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INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS BY A PENTACYCLIC TRITERPENES -RICH FRACTION FROM *Tabebuia hypoleuca* (C. Wright) Urb. LEAVES IN U-251 GLIOBLASTOMA CELLS

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ABSTRACT

Background: *Tabebuia* genera had been reported several pharmacological activities. Cuba has numerous endemic species in this genera, but only our group reported the anti-inflammatory, *in vitro* and *in vivo* antiproliferative properties from the specie *Tabebuia hypoleuca* (C. Wright) Urb. In vitro studies demonstrated that 786-0 (kidney), MCF-7 (breast) and glioma (U-251) were among the cancer cell lines most sensitive to ethyl acetate extract of leaves treatment.
Purpose: The aim of this study was to research the induction of cell cycle arrest and apoptosis by a pentacyclic triterpenes -rich fraction from *Tabebuia hypoleuca* leaves in U- 251 glioblastoma cells and identify the triterpene structures in this fraction.
Methods: A rich fraction of pentacyclic triterpenes were proceeded for the cell cycle arrest and apoptosis on glioblastoma cells using Annexin V and flow cytometry. The chemical composition was determinate by chromatography and spectroscopic methods.
Results: Our results showed that a pentacyclic triterpenes –rich fraction had the ability to cause cell arrest in G2/M phase and induce apoptosis. The elucidation of the chemical compounds in this fraction revealed a mixing of oleanolic, ursolic and betulinic acids. This finding about chemical composition in the first report by the genera.
Conclusion: Therefore, this study showed the presence of different pentacyclic triterpenes in *T. hypoleuca* and it have promising anticancer activity against glioblastoma.

Keywords: *Tabebuia hypoleuca*; cell cycle; apoptosis; pentacyclic triterpenes.

ABBREVIATIONS

U- 251 : *Glioblastoma cells*
G2/M : *Mitosis phase in cell cycle*
TMZ : *Temozolomide*
GBM : *Glioglastoma*
ZOL : *Zoledronic acid*
TH (3-6) : *Semi pure compounds from leaves*
MCF-7 : *Breast tumor cells*

INTRODUCTION

In the current decade, cancer has still remained the top listed death-causing disease despite of developments in tools of diagnosis, treatment and prevention and it is the most common death causing disease [1]. Cancer occurs when the cells proliferate without any control. The low efficacy of currently available drugs to cure cancer demands the identification of natural compounds for cancer prevention [2].

Therefore, search for effective anti-cancerous drugs with low toxicity is necessary for its treatment. Most of the anticancerous drugs currently used in chemotherapy are cytotoxic to normal cells [3]. The emergence of new anti-cancerous agents with fewer side effects has become an essential goal in cancer chemotherapy. Natural phytochemicals in medicinal herbs are one the most attractive strategies in cancer chemotherapy [4].

Recent studies have focused on the effects of plant-derived compounds on cell cycle regulatory and apoptotic pathways [5,6], yet little is known about their effects on non-apoptotic pathways e.g. autophagy, mitotic catastrophe, senescence leading to cell death, and programmed necrosis or ‘‘necroptosis’’ [7].

Cancer occurs when the balance between cell death and cell proliferation is deregulated. Apoptosis is an important physiological process essential for normal development and maintenance of tissue homoeostasis. This mode of cell death has been widely studied in the development of anticancer drugs. The advantage of apoptosis-inducing agents for the treatment of cancer is the elimination of tumor cells without causing inflammation [8].

Glioma is the most common primary malignant tumor of the intracranial tumors, accounting for approximately 45% of all intracranial tumors. The prognosis of glioma patients is generally poor, and the higher the malignant degree is, the worse the prognosis of glioma patients [9,10]. It has been reported that the median survival time of patients with grade IV polyglioblastoma is only one year with less than 5% of 5-year survival rate, which is one of the malignant tumors with the highest human mortality [11]. The treatment of glioma is mainly surgery, supplemented by radiotherapy and chemotherapy [12]. Despite the continuous development of medical technology in recent years, the improvement of survival rate of glioma patients is still limited. Many studies have reported that the high mortality rate of glioma is closely related to the excessive proliferation and invasion of tumor cells [13].

Doxorubicin, a familiar chemotherapeutic anticancer drug used in treating gliomas has many severe side effects that contribute to congestive heart failure. Additionally, this is further compounded by the growing menace of resistance by cancer cells to radiation and chemotherapy. Thus, there is an urgent need for the development of novel and efficient treatment options which should be able to overcome resistance and should have low toxicity to normal cells [14].

Temozolomide (TMZ), an alkylating agent which breaks the DNA double-strand,

thus causing cell cycle arrest and ultimately cell death, is a unique treatment regimen to glioblastoma (GBM), actually [15]. It is, by far, the most effective as it increases the median overall survival from 12 months (with RT alone) to 14.6 months, and the percentage of patients alive at 2 years increases from 10.4% to 26.5%. However, due to its short half-life, TMZ is administered at high dose, and prolonged systemic administration has resulted in a series of side effects. As a means to improve the efficacy of TMZ and reduce the side effects of chemotherapy, systemic TMZ administration using a biodegradable carrier such as nanoparticles is widely studied. Even though TMZ is the main drug choice for GBM treatment, other therapeutic agents have been explored, for example cucurmin, a bioactive compound derived from Indian spice *Cucurmin longa*; nimotuzumab has been used alone or concurrently in recurrent or relapsed brain tumors; Zoledronic acid (ZOL) is an aminobisphosphonate that has antitumor activity. Together, cucurmin, nimotuzumab, and ZOL are promising alternates to TMZ in the treatment of GBM. But, it is quite clear that there is no end to novelty and creativity in the development of new therapeutic agents to prolong the survival and improve the quality of life of GBM patients [16].

Tabebuia spp. have been traditionally used to treat syphilis, malaria, cutaneous infections, stomach disorders, cancer, inflammation, pain, bacterial and fungal infections, anxiety, poor memory, irritability, depression, and others [17,18].

β- lapachone, a quinone obtained from the bark of the lapacho tree (*Tabebuia avellanedae*) in South America, possess a wide range of pharmacological properties, and is a promising cancer chemopreventive agent. This compound induces apoptosis in HepG2 hepatoma cell line through induction of Bax and activation of caspase [19]. β-lapachone induced cell death was blocked by autophagy inhibitors, and ATG6 or ATG7 siRNA. These findings provide novel insights into the underlying mechanisms of β-lapachone-induced cell death, as well as offering a strategy for treatment of glioma, which are resistance to pro-apoptotic therapeutics [20].

