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Identification of Oleandrin and other Cardenolides with Anticancer Activity in Methanolic Extracts from Plants of the Apocynaceae Family

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19 de Junio de 2020, ciudad de Tena, Napo, Ecuador

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Tena, 19 de Junio de 2020

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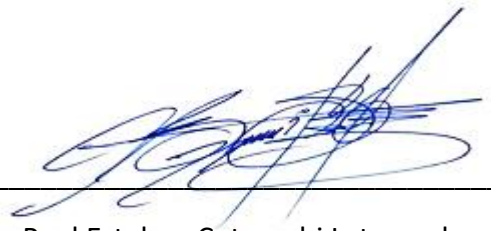

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APROBACIÓN DEL TRIBUNAL

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Resumen

Los cardenólidos son una familia de compuestos tipo esteroide, que debido a su actividad biológica han llegado a ser considerados como posibles agentes anticancerígenos. La familia Apocynaceae es la principal fuente de estos compuestos, de los cuales cerca del 25% presentan actividad anticancerígena. En esta investigación se consideró seis especies de la familia Apocynaceae presentes en la Amazonía ecuatoriana: *Himatanthus tarapotensis, Himatanthus bracteatus, cf. Aspidorperma excelsum, Rauvolfia praecox* y *Tabernamontana sananho*; siendo *Nerium oleander* la especie modelo. Se evaluó la presencia de oleandrina y otros cardenólidos, así como también la actividad citotóxica en dos líneas celulares de cáncer, HeLa y D-384, de los extractos metanólicos de estas seis especies. Se encontró que *H. tarapotensis, H. bracteatus* y *cf. A. excelsum* poseen cardenólidos, entre ellos oleandrina. Además, los extractos evaluados evidenciaron una fuerte actividad citotóxica en las dos líneas celulares sin producir efectos hemolíticos marcados. La actividad citotóxica mostrada por *H. tarapotensis, H. bracteatus* y *cf. A. excelsum* es superior a *N. oleander* y puede ser atribuida a un efecto aditivo o sinérgico entre cardenolidos y otra familia de compuestos como triterpeniodes, alcaloides de indol o iridoides reportadas en Apocynaceaes. Los resultados obtenidos ratifican el potencial que pueden presentar las especies vegetales en la búsqueda de nuevos agentes contra enfermedades complejas.

Palabras clave: Apocynaceae, Ecuador, cardenólido, anticancerígeno, oleandrina.

Abstract

Cardenolides are a family of steroid-type compounds, which due to their biological activity, have become consolidated as possible anticancer agents. The Apocynaceae family is the main source of these compounds, of which about 25% show anticancer activity. In this research, six species of the Apocynaceae family present in the Ecuadorian Amazon were considered: *Himatanthus tarapotensis, Himatanthus bracteatus, cf. Aspidorperma excelsum, Rauvolfia praecox* y *Tabernamontana sananho*; being *Nerium oleander* the model specie. In the methanolic extracts of these six species was evaluated the presence of oleandrin and other cardenolides, as well as the cytotoxic activity in two cancer cell lines, HeLa and D-384. It was found that the species *H. tarapotensis, H. bracteatus,* and *cf A. excelsum* possess cardenolides, among them oleandrin. Furthermore, these extracts showed strong cytotoxic activity in the evaluated cell lines without producing marked hemolytic effects. The cytotoxic activity shown by H*. tarapotensis, H. bracteatus* and *cf. A. excelsum* is greater than *N. oleander* and can be attributed to an additive or synergistic effect between cardenolides and another family of compounds such as triterpenoids, indole alkaloids or iridoids reported in Apocynaceaes. The results obtained confirm the potential that plant can present in the search for new agents against complex diseases.

Keywords: Apocynaceae, Ecuador, cardenolide, anticancer, oleandrin.

1. Introduction

Cardenolides are a family of steroid-derived compounds which are naturally found in plants either as glycosides or as free genins (lactone plus steroid) [1]. These compounds show several chemical structures. Although, they have similar characteristics such as: 23-carbon steroidal nucleus whose A/B and C/D rings are in *cis* conformation (unlike sex hormones and corticosteroids) [2], a γ-unsaturated α-lactone (furanone) in C17, β-hydroxyl group in C14, β-methyl in C10 and C13, and glycosidic portion in C3 [3]. In some cases there are substituents such as hydroxyl, formyl or acetyl groups in C16 (Figure 1) [4,5].

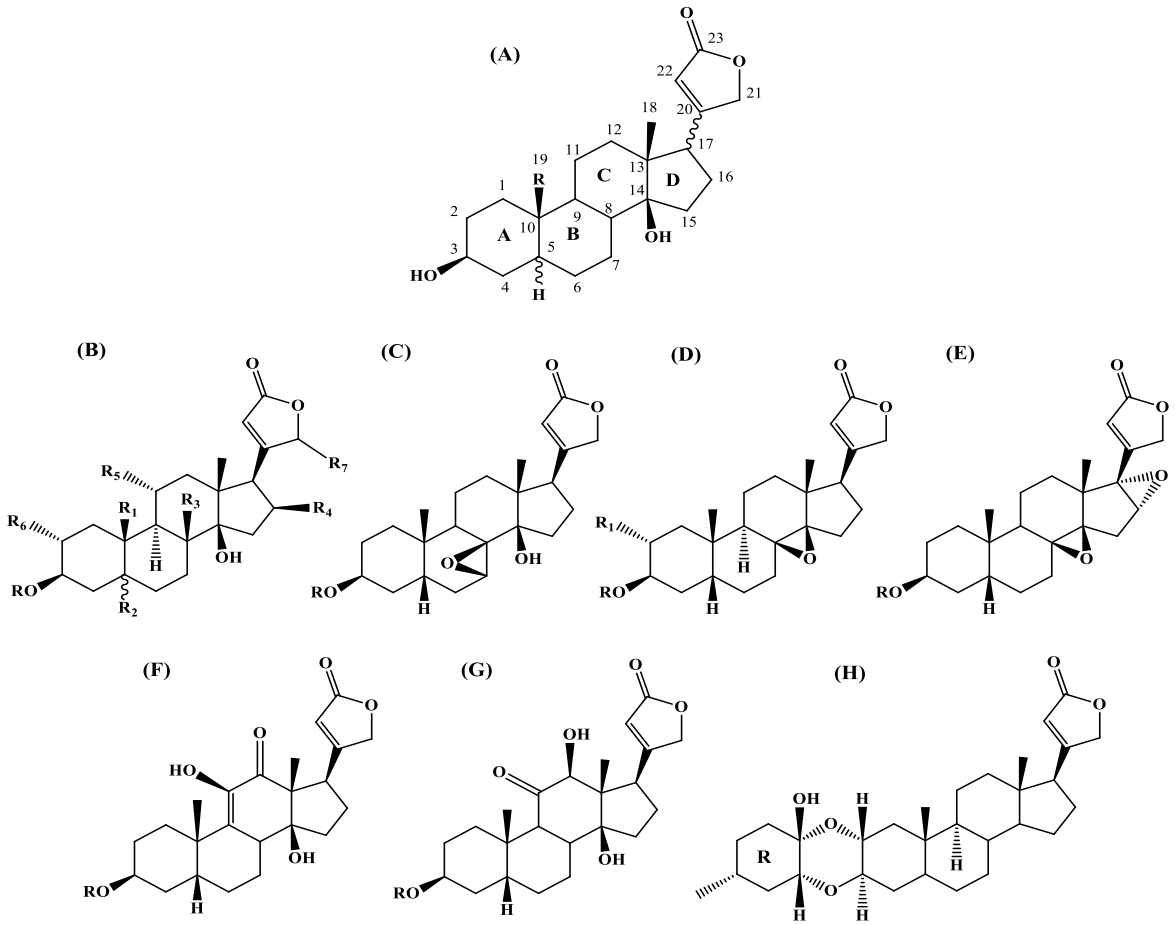


Figure 1. Chemical structures of cardenolides identified from the family Apocynaceae. **(A)** Basic skeleton of cardenolides. **(B)** Chemical structure present in greater quantity in cardenolides. **(C-E)** Cardenolides with an epoxy group. **(F-G)** Cardenolides with oxidation in the C ring of the steroid nucleus**. (H)** Cardanolide glycosides doubly linked. R represents the binding site of a sugar.

