tested extracts.

Selectivity test

The four extracts tested were less toxic to normal PBMCs than to THP-1 cancer cells (Table 1). The dichloromethane fraction of *Momordica charantia* L. exhibited the highest selectivity index (SI = 5.0), followed by the ethyl acetate fraction of *Launaea taraxacifolia* Willd. (SI = 2.9). The ethyl acetate fraction of *Momordica charantia* L. had an SI of 1.9, while the dichloromethane fraction of *Annona muricata* L. was the least selective (SI = 1.6). Furthermore, cisplatin showed no cytotoxic activity against PBMCs. The table 1 summarizes the CC_{50} values obtained and the resulting selectivity indices.

Phytochemical screening

Phytochemical screening of the four active extracts revealed the presence of multiple classes of bioactive compounds (Table 2). The AMFD fraction contained alkaloids, coumarins, flavonoids, anthracene glycosides, bitter principles, and terpenoids. Although they were not specifically investigated in this study, the potent activity observed may be attributed in part to acetogenins, a class of mitochondrial complex I inhibitors found in *Annona muricata* L., which are soluble in dichloromethane[24]. This is supported by several studies that have reported significant cytotoxic effects of acetogenins, also extracted from non-polar fractions on several types of cancer cell lines, such as Bel-7402 cells[25], MDA-MB-231, 4TI and BT-549[26], and DU-145[27]. The

Table 1. Selectivity index of the extracts

Extracts	CC_{50} (µg/mL)	IC_{50} (µg/mL)	Selectivity index
AMFD	226.4 ± 27.8	139.6 ± 39.6	**1.62**
MCFD	367.6 ± 62.5	72.89 ± 7.0	**5.04**
MCFA	160.9 ± 10.2	81.88 ± 12.6	**1.96**
LTFA	309.2 ± 34.0	106.3 ± 20.8	**2.91**
CIS	N-A*	7.916 ± 0.6	N-A*

*N-A: Not active.

Table 2. Phytochemical composition of the plants extracts.

Phytochemical group	AMFD	MCFD	MCFA	LTFA
Alkaloids	+	-	-	+
Cumarins	+	-	+	+
Flavonoids	+	-	+	+
Anthracene glycosides	+	+	+	+
Cardiotonic glycosides	-	-	-	-
Saponosides	-	-	-	-
Tannins	-	-	-	-
Lignans	-	+	+	-
Bitter principles	+	+	+	+
Terpenoids	+	+	+	+

+: Present; -: Absent.

potent activity of the dichloromethane fraction of *Annona muricata* (AMFD) may result from the presence of acetogenins, which inhibit mitochondrial complex I, leading to a collapse of the mitochondrial membrane potential and induction of apoptosis.

Both MCFD and MCFA fractions were rich in terpenoids, particularly cucurbitacins triterpenes with known anticancer activity through disruption of the cytoskeleton and inhibition of STAT3 signaling, triggering cell death[28]. Prior studies have shown that cucurbitacins exhibit cytotoxic effects on various malignant cell lines: A549 cells[29,30], CaSki, SiHa[31], and MCF-7[32].

As for the ethyl acetate extract of *Launaea taraxacifolia* Willd., it contained alkaloids, cumarins, flavonoids, anthracene heterosides, bitter principles and terpenoids (Table 2). Several other authors have found a similar phytochemical composition but with some differences, such as Agbessy *et al.* (2019)[33], and Koukoui *et al.* (2015)[34], of whom conducted studies in Benin. The few differences observed could be explained by the variability of the place and time of collection, as well as by the extraction method used. Furthermore, it remains difficult to attribute the cytotoxic activity observed to a particular phytochemical group.

Although this study demonstrated the cytotoxic potential of these extracts, several limitations should be noted. Cell viability was assessed *in vitro* on a single cell line, which may not fully reflect *in vivo* conditions, and selectivity tests used PBMCs from a single donor, limiting generalizability. Finally, the active compounds were not isolated or fully characterized. Preliminary phytochemical screening only provided an overview of extract composition, highlighting the need for more advanced analyses to identify the bioactive constituents responsible for the observed effects.

CONCLUSION

This study provides scientific evidence supporting the traditional use of *Annona muricata* L., *Momordica charantia* L., and *Launaea taraxacifolia* Willd., in cancer treatment. Among the twelve plant extracts tested, the dichloromethane fractions of *Annona muricata* L. and *Momordica charantia* L., as well as the ethyl acetate fractions of *Momordica charantia* L. and *Launaea taraxacifolia* Willd, demonstrated significant *in vitro* cytotoxic activity againstTHP-1 cell line, an monocytic leukemia cell line.

Importantly, these extracts exhibited moderate to high selectivity, showing reduced toxicity toward normal peripheral blood mononuclear cells (PBMCs). Phytochemical screening confirmed the presence of alkaloids, flavonoids, and terpenoids, secondary metabolites known for their anticancer potential.

This work employed flow cytometry, allowing for a more accurate and direct assessment of cell viability. This methodological approach strengthens the reliability of the cytotoxicity data obtained and provides clearer insight into the actual cytotoxic potential of the extracts.

These findings justify further investigation of these species, particularly through bioassay-guided fractionation, compound identification via LC-MS/MS or NMR, as well as *in vivo* studies. Ultimately, this study contributes to the scientific valorization of the Beninese pharmacopoeia and highlights the promise of local medicinal plants as a valuable source of novel anticancer agents.

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