Previously, we reported *in vitro* and *in vivo* antiproliferative activity of extracts and fractions of leaves and stems from *Tabebuia hypoleuca* and the presence of different pentacyclic triterpenes not reported before in the literature by this genera. The most active fraction from leaves (TH 3-6) showed better *in vitro* antiproliferative activity towards kidney, glioma, breast, lung, prostate, ovary, colorectal and leukemia cancer cell lines. Additionally, TH 3-6 reduced the Ehrlich solid tumor growth, showing effectiveness at a low dose (25 mg/kg) with no side effects and its fraction reduced 43% of the cell proliferation of tumor cells MCF-7 on Hollow fiber model.

Based on these significant *in vitro* and *in vivo* activities, the present study sought to elucidate the mechanism of action of TH 3-6 fraction in the cell death of the human tumor cell lines U- 251. The aim of the present study was to demonstrate the cell cycle arrest and apoptosis actions from this fraction and to identify the chemical compounds present associated with the activity.

MATERIALS AND METHODS

Chemicals and Equipment

Silica gel, hexane, dichloromethane and methanol (P.A. grade) were supplied by Merck (Germany). Guava Nexin Reagent,

Guava Multicaspase kit and Guava Mitosox Red were supplied by Millipore (California, USA). Flow cytometry experiments and analysis were performed using Guava EasyCyte Mini Flow Cytometry System, Millipore (Billerica, Massachusetts, USA). A total of 5.000 events per duplicate were acquired.

LC-908 Recycling preparative HPLC Hitachi Company (Japan), Column: Jaigel-2H and 1H (600 x 20). Reverse column chromatography by LiChroprep RP-18, 40-60 µm (Merck). 1H-NMR spectra were recorded on a Bruker Avance AV 300 but 13C-NMR and 2D-NMR spectra were recorded on a Bruker Avance AV 600 NMR instrument, using CDCl3 as solvent. The mass spectra (EI and HREI-MS) were measured in an electron impact mode on Varian MAT 112 or MAT 312 spectrometers.

Isolation of Pentacyclic Triterpenes-Rich Fraction from *Tabebuia hypoleuca* (C. Wright) Urb Leaves

Pentacyclic triterpenes – rich fraction was isolated from *Tabebuia hypoleuca* (C. Wright) Urb leave. *T. hypoleuca* were collected at the National Botanical Garden (JBN) in June 2017, Havana Province, Cuba. The identification of the plant was confirmed by the botanist Dr. Eldis R. Becquer and a sample was deposited in the herbarium of the experimental station with the number HFC-88204. Its leaves were separated and dried at room temperature and milled at 40 mesh. Solid-liquid successively extraction in Soxhlet increasing the polarity of the solvent were used with n-hexane, ethyl acetate and methanol. Each extract were filtered and concentrated using rotary evaporation, providing the crude hexane (THH, 1.15% yield), ethyl acetate (THA, 2.59% yield) and methanol (THM, 4.09% yields). *In vitro* cytotoxic assay monitored the extracts activity, showing that THA was active. An aliquot (2.5 g) of THA was chromatographed over silica gel (80 g), eluted first with hexane. The eluent polarity was increased by gradients of ethyl acetate and then methanol, providing sixteen fractions (100 mL). They were grouped according to the thin layer chromatography (TLC) profile, visualized with anisaldehyde reagent (50 mL acetic acid, 0.5 mL sulfuric acid, and 0.5 mL anisaldehyde) followed by heating at 110°C. It was observed a high quantity of solid in fractions elute with n-hexane- ethyl acetate mixture (THA 3 to7). This fraction was named as a fraction rich in pentacyclic triterpenes.

This fraction was proceeding by Exclusion Chromatography with Sephadex LH-20 with the elution with dichloromethane-methanol at 5% collecting 44 fractions of 2 ml each. Fraction 38- 44 was made a new flash chromatography separation and semi-preparative chromatography (reverse phase RP18, flow= 4 ml/min, elution MeOH- H2O, 80:20). The pure compounds were elucidated by NMR ((1H and 13C NMR) and EI Mass Spectrometry.

Annexin V-PE/7-AAD

U-251 cells were seeded at 5 x 10^5 cells/ml in 6 wells microplate and incubated for 24 hrs, followed by treatment with fraction rich in pentacyclic triterpenes (TH 3-6) at various concentrations (2.5, 5, 10 and 20 µg/ml). After 24-h incubation, medium and floaters from each well were collected and cells were rinsed with PBS and incubated with trypsin/EDTA 0.25% until 80% of the cells were detached. All cell suspensions were collected and centrifuged. Following centrifugation, the supernatant was removed and pellet was washed with ice-cold PBS and re-centrifuged. The supernatant was discarded and the pellet

was mixed with 100 µl of Guava Nexin Reagent (annexin-V PE/7-AAD) for 20 min at room temperature and in the dark. Flow cytometry analysis was performed using Software Guava®Nexin. The stained samples were analyzed within an hour.

Cell-cycle Analyses

Cells cycle analyses were performed with the Guava Cell Cycle reagent (Guava Technologies, Hayward, CA) in accordance with the manufacturer's instructions. U-251 cells were deprived for 24 h and then treated with TH 3-6 at various concentrations (0.5, 2.5, 5 y 10 µg/ml) for 24 h. It was used a positive group with colchicine at 0.5 µg/mL. The cells were then harvested and resuspended at a density of 1x 10^5 cells in phosphate-buffered saline (PBS; 100 µL). The binding buffer containing propidium iodide (PI) was added to the cells (100 µL), and the suspension was incubated in the dark for 20 min at room temperature. The cells were then analyzed with a Guava Easycyte Mini flow cytometer (Guava Technologies, Hayward, CA).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments with duplicate samples. Statistical analyses were performed with Infostat 2016 [21], followed by Kruskal-wallis test was used, and p-values less than 0.05 were regarded as significant.