Currently, it is known that the genin or aglycone portion is the pharmacologically active region or pharmacophore [1]. However, a cardenolide shows its activity when it presents at least one hydroxyl group or a sugar containing one to five monosaccharides in C3 [2]. The sugars that can be found are pentoses, hexoses, deoxy sugars, or another special sugars

[4,5]. Although glycosidic portion is not pharmacologically active, it does affect the pharmacodynamic and pharmacokinetic profile of this family of compounds [1,4,6]. Furthermore, the attached type of sugar influences the potency of the compound making it decrease from monosaccharides, disaccharides, trisaccharides to aglycones with the 3β-hydroxyl group; being the monosaccharides, those that have higher activity [4,5,7].

Since the 1980s, cardenolides have been used in the treatment of heart failure and arterial arrhythmia [1,8]. Nowadays, the study of these compounds in the field of prevention and treatment of cancer has aroused great interest due to the ability to regulate both at the molecular and cellular levels the survival and death of cancer cells through multiple signaling pathways [9,10]. Among the mechanisms by which the proliferation of tumor cells is modulated are: inhibition of Na^+/K^+-ATPase; alteration of membrane fluidity; decreased activation of nuclear transcription factors NF-κB, JNK, and AP-1; increase in intracellular calcium; production of reactive oxygen species (ROS); oxidative lesion and mitochondrial lesion; FGF-2 inhibition; deregulation of IL-8 receptors; modulation of the MAPK/ERK pathway; inhibition of HIF1α, mTOR, and ERK1/2; and regulation of the Notch signaling pathway [11–14].

The leaves of *N. oleander* contains several cardenolides, being the most abundant, oleandrin [15,16]. This compound has the highest anticancer activity and it nowadays has been reported in *Nerium oleander, Nerium odorum, Beaumontia grandiflora, Thevetia peruviana,* and *Plumeria obtusa* [30]. The multiple targets that can be affected by oleandrin and other cardenolides, they have stimulated the search of biological sources where it will be possible to extract them in significant quantities [9]. Several investigations have been carried out to clarify the mechanism of action of oleandrin on cancer cells [17]. As a result of these investigations, it has been possible to establish that oleandrin induces the onset of cell death by regulating the Notch signaling pathway through an apoptosis-inducing ligand related to tumor necrosis factor, TRAIL [13,18].

Based on the evidence of oleandrin as an antitumor agent in various tumor cell lines, they have been developed drugs such as Anvirzel [19], (aqueous extract of *N. oleander* approved by FDA) it is comprised primarily of oleandrin and oleandrigenin, and PBI-05204 [20] (supercritical CO2 extract of *N. oleander*). These botanical drugs are in the clinical phase I and II trial, respectively. Furthermore, in the case of Anvirzel and oleandrin, the synergy

produced with cisplatin (agent used in chemotherapy) has shown to improve the susceptibility and induction of cell death by apoptosis in against a wide range of tumors [19,21]. On the other hand, the synergy between PBI-05204 and Temozolomide (agent used in chemotherapy) shows better efficacy in the treatment of glioblastomas and it inhibits growth of human pancreatic cancer [20,22]. Other cardenolides such as ceberin, digitoxin, digoxin, ouabain, lanatoside C, digitoxygenin, digitonin, have shown anti-cancer properties in various cell lines of solid tumors (sarcomas, carcinomas, and lymphomas) under in vitro conditions [23]. This makes possible the potential use of cardenolides as chemoadjuvants in radiotherapy and chemotherapy, which it would contribute to an effective treatment against cancer [14] . It is worth noting that, the mechanisms by which these compounds induce cell death are varied and they depend of the structure of the compound. the type and stage in which the cancer was diagnosed, making it difficult to understand the biochemical basis of pleiotropism of cardiotonic glycosides [24].

Cancer will remain a top priority disease for a long time, since it is the second cause of death around the world. In 2018 around 9.5 million deaths were due to this disease and 18 million new cases were reported worldwide; being most frequent the cancers of lung, breast, colorectal, prostate and stomach [25,26]. On the other hand, in Ecuador, in the same year, about 28 thousand new cases were reported and around 15 thousand deaths were attributed to prostate, breast, stomach, colorectal and cervical cancers [27]. These data reveal that, for every two new patients diagnosed one dies; for this reason, it is of great importance to find new alternative treatments and cardenolides could be useful molecules in this field.

Considering the capacity of these substances to exert biological and therapeutic effects, it is important to establish the natural distribution of these compounds. Cardenolides can be found in greater proportion in leaves of plants of the Scrofulariaceae, Apocynaceae, Liliaceae, Ranunculaceae, and Moraceae families [11]. In this case, the Apocynaceae family, which is among the five families with the largest number of medicinal plants worldwide [28], it has the largest number of species with the presence of cardenolides. These compounds can be found mainly in *Nerium*, *Apocynum*, and *Strophanthus* genus from Apocynoideae subfamily, and also *Thevetia* and *Cerbera* genus from Rauvolfioideae subfamily [4]. In these subfamilies, in addition to cardenolides, alkaloids with biological

activity and ethnomedical use have been reported, among them cancer treatment [33]. In Ecuador, at least 65 species of Apocynaceae family have been reported with food or ethnopharmacological use. Therefore, if this family is considered as the main source of cardenolides, it is possible that within this group species any of the 109 identified cardenolides could be found, of they which 25% of them have shown anticancer activity [4].

In this sense, the wide diversity and distribution of this type of compounds, it added to the biodiversity of the Ecuadorian Amazon, it would make suppose that they exist species of the Apocynaceae family where the presence of cardenolides or anticancer potential has not yet been reported. For this reason, the main goal of this study is to evaluate the anticancer activity of methanolic extracts of *Tabernaemontana sananho, Rauvolfia praecox, Himatanthus tarapotensis, Himatanthus bracteatus* and cf. *Aspidosperma excelsum*, as well as the identification of oleandrin and other cardenolides. Since those that are considered pharmacologically active, they can hardly be extracted from plants in great quantities and their structural complexity prevents their synthesis by chemical methods [29].

2. Materials and Methods

2.1. Chemicals and Reagents

Oleandrin standard was obtained from Sichuan WeiKeqi Biological Technology Co., Ltd. Methanol, lead acetate, formic acid, monopotassium phosphate, picric acid, HPLC methanol, acetonitrile (ACN, grade HPLC), and LC-MS acetonitrile were purchased from Sigma Aldrich. Chloroform from Loba Chemie Pvt. Ltd. Isopropanol from Fisher scientific. NaOH from Merck KGaA, Darmstadt, Germany. Saline phosphate buffer (PBS) from ChemCruz, Santa Cruz Biotechnology and Triton® X-100 from Promega.

2.2. Preparation of the extracts

The species used in this study, based on their phylogeny, all have to belong to the Rauvolfioideae subfamily. In the case of *H. bracteatus* and *H. tarapotensis* both belong to the Plumerieae tribe, the same to which the *Plumeria* and *Thervetia* (source of oleandrin) belong [31]. As for *T. sananho*, (named “Sikta” by Kichwa communities in Ecuador) , it is used as an antipyretic and healing agent, and its fruit is edible [32]. For *R. praecox* and *cf. A. excelsum*, ethnomedical uses reported in the genus to which they belong were taken

into consideration. At the same time, it was considered that Ghosh, and collaborators [33] mention that the subfamily of Apocynaceae, Rauvolfioideae, is source of alkaloids and cardenolides in their laticiferous tissues, which are traditionally used in the treatment of stomach ulcers, fever, asthma, whooping cough, and cancer.

The leaves of the species *T. sananho* (1°03'55.5"S 77°38'18.8"W), *R. praecox* (1°04'19.2"S 77°36'05.8"W), *H. tarapotensis* and *H. bracteatus* (1°03'48.9"S 77°36'48.1"W) were collected in the Ahuano parish near the Jatun Sacha Biological Station (1°03'48.9"S 77°36'48.1"W, 426 m.a.s.l). *cf. A. excelsum* species was collected in Alto Tena (0°56'36.1"S77°53'33.3"W), all in the province of Napo-Ecuador. *N. oleander* was collected in El Tingo parish (0°17'17.4"S78°26'31.9"W, 2.613 m.a.s.l) province of Pichincha-Ecuador. A sample of each species was deposited and identified in the Amazonian Herbarium of Ecuador (ECUAMZ). The collected leaves of each species were dried in an oven (Esco OFA-54-9) with airflow at a temperature of 37°C for 3 days and were then crushed in a mortar. To obtain the extract, 30 g of dried leaves were used in 150 mL of 70% methanol. Extraction was carried out in an ultrasonic bath (Branson 3800) for 45 minutes at 65°C. The extract was concentrated at 50 mbar and 37°C on a rotary evaporator (Buchi R-300) and stored at 4°C.