RESULTS

Structures of Pentacyclic Triterpenes Isolated from Rich Fraction TH 3-6

Structure of compound 1, 2 and 3 (Fig. 1) were confirmed as oleanolic acid, ursolic acid and betunilic acid by comparing NMR and mass spectroscopic data of these three compounds with reported data [22,23].

Compound 1 was obtained as white powder with the positive EI-HRMS of the molecular ion peak at m/z 456.36 indicative of molecular formula of $C_{30}H_{48}O_3$ (calcd. 456.3603) that was in accordance with the number of carbons and hydrogens counted in NMR data. The seven degree of unsaturation, ^{13}C-NMR and DEPT spectral data, suggested five rings plus one acidic carbonyl an olefinic group in the molecule. Mass spectrum showed prominent peaks at m/z 248 $[C_{16}H_{24}O_2]$+ and 207 $[C_{14}H_{23}O]$+ retro-Diels-Alder (RDA) fragments characteristic for Δ^{12}-amyrine series with COOH group (6), 203 $[C_{15}H_{23}]^+$ due to the loss of COOH from *m/z* 248 along with other fragments at m/z 438 $[M - H_2O]^+$, 410 $[M - HCOOH]^+$, 392 $[410 - H_2O]^+$, 189 $[C_{14}H_{21}]^+$, 175 $[C_{13}H_{19}]^+$, 133 $[C_{10}H_{13}]^+$, 119 $[C_9H_{11}]^+$ and 69 $[C_5H_9]^+$ in Fig. 2. The 13C- NMR and DEPT spectra confirmed presence of thirty carbon consisted of eight quaternary, five tertiary, ten secondary carbons and seven methyls. 1H-NMR showed signals for an olefinic proton at δ_H of 5.26 (t, J = 3 Hz), a carbonilic proton at δ_H of 3.20 (dd, $J_{ax,\ ax}$= 11.5, $J_{ax,eq}$ = 4 Hz) suggesting it´s axial and α orientation and δ_H of 2.81 (dd, J = 13.5, 3.5 Hz) along with seven singlet methyls at δH of 1.23, 1.11, 0.97, 0.91, 0.89, 0.88 and 0.77. All the above data of compound 1 identified it as oleanolic acid [22].

Compound 2 was obtained as white powder with the positive EI-HRMS of the molecular ion peak at *m/z* 456.3622 indicative of molecular formula of $C_{30}H_{48}O_3$ (calcd. 456.3603) that was in accordance with the number of carbons and hydrogens counted in NMR data. The seven degrees of unsaturation, ^{13}C-NMR and DEPT spectral data, suggested five rings plus one acidic carbonyl and one olefinic group in the molecule. Mass spectrum showed peaks at

m/z 248 as a strong peak and m/z 207 in smaller extent as characteristic for 12- 13 double α or β- amyrine series with COOH group that was confirmed by m/z 203 [248 - COOH] as a prominent peak with other fragments at m/z 438 $[M - H_2O]^+$, 410 $[M - HCOOH]^+$, 189 $[C_{14}H_{21}]^+$, 133, 119 and 55. ^{1}H-NMR spectrum shown olefinic proton at δ_H of 5.27 (t, J = 3.3 Hz), a proton germinal to hydroxyl group at δ_H of 3.19 (dd, $J_{ax,ax}$ = 10.8, $J_{ax,eq}$ = 5.1 H_z) inferring its α – and axial orientation δ_H of 2.81 (br-d, J= 9 Hz) five singlet methyls at δ_H of 1.23, 1.12, 0.97, 0.90 and 0.76 (H-23, 27, 26, 24 and 25), two doublet methyls at δ_H of 0.84 (d, J = 6.6 Hz, Me30) and 0.79 (d, J = 6.9 Hz, Me29) which were characteristic for ursane skeleton. The ^{13} C- NMR and DEPT spectra confirmed the presence of thirty carbon consisted of seven quaternary, seven methyls that were in accordance with ursolic acid reported in the literature [24].

Compound 3 was obtained as colorless needles. Spectrometry (EI-HRMS) of the molecular ion peak at m/z 456.3587 matched with the number of carbons and hydrogens counted in NMR data. With regard to the seven degrees of unsaturation, 13C-NMR and Distortion less Enhancement by Polarization Transfer (DEPT) spectral data, five rings, one acidic carbonyl and one double bond were detected in the molecule. ^{1}H-NMR revealed six singlet methyls at δ_H of

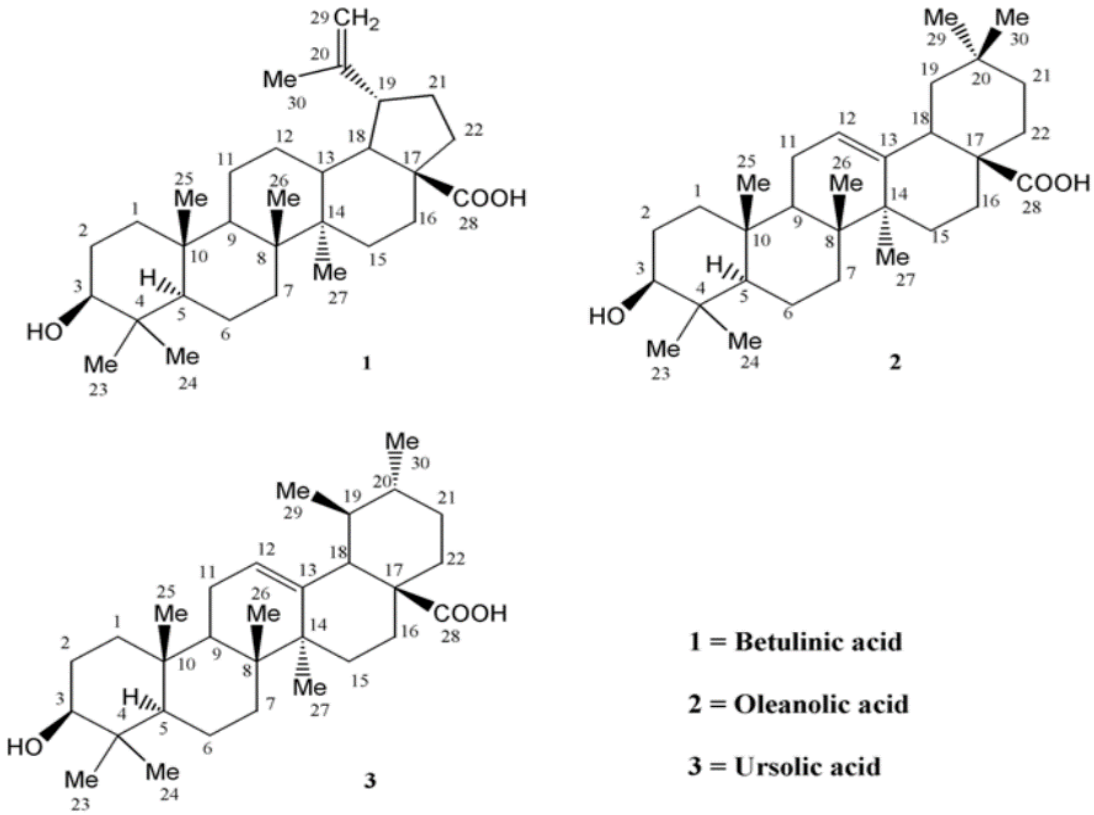


Fig. 1. Pentacyclic triterpenes from *Tabebuia hypoleuca* (leaves)

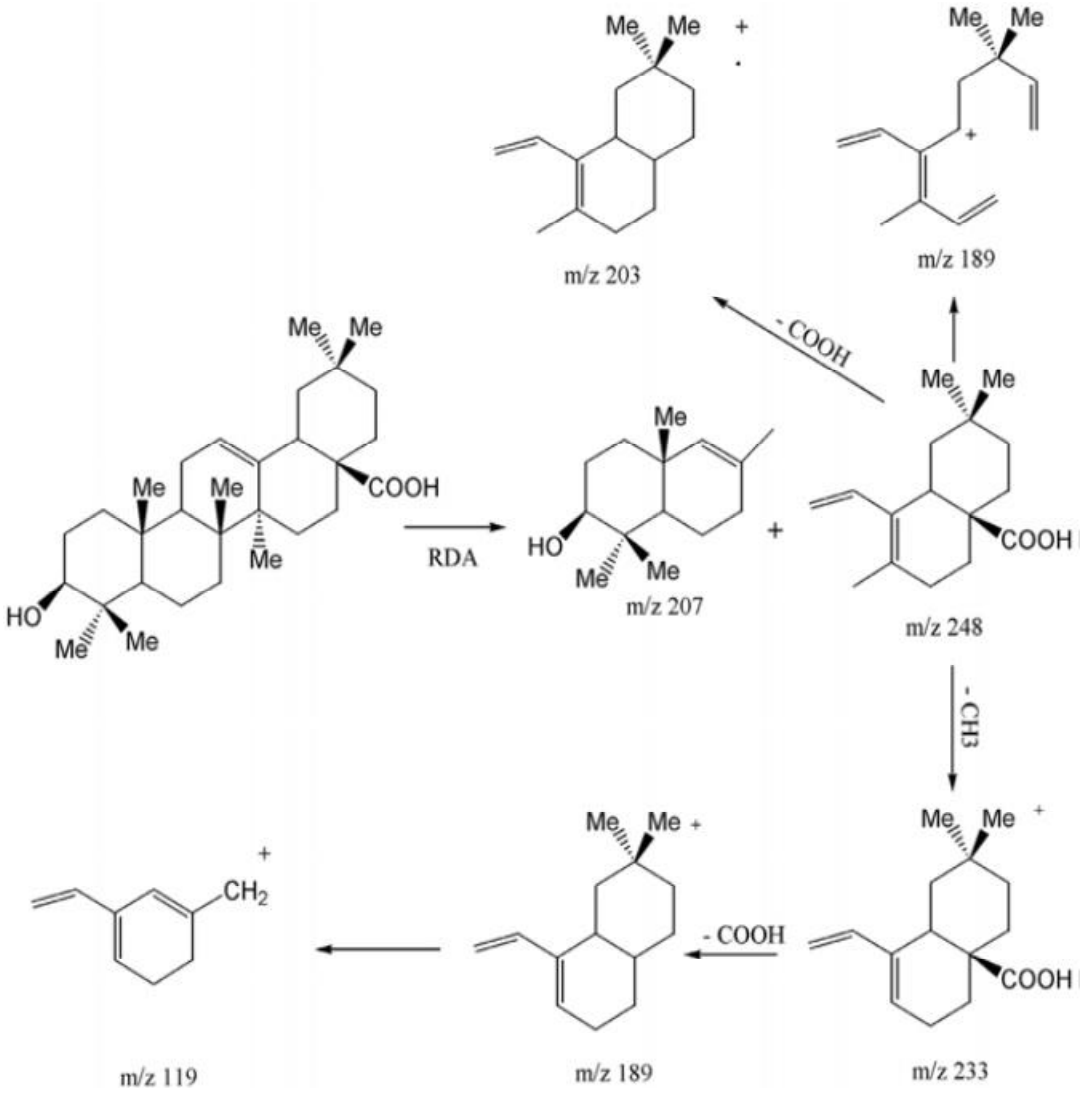


Fig. 2. EI-Mass fragmentation pattern of oleanolic acid

1.67 s, 0.95 s, 0.94 s, 0.91 s, 0.80 s and 0.73 s, a pair of olefinic protons at δ_H of 4.71 and 4.58 (each one H, br-s) characteristic of exocyclic methylene group, a carbonilic proton at δ_H of 3.17 (dd, $J_{ax,ax}$ = 10.8, $J_{ax,eq}$ 5.1 Hz, H3) referring s axial and α orientation, δ_H of 2.95 (dt, *J* = 11.1, 3 Hz, H19), 2.24 (br-d, *J* = 12.3 Hz, H16a), 2.16 (brt, *J* = 10.4 Hz, H13) and 1.95 (dd, *J* = 11.1,4.5 Hz, H21a) which were characteristic for lupine triterpenes. Further information about the compound was obtained from typical EI-Mass related to the fragmentation pattern of lupane type triterpenes (Fig. 3) through the presence of m/z 438 [M-H_2O], 411 [M-COOH], 248

$[C_{16}H_{24}O_2]$, 203 [248 - COOH], 220 $[C_{15}H_{24}O]$, 203 [220 - OH], 220 $[C_{14}H_{20}O_2]$, 175 [220 - COOH], 207 $[C_{14}H_{23}O]$, 189 [207 - H_2O] 205 and 207 [M - $C_{16}H_{27}$] characteristic series for betulinic acid [25]. ^{13}C-NMR (BB and DEPT) spectra showed thirty carbons comprised of seven methyls, eleven methylenes, six methines and six quaternary carbons. Based on above observations, the spectral data of compound 3 was similar to those previously reported of betulinic acid.