2.3. Cardenolides extraction

The methanolic extract (2 g) was placed into 50 mL conical tubes, dissolved with 10 mL of 70% methanol and centrifuged (Centrifuge: Sorvall ST4OR, Thermo Scientific) at 4 700 rpm for 15 minutes. Afterward, the pellet was discarded, 2.5 mL of 15% (w/v) lead (II) acetate was added and the resultant solution was centrifuged to the initial conditions. Likewise, the pellet was discarded, 5 mL of 4% (w/v) monopotassium phosphate were added and again, the resultant solutions was centrifuged under the same conditions. The supernatant was transferred to 50 mL conical tubes and filled until a final volume of 20 mL with distilled water and centrifuged under the same conditions as previously done. Then, the supernatant was mixed with 5 mL of chloroform: isopropanol (3:2) and centrifuged for 10 minutes at 4 700 rpm and the organic phase was transferred to 50 mL conical tubes. To the remaining methanolic phase, 5 mL of the chloroform: isopropanol solution (3:2) were added, centrifuged at room temperature at 4 700 rpm for 8 minutes and the organic phase was taken [34,35]. The two extractions were combined and concentrated in a rotary

evaporator (Buchi R-300) at 250 mbar and 60 rpm. Finally, they were dissolved in 5 mL of methanol grade HPLC. The obtained samples were used both in the analytical and biological tests.

2.4. Determination of total cardenolides similar to oleandrin by UV-Vis-NIR spectrophotometry using Baljet's reagent

The calibration curve was performed using 1:1 solutions between oleandrin standard at concentrations of 5, 10, 30, 40, 50, 100, and 200 µg/mL and Baljet's reagent (1:1 solution of picric acid 1% and NaOH 5%) freshly prepared. These were kept at room temperature for 30 minutes and they measured their absorbance at 490 nm using quartz cuvettes in a UV-Vis-NIR spectrophotometer (SHIMADZU, UV-3600 Plus) and analyzed with UVProbe 2.62 software. Later, to determine cardenolides in samples, 1:1 solutions of purified extract and Baljet's reagent were prepared and leaved at room temperature for 30 minutes. Finally, the absorbance was measured at 490 nm. A 1:1 solution between Baljet's reagent and methanol HPLC grade was used as blank in both cases [36,37].

2.5. Determination of oleandrin by HPLC

An aliquot of each sample was transferred to a 2 mL vial. Chromatography was performed at a flow rate of 1.2 mL/min with a binary HPLC pump solvent supply system (Waters 1525), a dual λ absorbance detector (Photodiode array detector) at 220 nm and 350 nm. 20 µL of each sample was injected using an automatic injector (Autosampler Waters 2707). One column, Shim-pack GIS C18, 4 µm, 4.6 I.D. x 150 mm for reverse-phase (RP)-HPLC, was used. The elution gradient was made with water (mobile phase A) and acetonitrile (mobile phase B) as follows: 0-20 min 20% (A), 80% (B); 20–27 min 32% (A), 68% (B); 27-35 min 58% (A), 42% (B); 35-40 min 60% (A), 40% (B); 40-60 min 0% (A), 100% (B); 60-65 min 20% (A),80% (B). The calibration curve was performed using oleandrin standard solutions at concentrations of 15, 25, 50, 100, and 150 µg/mL [35].

2.6. UPLC-QTOF-MS analysis to identify other cardenolides in methanol extracts

This method was used for identifying other cardenolides that could be present in the extracts obtained. The analysis was performed based on protocol presented by the group of Ravi [30] using a Waters Xevo G2-XS QTOF mass spectrometer with a UPLC I class Waters Acquity, operated in the positive ion mode for electrospray ionization (+ESI). Chromatographic separation was obtained using a column, ACQUITY UPLC BEH C18, 1.7

µm, 2.1 I.D. x 100 mm, Waters. The elution was made with 0.1% formic acid (mobile phase A) and acetonitrile-formic acid 0.1% (mobile phase B). Gradient elution was carried out at a flow rate of 0.2 mL/min, with a linear gradient of 0-12 min 90% (A), 10% (B); 12-13 min 5% (A), 95% (B); 13-16 min 90% (A), 10% (B) and balanced for 3 min before the next injection. The sample injection volume was 20 µL. The MS parameters were established as follows: desolvation temperature: 500°C; scan range 50-1150 Da; sampling cone: 30V; source temperature: 120°C; capillarity voltage: 1kV; resolution: 17500, 4.0 m/z isolation window; collision energy: 15V and maximum injection time set to automatic. Data processing involved extraction of the precursor ions from the cardenolides suspected were determined by the software MZmine with nominal mass and identified by the peak of the adducts $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ above 1×10^5 a.u. In order to validate the method and confirm the presence of oleandrin in the extracts, two samples of oleandrin standard (500 and 1000 ppb) were injected.

The identification of cardenolides and isomers was performed by reviewing the molecular masses based on steroid glycoside spectroscopic data. Masses with adducts ($[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$) of the cardenolides reported in other species of the Apocynaceae family were reported (Tables 4-9).

2.7. Cell Culture Procedures

Two human cancer cell lines were used: HeLa and D-384. The cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimicotic solution (100 units/ml penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B), and 1% L-glutamine (2 mM). The cells were incubated at 37°C in a 5% CO_2 atmosphere and monitored in the Neubauer chamber by inverted microscopy.

2.8. Cell Viability Analysis via MTS Assay

The inhibitory effects of the extracts obtained were evaluated using the MTS (5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfo-phenyl)-2H-tetrazolium inner salt) cell viability assay. $3–5\times10^3$ cells/well were seeded into 96-well plates and incubated for 24 h. The cells were then treated in triplicate with 50 µg/ml of extract to yield a final volume of 2 ml. Additionally, an treatment with 1 µM oleandrin was performed to verify its cytotoxic effect. Dimethyl sulfoxide (DMSO) 0.1% v/v was used as negative control and 1 µM doxorubicin as positive control. The cells were incubated with the treatments for

48 h, after which 20 µL MTS (5 mg/mL) was added and further incubated for 4 h at 37°C. The absorbance was measured at 490 nm. The absorbance data obtained with cells treated with DMSO were considered to represent 100% viability.

2.9. Hemolysis test

Human blood (4 mL) was extracted into vacutainer tubes. The sample was carefully placed in 50 mL conic tubes. It centrifuged (Centrifuge: Sorvall ST4OR, Thermo Scientific) at 1200 rpm for 5 minutes at 4°C and the supernatant (plasma) was discarded. Three washes with 10 mL of sterile 1X saline phosphate buffer (PBS) were performed by gentle agitation for 3 minutes to avoid hemolysis. It is worth mentioning that for each wash, it was centrifuged at 1000 rpm for 5 minutes at 4°C and the supernatant was discarded. After that, a 4% solution was prepared with 2 mL of erythrocytes and 50 mL of sterile PBS. In 1.5 mL tubes, were placed 200 µL of erythrocyte solution and they treated with serial solutions of oleandrin and each methanolic extract at concentrations of 200, 100, 50, 25, 12.5, and 5 µg/mL in a final volume of 400 µL. Each treatment (samples and controls) was performed in triplicate and incubated for 2 hours at 37°C. Later, samples were centrifuged and supernatants were transferred to a 96 well plates. Absorbance was measured at 560 nm in a plate reader (GloMax® Explorer Multimode Microplate Reader). For negative control, 200 µL of PBS 1X was added to 200 µL erythrocyte solution, and for the positive control, a 200 µL erythrocyte solution, 195 µL PBS 1X and 5 µL Triton X-100 (Promega) was employed. The data obtained with the triton treatment were considered as 100% hemolysis. The percentage of hemolysis was calculated using the formula:

$$\%\mathbf{Hemolysis} = (\overline{\mathrm{X}} - \mathrm{C}^{-})/(\mathrm{C}^{+} - \mathrm{C}^{-})\ \mathrm{x}100$$

$$\mathbf{Where}:\ \overline{\mathrm{X}} = \text{average}$$

$$\mathrm{C}^{-} = \text{negative control}$$

$$\mathrm{C}^{+} = \text{positive control}$$

2.10. Statistical Analysis

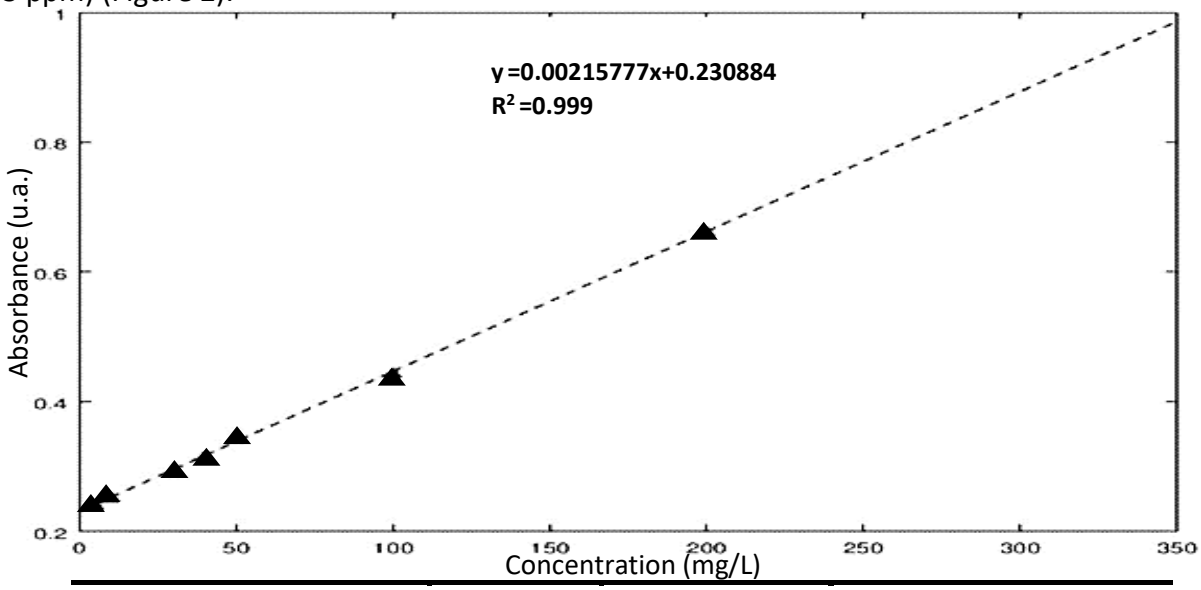
The data obtained were reported as means ± standard error of the mean (SEM) of three independent experiments. The nonlinear regression was determined by GraphPad Prism 8.4.0 (GraphPad Software, LLC). The detection limit (LOD) was defined as 3.3 folds of the lowest deviation standard deviation (σ) divided by the slope of the linear calibration curve (S), or LOD = 3.3 σ/S. The limit of quantification (LOQ) was calculated as LOQ = 10 σ/S.

3. Results and discussion

The search of anticancer agents with greater effectiveness and selectivity is still a challenge for the world, due to the complexity of this disease. In that sense, a successful strategy in the treatment of systemic and metastatic malignancies is the combination of chemical agents and the nature becomes a fundamental source of anti-cancer compounds, as nearly 50% of the FDA-approved drugs are inspired by it [36]. In this context, cardenolides have shown promising anticancer effects.

3.1. Quantification of total cardenolides by UV-Vis-NIR spectrophotometry

The total cardenolide content was determined by the Baljet reagent measured at 490 nm using oleandrin as standard. It has been reported that the Baljet reagent acts decomposing the lactonic ring giving an orange coloration that can be detected at 490 nm [37]. This assessment was used to determine the presence of cadenolides in the samples. In this way, it was obtained that *N. oleander* (345.013 ± 6ppm) contains a greater quantity of cardenolides followed by *H. bracteatus* (164.886 ± 6 ppm) and *H. tarapotensis* (158.665 ± 3 ppm) (Figure 2).



Sample	Absorbance λ=490	ppm Cardenolides	Content of cardenolides (µg/g in dried matter)
Nerium oleander	0.975	345.012 ± 6	345.012 ± 30
Himatanthus tarapotensis	0.573	158.665 ± 3	158.665 ± 15
Himatanthus drasticus	0.587	164.886 ± 6	164.886 ± 30
Tabernaemontana sananho	0.071	< L.O.D.	-
Rauvolfia praecox	-	< L.O.D.	-
cf. Aspidosperma excelsum	-	< L.O.D.	-

Figure 2. Calibration curve with fit linear (First Order) made with oleandrin standard solutions. Determination of total cardenolides similar to oleandrin by UV-Vis-NIR spectrophotometry using Baljet's reagent.

Table 1. Parameters for the calibration curve of cardenolides by UV-Vis-NIR spectrophotometry using oleandrin as standard.

Parameter	Value
Slope	0.002
Intercept	0.230
R^2	0.999
L.O.D.	19.838 ppm
L.O.Q.	6.546 ppm

LOD: detection limit; LOQ: limit of quantification

The *T. sanaho* extract showed a small absorbance, but it was not possible to be quantified because it is below the detection limit (LOD), Table 1. As for the other species, they do not show quantifiable results by UV-Vis-NIR spectrophotometry, Figure 2. When these results are compared with those obtained by HPLC (see in the next section), it is possible to observe that the trend in terms of the amount of oleandrin and total cardenolides is maintained, Figure 2 and 4. Furthermore, it could be observed that *N. oleander* has a considerably higher amount of cardenolides, in relation to the rest of samples. The high content of these compounds would explain why *N. oleander* is the most studied specie.

As for the plants of the genus *Himatanthus*, it has been shown that according to their phylogeny, these plants should have cardenolides, in addition, to iridoid and terpenoid glycosides [31,38]. However, there are no reports about cardenolides in *H. bracteatus* and *H. tarapotensis* (originating in the tropical regions of South America). It has been reported the alcoholic extracts of *H. drasticus* show anti-inflammatory, antinociceptive, antitumor and gastroprotective properties [39]. On the other hand, for *T. sananho* specie, there are qualitative reports that suggests the presence of cardiac glycosides [40]. At the genus level, in *Tabernaemonta, Apisdosperma,* and *Raulvolfia*, several species are reported where by means of qualitative analyzes (Baljet test) the presence of cardiotonic glycosides and indole alkaloids is shown. Some examples are: *T. cymosa* [41], *A. macrocarpon* [42], *A. pyrifolium* [43], *R. caffra* [44], and *R. serpentina* [45] among the main ones.

3.2. Identification and quantification of oleandrin by HPLC

The content of oleandrin was determined in MeOH extracts (2×10^4 $\mu g/mL$) of *N. oleander, T. sananho, H. tarapotensis, H. bracteatus, cf. A. excelsum,* and *R. praecox*, finding this cardenolide in the first four mentioned species. According to the results obtained by

HPLC, *H. bracteatus* shows a higher oleandrin content 142.50 ppm, compared to the rest of the studied species *T. sananho*: 52.90 ppm, *H. tarapotensis*: 84.30 ppm and *N. oleander*: 112.40 ppm, as shown in Figure 4. In the evaluated extracts oleandrin eluted at 35.543 minutes with a maximum difference in retention time of 0.050 minutes with respect to the standard and overlapping peaks were not observed, Figure 3.