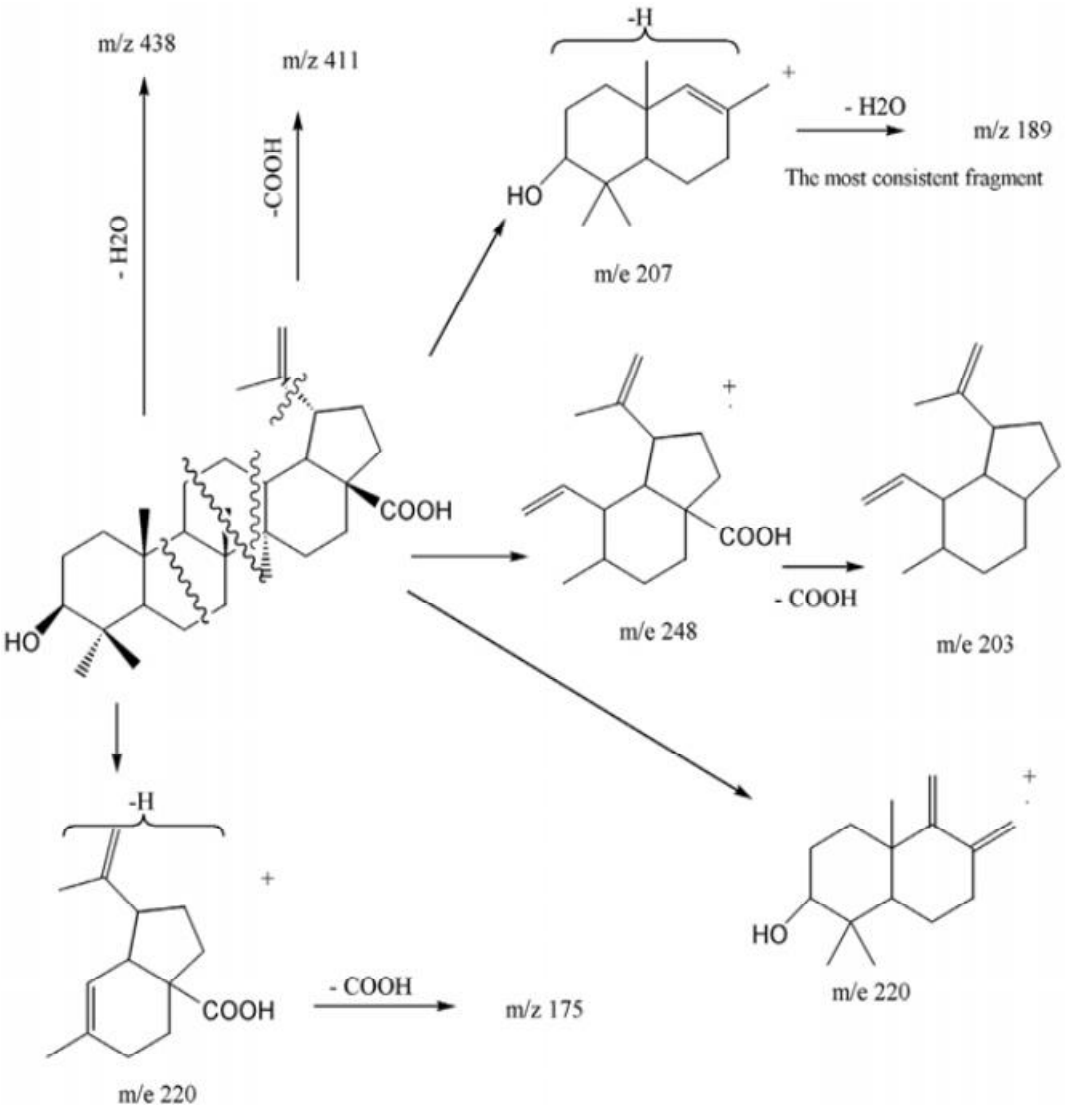


Fig. 3. EI-Mass fragmentation pattern of betulinic acid

Annexin V-FITC/PE

Considering that the apoptosis induction is one of the main mechanisms of the action of antitumor drugs, we evaluated the ability of triterpene acids to induce apoptosis in the cells of glioma cell line U-251 using an Annexin V-FITC/PE kit. Annexin V, a Ca^{2+}-phospholipid binding protein, has a high affinity to phosphatidylserines, which are localized on the inner side of the membrane in living cells, and when apoptosis is induced, they translocate to the outer side of the membrane, staining with Annexin V-FITC. For assessment of PS exposure, we used the double staining with Annexin V and 7-amino-actinomycin D (7-AAD). Annexin V binds to PS translocated to the outer face of the cell membrane during the initial process of cell death while 7-AAD binds to the DNA only after loss of cell membrane integrity. This way, cells stained only with annexin-V-PE could be considered as in early stage of cell death subroutine. The results of an analysis of the proapoptotic effect of TH (3-6), fraction of pentacyclic triterpenes from *Tabebuia hypoleuca* leaves are presented in Table 1 and Fig. 4. This fraction promoted a pronounced proapoptotic effect, with an increase in early and late stages of U-251 apoptosis in comparison with the control group. This effect was doses-dependent. At 20 µg/mL (data not shown) more than 50% of the tumor cells had died.

Results are presented as the mean ± SD of three independent experiments with duplicate samples. Kruskal-wallis test, $p < 0.05$ significant difference relative to the untreated cells. The letters give the significant by each column, independent.