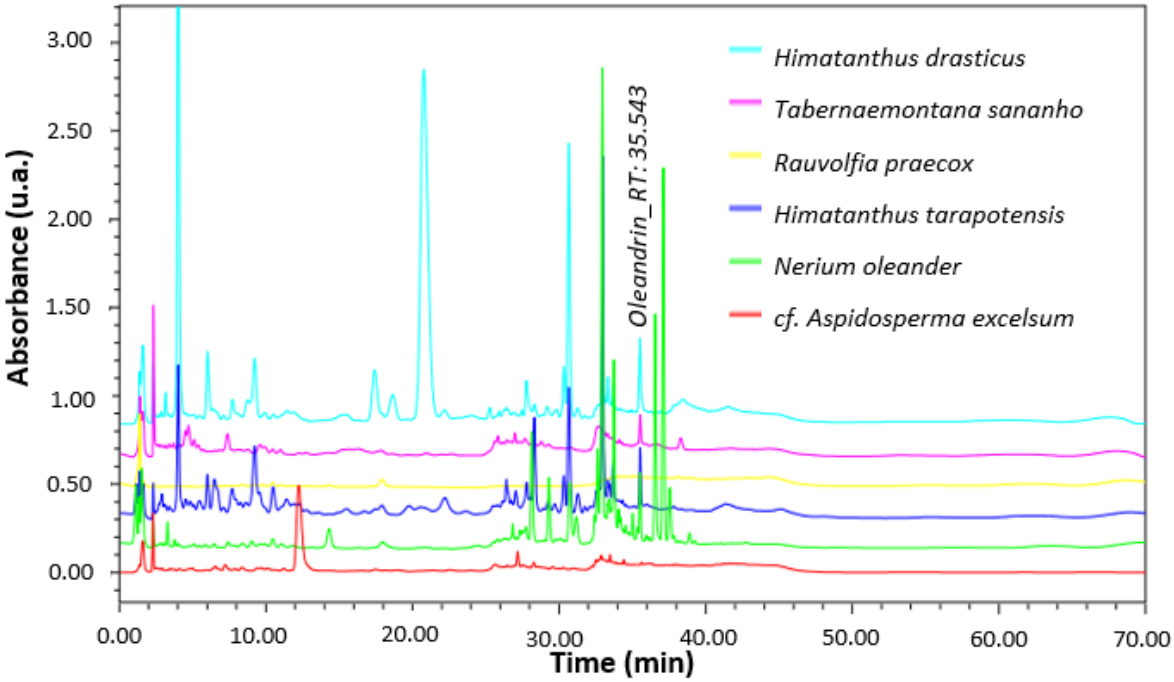
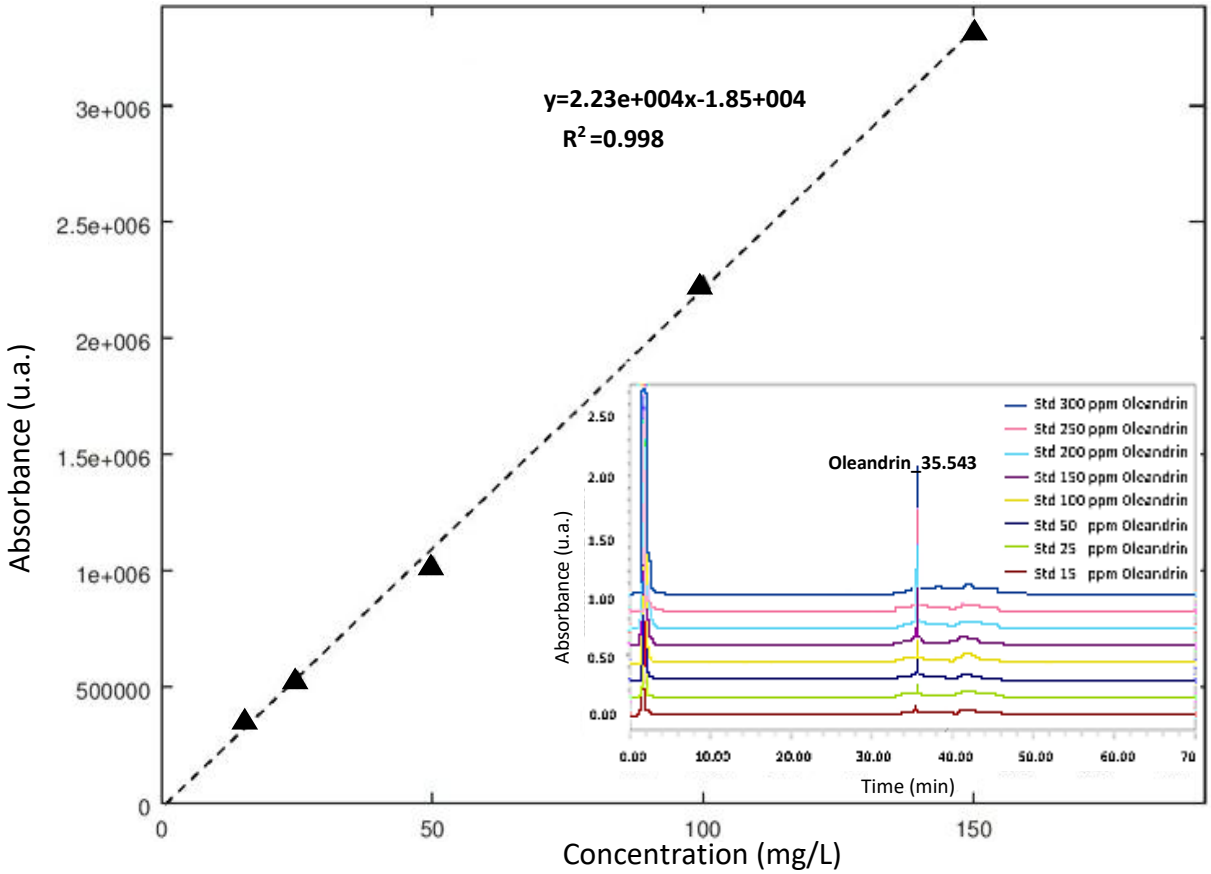


Figure 3. Spectra with oleandrin identification in samples of methanolic extracts.

As it has been reported by the group of Jiang [4] *N. oleander* contains oleandrin as the main cardenolide even though this hypothesis was not corroborated in the present investigation. The measurement of the oleandrin standard was carried out at λ = 220 nm [46], obtaining the parameters shown in Table 2. The strong ultraviolet absorption of many cardiac steroid glycosides is associated with a conjugated unsaturated cyclic lactone at position C-17, Figure 1-A. Although, γ-lactone show a maximum UV absorption at 217 nm, it has been establish that at λ = 220 nm furanone γ-lactone cannot be considered an impurity [47], Figure 4. In addition, the treatment with lead acetate and monopotassium phosphate allows removing pigments (specially chlorophylls), lipids and other compounds that could cause interference in reading [48].



Sample	Retention time (min)	Peak area	ppm Oleandrin	Content of oleandrin (µg/g in dried matter)
Nerium oleander	35.590 + 0.05	6591673	112.4	281.0
Himatanthus tarapotensis	35.600 + 0.05	3405602	84.3	210.8
Himatanthus drasticus	35.577 + 0.05	3685364	142.5	356.6
Tabernaemontana sananho	35.595 + 0.05	1882116	52.9	132.3
Rauvolfia praecox	-a		< L.O.D.	-
cf. Aspidosperma excelsum	-a		< L.O.D.	-

[-a] **Not was observed any peak in this retention time**

Figure 4. Calibration curve of oleandrin with fit linear (First Order) and an insert of the chromatograms belonging to different concentrations of oleandrin standard; Quantification of oleandrin on methanolic extracts by HPLC

Table 2. Parameters for the calibration curve of oleandrin by HPLC using oleandrin as standard.

Parameter	Value
Slope	0.0002
Intercept	-0.0001
R^2	0.998
L.O.D.	36.599 ppm
L.O.Q.	12.077 ppm

LOD: detection limit; LOQ: limit of quantification

3.3. Identification of oleandrin and another cardenolides by UPLC-QTOF-MS

UPLC-QTOF-MS analysis for the detection of cardenolides shows the possible presence of cardenolides in the methanolic extracts of the species analyzed in this study. Both the oleandrin standard (1000 and 500 ppb) and the extracts from the samples were subjected to this experiment. The data was processed with a mass window filter of ± 115 mDa with respect to the calculated mass. In addition, a window filter of ± 0.03 min was used when oleandrin was determined. A peak of 599.254 Da and retention time 7.54 minutes corresponding the m/z of the sodium adduct $[M+Na]^+$ of theoretical mass 599.319 Da was observed. In addition, the m/z of 615.225 corresponding the potassium adduct $[M+K]^+$ was also observed, Figure 4. The results related to the oleandrin peak in the samples were presented with a difference in retention time of ± 0.02 minutes (Figure S1-S5) in relation to the standard, Table 3 and Figure 5-9.

Table 3. Identification of oleandrin by HPLC-QTOF-MS

Compound/ Species	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z theoretical	m /z observed	peak intensity (u.a.)	± error (ppm)
Oleandrin standard	7.54	$C_{32}H_{48}O_9$	576.329	$[M+Na]^+$	599.319	599.254	692972	108.60
Nerium oleander	7.57	$C_{32}H_{48}O_9$	576.329	$[M+Na]^+$	599.319	599.309	255709	16.730
Himatanthus bracteatus	7.52	$C_{32}H_{48}O_9$	576.329	$[M+Na]^+$	599.319	599.269	3203976	83.440
Himatanthus tarapotensis	7.52	$C_{32}H_{48}O_9$	576.329	$[M+Na]^+$	599.319	599.264	3008844	91.790
cf. Aspidosperma excelsum	7.52	$C_{32}H_{48}O_9$	576.329	$[M+Na]^+$	599.319	599.354	102247	58.520

In high resolution mass analysis, errors with two or three significant figures are recommended, one of them being a decimal figure. Measurements with four or more significant figures were obtained (e.g. Table 3), this means that there is uncertainty regarding the precision of the mass scale. Which is indicative of a deficient or failed calibration of the mass. In these cases, it is recommended to use masses with a single decimal figure and to call them nominal masses [49]. In other words, with a deficient calibration is not possible to use exact masses. In addition, It should be noted that in TOF calibration the intensities of the measured peaks have a significant influence on the magnitude of the mass error with high-intensity peaks resulting in detector saturation

displaying larger mass errors [50]. Therefore, in this work the nominal mass of the peaks was used to make comparisons with the masses of known cardenolides compounds other than oleandrin, in order to detect them in plant samples. It should be noted that this detection method does not allow to distinguish between isomers, so that two or more compounds could have the same mass.

Tables 4, 5, 6, 7, 8, and 9 show the results of the mass adducts obtained for either $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ with a peak relative intensity equal or greater in magnitude than the sample of standard of oleandrin and with an error not higher than 110 ppm; as well as the species of the Apocynaceae family where they have been identified. In this sense, we can observe that in Table 5, corresponding to *N. oleander*, 19 different nominal masses were identified at an acceptable peak intensity, one of them being oleandrin, (entry 17, Table 5). Additionally, it is shown that five of them have more than one isomer. Regarding cf. *A. excelsum*, it exhibits the presence of an oleandrin-like peak, (entry 13, Table 6); as well as seven of the 13 masses present one or more isomers possibly belonging to cardenolides. The results for *H. bracteatus* (entry 15, Table 7) and *H. tarapotensis* (entry 12, Table 8) shown a peak belonging to oleandrin. Similarly, for *H. bracteatus*, of 19 nominal masses for cardenolides, of which nine of them show one or more isomers. In *H. tarapotensis,* 21 nominal masses were obtained for cardenolides; where nominal twelve masses have one or more isomers. For *R. praecox*, 14 nominal masses for cardenolides were detected; where five of them show one or more isomers. *T. sananho* presents seven nominal masses for cardenolides, six of which have one or more isomers. For *T. sananho*, was obtained one nominal mass equal to the oleandrin (entry 3, Table 10), but it differs in retention time, which is 4.5 min, therefore, it can be assumed that it is another compound.

Additionally, nominal mass entries are presented with reports of cardenolides with cytotoxic activity, without considering oleandrin. Therefore, we have for *N. oleander*, *cf. A. excelsum* and *R. praecox* are shown seven nominal masses covering cardenolides with reports of cytotoxic activity. Within this framework, *H. bracteatus* presents seven masses and in *H. tarapotensis* nine nominal masses with reports of cardenolides can be seen. Finally, *T. sananho* presents four nominal masses that correspond to known compounds shown cytotoxic activity. It should be noted that the number of compounds does not represent the total abundance of cardenolides. Thus, for example, in *N. oleander* fewer

mass reports are shown in relation to *H. bracteatus* and *H. tarapotensis*, however, the concentration of cardenolides detected by UV-Vis-NIR spectrophotometry is greater in *N. oleander*. Considering that the cardenolides only were detected by UPCL-QTOF-MS, it is possible that species such as *cf. A. excelsum, R. praecox,* and *T. sananho* have low amounts of cardiotonic glycosides.

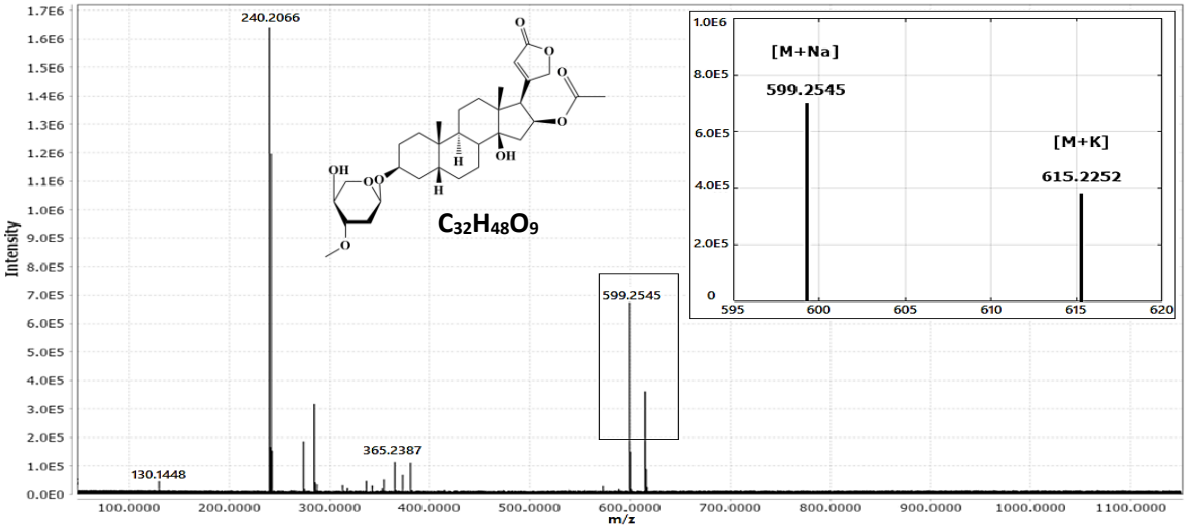


Figure 5. Mass spectrum of oleandrin standard by LC-MS analysis. Ions identified: $[M+Na]^+$ 599.2545 and $[M+K]^+$ 615.2252 at retention time: 7.54 min.

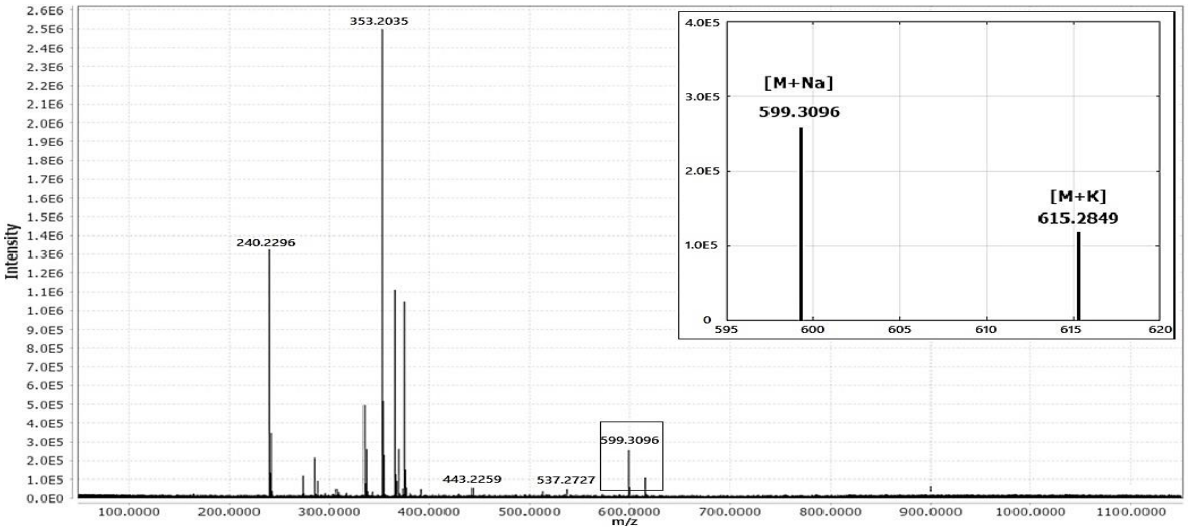


Figure 6. Oleandrin in *N. oleander* extract by LC-MS analysis. Ions identified: $[M+Na]^+$ 599.3096 and $[M+K]^+$ 615.2849 at retention time: 7.57 min.