Cell-cycle Analyses

Flow cytometry was used to determine the effects of TH 3-6 fraction on cell cycle distribution and apoptosis; the results are shown in Fig. 5. Colchicine (0.5 µg/mL), was employed a positive control, as it promotes a cell cycle arrest on G2/M phase. Colchicine is a natural product that binds to tubulin leading microtubule destabilization and prevents mitosis progress [26]. The cell cycle distribution of treated U-251 cells is presented in the Fig. 5. As expected, colchicine increased the population of U-251 cells in G2/M phase (65.1 ± 5.98%) followed by a decreasing on G1 phase cell population (17.61 ± 3.16%) in comparison to vehicle treated cells (Fig. 5). At these experimental conditions, TH 3-6 increased the population of cells in S and G2/M at 5 µg/mL concentration (17.06% and 32.19%, respectively). At 10 µg/mL, there was an increase of sub-G1 phase cells (4.95%), what means cells were in process of cell death by apoptosis, corroborating with Annexin-V/7AAD data. This concentration has culminated in cell cycle arrest at G1 phase (66.89%).

DISCUSSION

We had previously reported the in vitro antiproliferative activity from TH 3-6, a rich fraction of pentacyclic triterpenes isolated from ethyl acetate extract of *Tabebuia hypoleuca* on different tumor cell lines, with a TGI of 11.16 µg/mL on glioma cells (U-251) and in vivo anticancer effect on two experiments: Ehrlich´s test and Hollow fiber assay with human breast cell (MCF- 7) [27].

In the present study we proceeded to the isolation of the active compounds present in this fraction. We identified three pentacyclic triterpenes acids, with the major proportion of oleanolic acid, following for ursolic and betunilic acids. With this finding we could show that our Cuban endemic plants have as secondary metabolites a high concentration of pentacyclic triterpenes. Our finding are the first report about the presence of pentacyclic triterpene acids in *Tabebuia* spp.

Table 1. Apoptosis stages (in percent) of U-251 cell line after treatment with a pentacyclic triterpene rich fraction from *Tabebuia hypoleuca* leaves

Samples	Doses µg/ml	Living cells (Q3)	Early apoptosis (Q4)	Late apoptosis (Q2)	Necrosis (Q1)	General apoptosis
DMSO	1	91,03 ± 1,37 A	0,73 ± 0,51 A	4,43 ± 0,85 A	3,80 ± 1,18 A	5,20 ± 1,32 A
TH 3-6	2,5	86,17 ± 2,58 AB	3,97 ± 0,96 AB	6,37 ± 1,03 AB	3,53 ± 0,67 A	10,33 ± 1,99 AB
	5	75,87± 1,83 BC	7,13 ± 0,42 BC	11,57 ± 1,17 BC	5,47 ± 0,40 AB	18,67 ± 1,59 BC
	10	51,23± 1,17 C	13,87 ± 0,31 C	27,20 ± 1,47 C	7,73 ± 0,60 B	41,13 ± 1,76 C

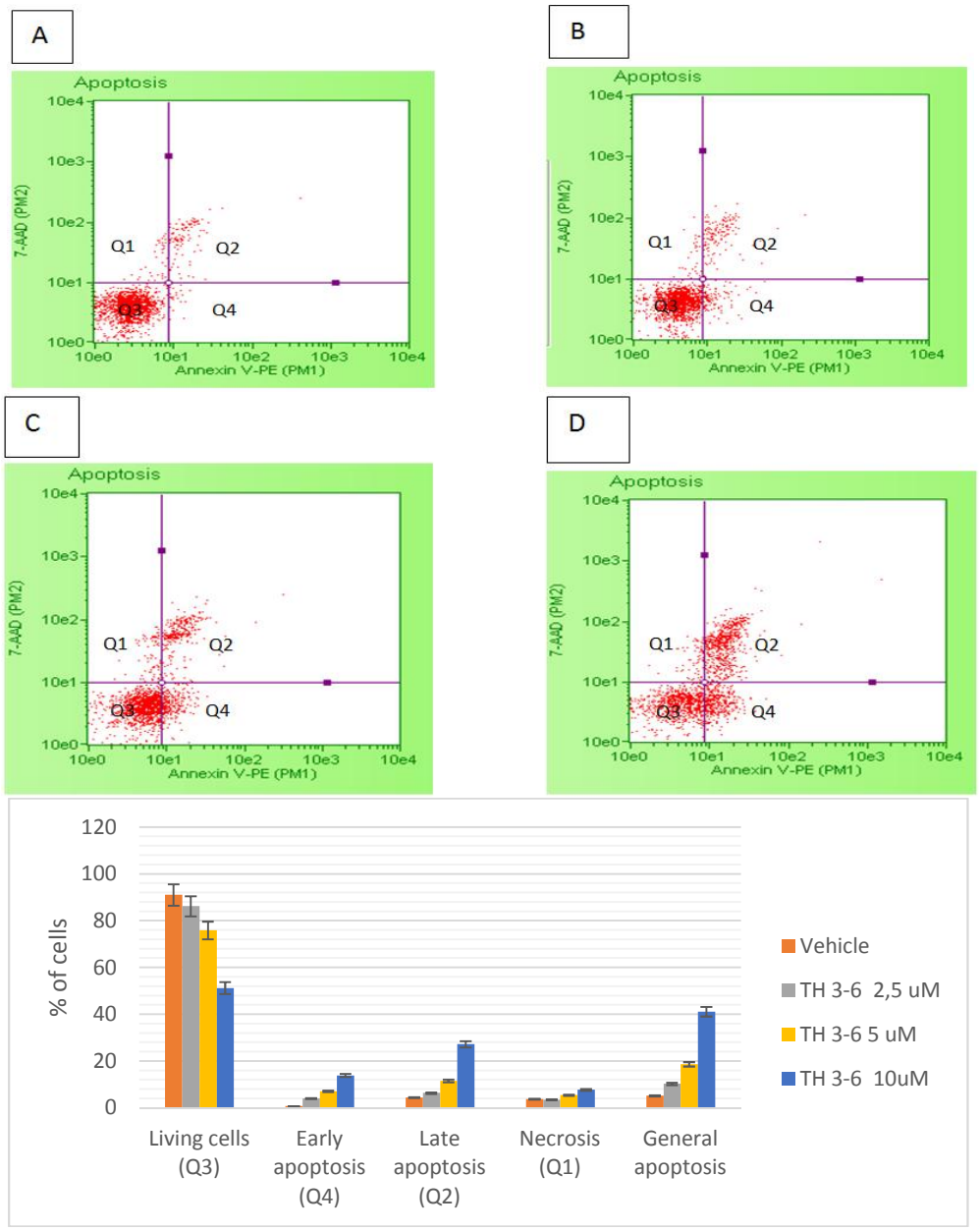
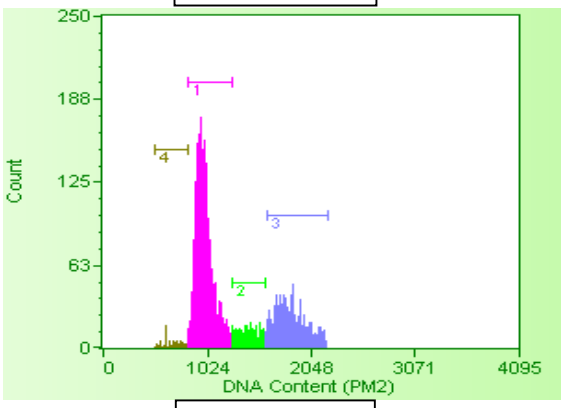
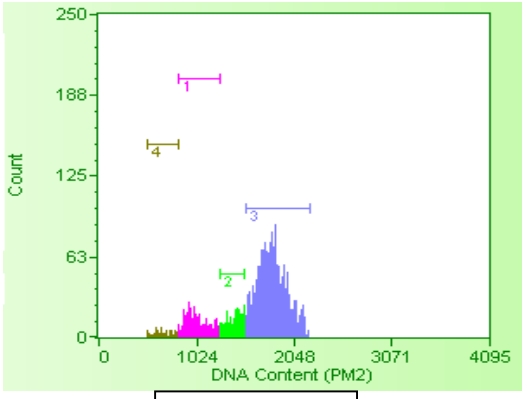