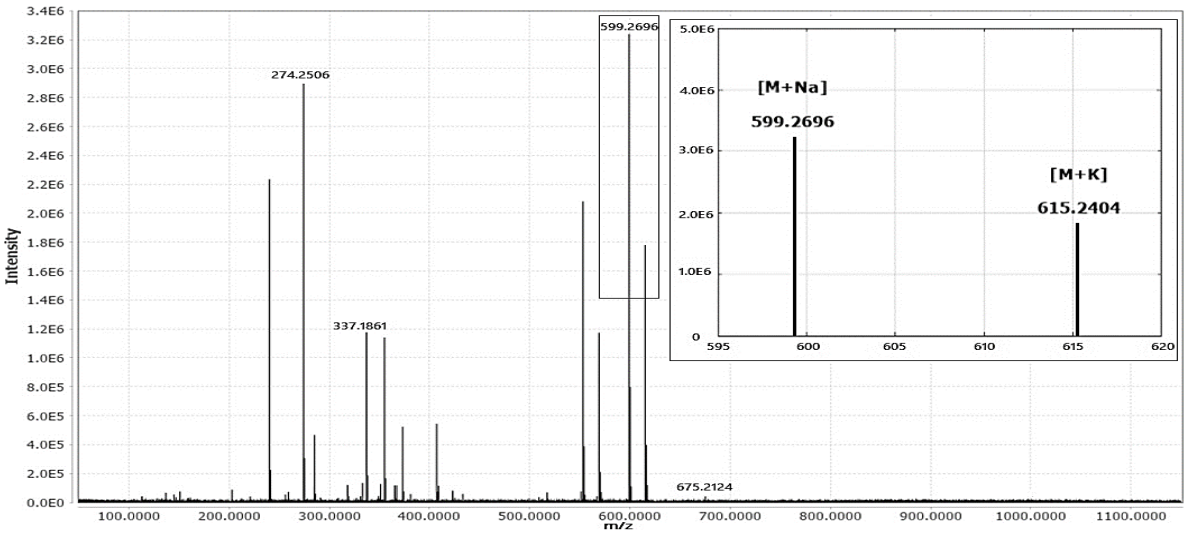


Figure 7. Oleandrin identification in *H. bracteatus* extract by LC-MS analysis. Ions identified: $[M+Na]^+$ 599.2696 and $[M+K]^+$ 615.2404 at retention time: 7.52 min.

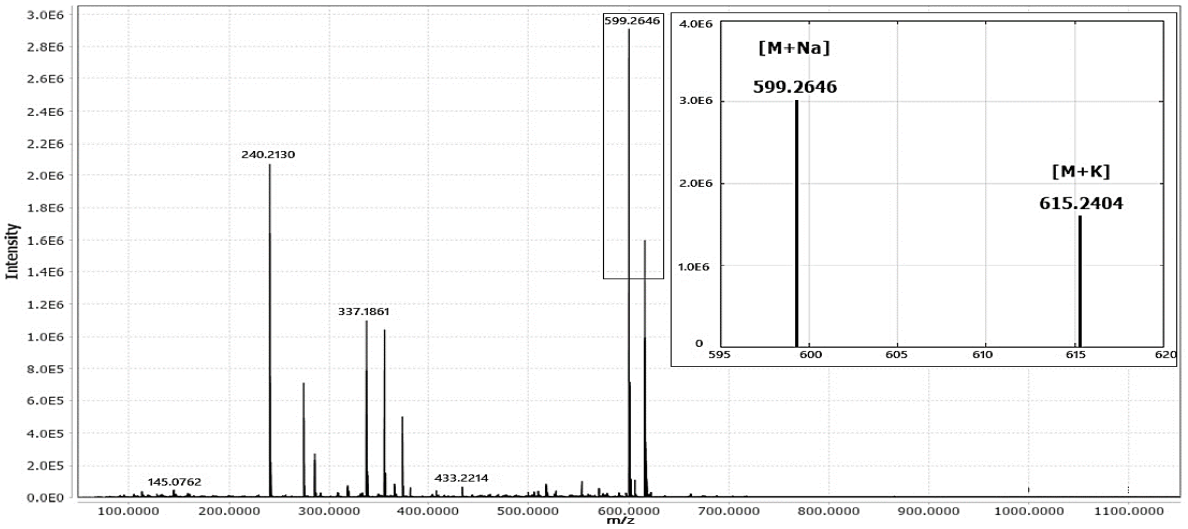


Figure 8. Oleandrin identification in *H. tarapotensis* extract by LC-MS analysis. Ions identified: $[M+Na]^+$ 599.2646 and $[M+K]^+$ 615.2404 at a retention time: 7.52 min.

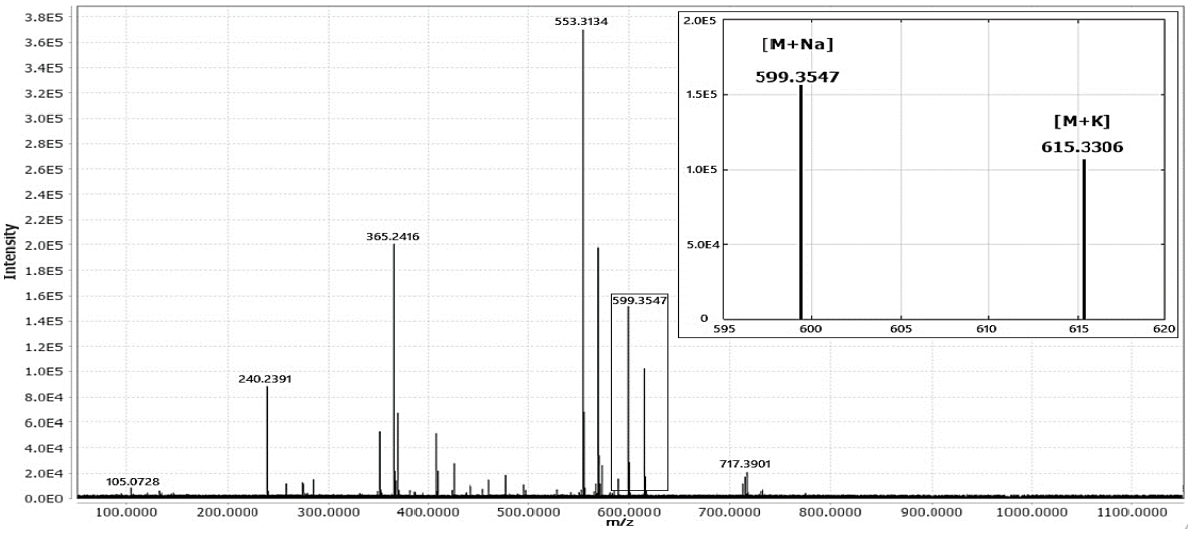


Figure 9. Oleandrin identification in *cf. A. excelsum* extract by LC-MS analysis. Ions identified: $[M+Na]^+$ 599.3547 and $[M+K]^+$ 615.3306 at retention time: 7.52 min.

3.4. Antitumor activity of methanolic extracts on human cell lines.

The *in vitro* antitumor activity (cytotoxicity) of the methanolic extracts (50 µg/mL) was tested in cervical cancer (HeLa) and medulloblastoma (D-384) cell lines through the MTS assay. In the case of *H. tarapotensis*, *H. bracteatus*, *cf. A. excelsum*, and *T. sananho* methanolic extracts exhibit high potency against the cervical cancer cell line, 98.28%, 96.97%, 88.440%, and 60.32% respectively, while the extracts of *N. oleander:* 34.140%, *R. praecox:* 30.55%, and 1µM oleandrin (0.577 µg/mL): 40.56% exhibit moderate activity in HeLa line cell (Figure 10-A). On the other side, in D-384 cell line, high percentages of cytotoxicity are maintained in *H. bracteatus*: 93.62% and *H. tarapotensis*: 95.20%, while in *cf. A. excelsum* cytotoxic activity decreased about 40% in relation to what was shown in HeLa line cell. In D-384 cell line, the cytotoxicity shown by *N. oleander* is 5%, while that the sample of 1µM oleandrin (0.577 µg/mL), *R. praecox,* and *T. sananho* do not present cytotoxic activity (Figure 10-B).

Furthermore, the data obtained from the hemolysis test show that cytotoxic damage is low. In this sense, hemolytic results at the highest tested concentration (200 µg/mL) show cell damage percentages in *N. oleander*: 25.814%; *H. tarapotensis*: 18.267%; *H. bracteatus*: 20.154%; *cf. A. excelsum*: 15.887; *R. praecox*: 13.279%; and *T. sananho*: 19.929%. On the contrary, when compared with the cytotoxicity and hemolysis data at the same concentration (i.e. 50 µg/mL), the cell damage by hemolysis does not exceed 15% and the oleandrin standard did not show hemolytic activity at any concentration (Figure 10-C).