Fig. 4. Apoptosis stages of U-251 cell line after treatment with a pentacyclic triterpenes rich fraction from T. hypoleuca (as percentage of the whole cell population): (a) cells untreated (control); (b, c, d) treatment with the fraction: 2.5; 5 and 10 µg/mL), respectively. Diagram captions: Q1 is necrosis; Q2 is late apoptosis; Q3 is normal cells; Q4 is early apoptosis

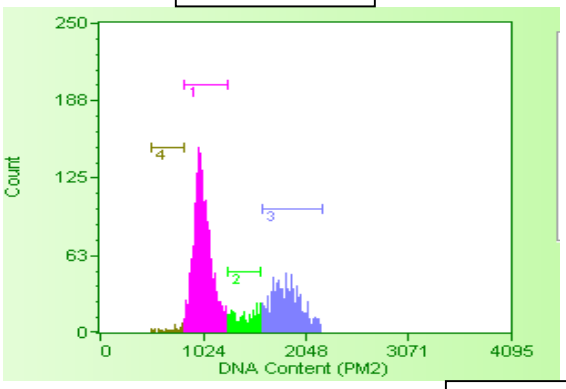
Control (DMSO)



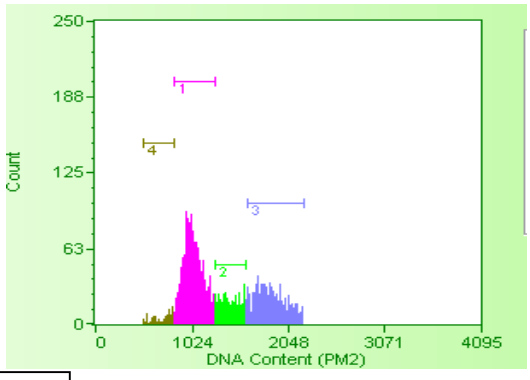
Colchicine 0,5 µg/mL



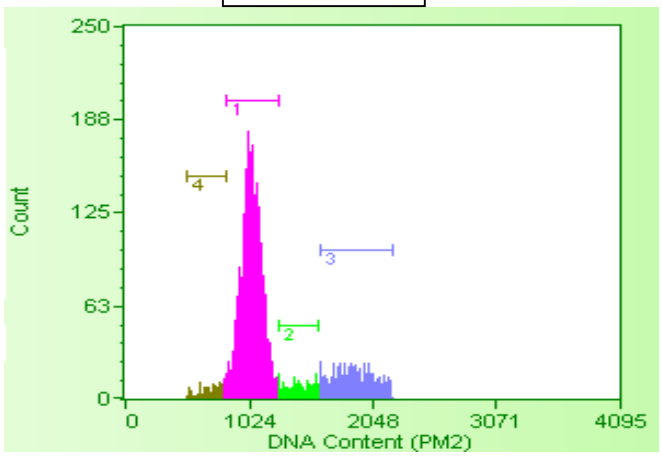
TH (3-6)2,5 µg/mL



TH (3-6) 5 µg/mL



TH (3-6) 10 µg/mL



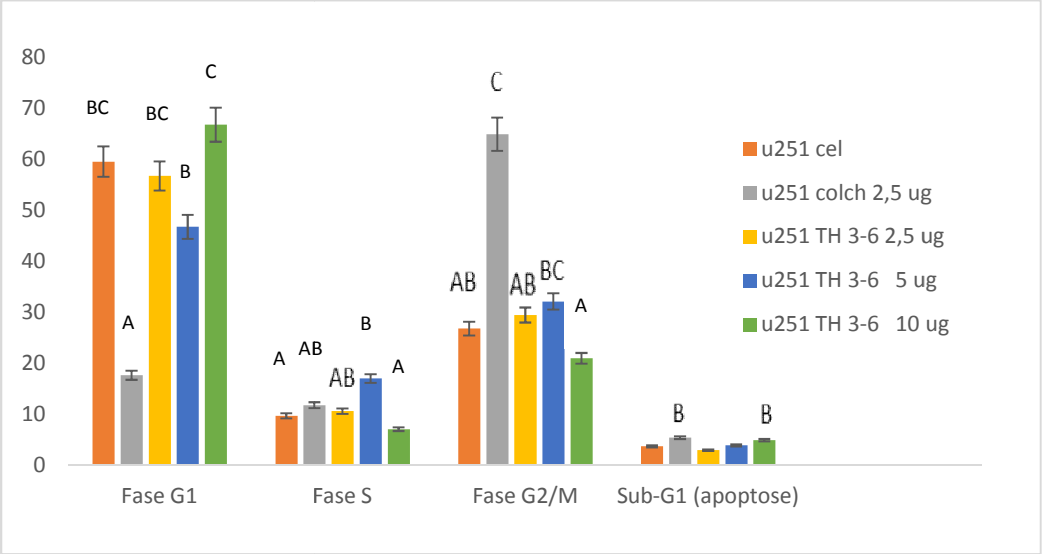


Fig. 5. Effect of a rich pentacyclic triterpene fraction from *Tabebuia hypoleuca* on cell cycle progression in glioma cells. U-251 cells were exposed to various concentrations of TH 3-6 fraction (2.5; 5; 10 µmol/L) for 48 h and then analyzed using flow cytometry. B. The percentages of the total cell population in the sub-G1, G1, S, and G2/M phases of the cell cycle are calculated. The results are represented by the mean± SE, in percentage of cells; p<0.05, statistical analysis by Kruskal - wallis test. Letters give the significant by each column, independent

The deregulation of apoptosis has frequently been observed in various cancers; thus, the induction of apoptosis has been proposed as an important strategy to treat cancer [28]. Generally, there are two major pathways that control the initiation of apoptosis: the death – receptor - induced extrinsic pathway and the mitochondria-apoptosome mediated intrinsic pathway, both of which ultimately activate a series of effector caspases and apoptosis effector molecules [29].

Cell cycle arrest and apoptosis are the two main causes of growth inhibition. Many anticancer agents exhibit their activity by inhibiting cell cycle progression at a particular checkpoint such as G0/G1, S, or G2/M and there by induce apoptosis [30].

Many clinically used antitumor drugs were reported to be able to induce apoptosis on tumor cells, so the novel compounds that can induce apoptosis in tumor cells can be novel antitumor drugs [31,32].

The fraction rich in pentacyclic triterpenes, at highest concentration evaluated, has led to apoptosis on glioblastoma U-251 cells. Moreover, the intermediate concentration caused an increase of cells in S and G2/M phases and the highest concentration led to cell cycle arrest at G1 phase, with a great increase of cells at sub-G1 phase, highlighting its apoptotic potential.

This fraction is a mix of ursolic, oleanolic and betunilic acids, three compounds with

preview reports of antiproliferative activity against some tumor cells. According to Wang et al. [33], ursolic acid (UA) induces a decrease in the viability of a variety of cell types, including colon, prostate, gastric and breast cancer cells and that the mechanisms of UA-induced apoptosis are associated with inhibition of the initiation of DNA replication, up-regulation of MMP family gene expression, induction of p53, activation of JNK1/2, caspase-3, caspase-8 and caspase-9 and decrease in the expression of Bcl-2 family proteins. These authors revealed that UA inhibits cell proliferation and induces apoptosis in human glioblastoma cell lines U-251 by suppressing TGF-b1/miR-21/PDCD4 pathway. By accumulated results reviewed by Navin et al. [34] suggest that UA causes apoptosis in cancer cells by activating cell cycle arrest and altering related molecular targets that promote its anti-cancer properties. For also, UA can reportedly be used as a potential drug when integrated with modern technological advancements to treat cancer and other diseases [34].

Prasad and colleagues [35] described the chemosensitizing properties of UA against different cancers and to chemotherapeutic drugs. With relatively low concentrations of UA cancer cells were sensitized to the chemotherapeutic agents Taxol or cisplatin through the suppression of NF-κB activity. Interestingly, the chemo-sensitization by UA was observed only in cancer cells, not in primary normal cells.

Guo et al. [36] demonstrated that oleanolic acid (OA) inhibit migration and invasion of malignant glioma cells (U-87 MG) by inactivating MAPK/ERK signaling pathway.

An article published by Wang and co-workers revealed that oleanolic acid (OA) inhibited tumor growth in mice and HepG2 cell proliferation. In both tumors and cells, OA induced apoptosis involving increase of Bax, decrease of Bcl-2, release of mitochondrial cytochrome c into the cytosol and subsequent activation of caspase-9 and -3, followed by cleavage of PARP, which was consistent with previous findings of antitumor effect of OA in other cells. Moreover, OA induced G2/M cell cycle arrest and the authors suggest that P21-mediated inhibition of cyclin B1/cdc2 may be involved at this result [37].

OA also presented anti-tumor activity on pancreatic cancer cells (Panc-28) with cell cycle arrest in S phase and G2/M phase and remarkable apoptosis, evidenced by an increased percentage of early/late apoptotic cells, DNA ladder and nuclear morphology change [38].

Other reports shown that OA treatment prostate DU145 cells showed enhancement in the percentage of cells in G2 compared to the control cells ($P < 0.05$). Treatment with 50 and 100 µg/mL OA resulted in 23.15% and 27.62% of cells arrested in the G2 phase, respectively. Conversely, breast MCF-7 cells treated with the same growth suppressive concentrations of OA reported a decrease in the percentage population (64.62% and 67.21%, respectively), and a decrease in the S and G2 populations compared to those in the control. MCF-7 and glioblastoma U87 cells were arrested the G1 phase at 100 µg/mL concentration. These observations suggested that OA impacted cell cycle progression based on cell types [39].

One betunilic acid –rich fraction from *Dillenia suffruticosa* root inducted cell arrest of G0/G1 phase and apoptosis in MCF-7cells involved p53/p21 and mitochondrial signaling pathway [40]. Zhan et al. [41] that

the treatment with betulinic acid was able to inhibit the colony formation potential in a dose-dependent manner. A lower cytoxicity by betulinic acid against normal human epithelial FR2 cells was observed compared with H460 cells. The betulinic acid exerted anticancer activity via the induction of apoptosis by regulating the Bcl-2/Bax signaling pathway. Additionally, treatment with betulinic acid resulted in cell cycle arrest of paclitaxel-resistant lung cancer H460 cells at the G2/M phase. Betulinic acid was also reported to cause reductions in the mitochondrial membrane potential in a dose-dependent manner. So, betulinic acid may be a useful drug candidate for the management of drug-resistant lung cancer [41].

These findings support the relevant antiproliferative properties presented by this fraction rich in pentacyclic triterpenes and reinforce the need for further studies on its mechanism of action. This work encourages investigations on the anticancer property of this fraction, using animal models for gliomas.

CONCLUSION

Therefore, this study showed the presence of different pentacyclic triterpenes in *T. hypoleuca* as the first report of these compounds in this genera and, it have promising anticancer activity against glioblastoma. We recommend further the study of these fraction by the treatment of glioblastoma.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests regarding the publication of this paper.

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