Considering that oleandrin, up to now, is the most potent cardenolide producing apoptosis in HeLa cells [15,51]. The moderate cytotoxic activity shown suggests that the extracts that present greater cytotoxic activity (*H. tarapotensis, H. bracteatus*, and *cf. A. excelsum*) could contain compounds that by themselves improve cytotoxic activity or could generate an additive effect (additive synergy) or synergistic effect (empowerment synergy) with other cardenolides [52,53]. However, to verify this hypothesis, a phytochemical screening should be carried out and the anticancer capacity of each isolated molecule should be evaluated. In *cf. A. excelsum* the cytotoxic activity may be occurring mainly due to the presence of oleandrin (entry 13, table 6) and ouabain (entry 6, table 6), two potent cardenolides used against several types of cancer even at micromolar concentrations [54,55]. Therefore, it is

possible that the low concentrations reported, Table 6, are enough to generate the percentage of cytotoxicity reached, however, the presence of other compounds that are contributing to it cannot be ruled out. As it was evident in the results obtained by UPLC-QTOF-MS, thought several cardenolides are found, not all of them have anticancer activity. Some specific residues in the structure of a cardenolide (Figure 1, Table 4) fulfill a determining role in the pharmacokinetic profile. Thence has been obtained that, the *cis* conformation between the A/B and C/D rings of the steroid nucleus of cardiotonic compounds is responsible for the inhibition of Na^+/K^+-ATPase [24]. The 16-OH acetylation of aglycons potentiates the activity of cardenolide [56]. The double bond between position C8/C9 and C16/C17 weakens cytotoxicity. In addition, when the ring opens or forms an epoxy structure between positions C14 and C8, the activity will disappear [15,24,56]. Therefore, a high content of cardenolides does not necessarily mean a higher anticancer activity. Consequently, it is possible that other different compounds than cardenolides could cause the cytotoxicity in D-384 since both oleandrin and *N. oleander* (species-rich in cardenolides) lose this property in this cell line whereas *H. bracteatus* and *H. tarapotensis* keep it. It is based on the fact that the cardenolides show specificity for the α3 subunit of Na^+/K^+-ATPase, overexpressed in human tumor cells, [57,58] and this is not reproduced by oleandrin or the crude extract of *N. oleander* in D-384. In this case, it could be considered a synergistic effect in the extracts of the species *Himantantus* and *cf. A. excelsum*. This according to the observed effect which is greater than the sum of the partial effects shown by oleandrin and oubain in *cf. A. excelsum*. Similarly, an additive or synergistic effect cannot be ruled out between cardenolides, indole alkaloids [59], iridoid, and terpenoid [60] (all compounds show cytotoxic properties) that may be present in the extracts.

On the other hand, it should be noted that pure oleandrin presented null results in hemolysis tests, agreeing with which was reported by the group of Newman [61], where it shows that at therapeutic concentrations, oleandrin does not present damage or toxicity in normal cells [22,61–63]. In the same way, being those extracts a pool of unknown metabolites, there is the possibility of purifying them and thus lowering the percentages of hemolysis shown in Figure 10-C, as well as increasing cytotoxicity. This strengthens the hypothesis that a compound within of the extracts could have a potent and selective

cytotoxic activity for cancer cells, mainly in *H. tarapotensis*, *H. bracteatus*, and *cf. A. excelsum* extracts.

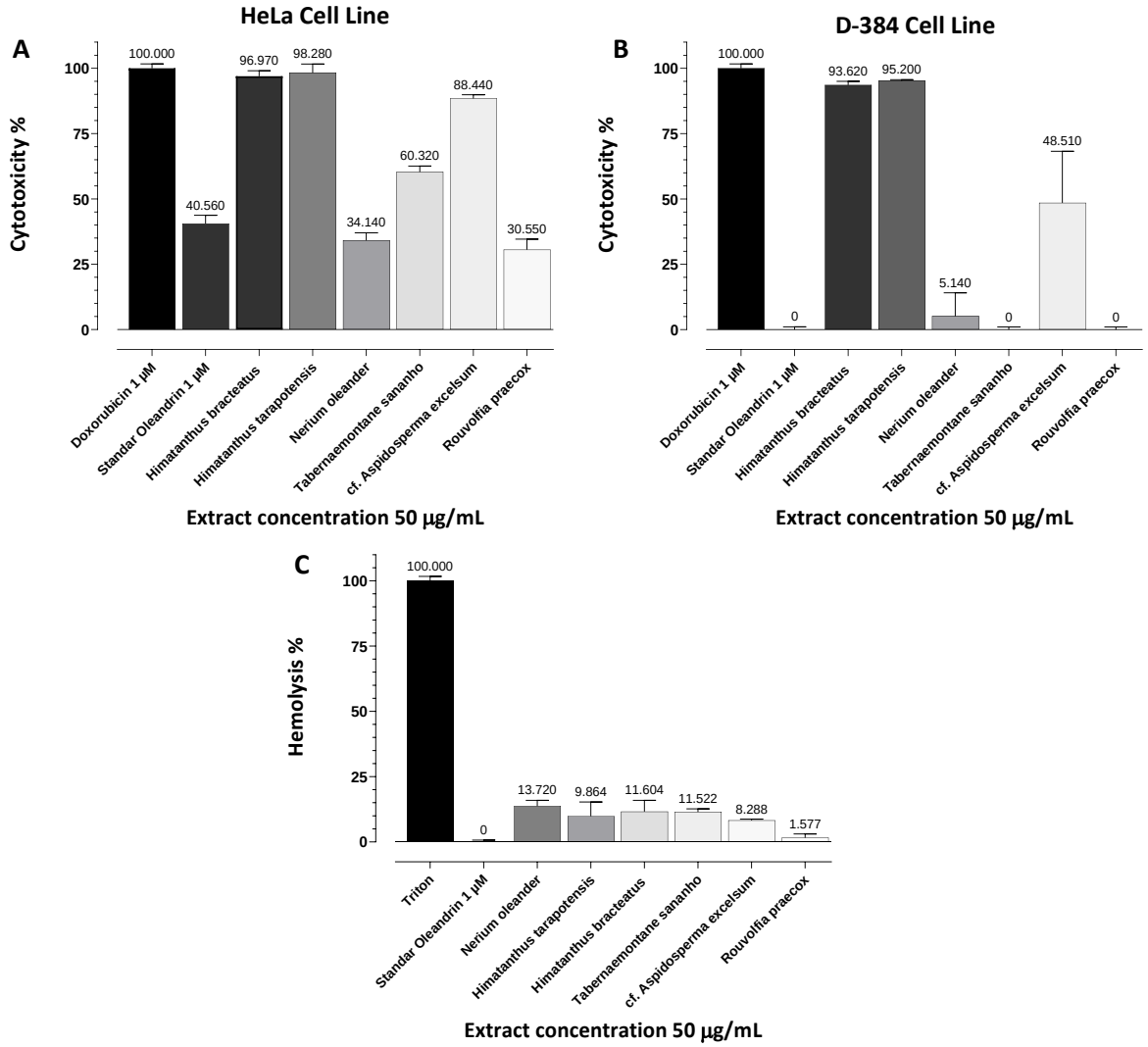
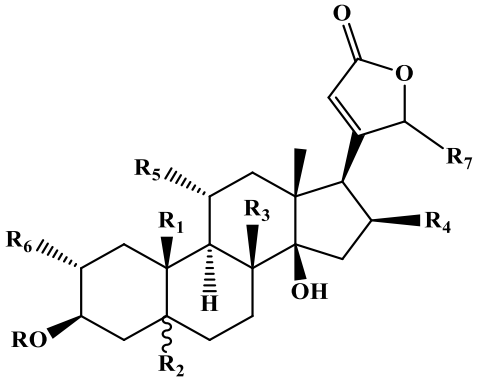


Figure 10. Cytolytic effects of methanolic extracts and oleandrin on HeLa, D-384 cell line and erythrocytes. A) HeLa cell culture treated with concentrations of extract to 50 µg/mL and oleandrin 1µM (0.577 µg/mL), its cytotoxicity was evaluated in comparison with DMSO 1% (100% viability). B) D-384 cell culture treated with concentrations of extract to 50 µg/mL and oleandrin standard 1µM (0.577 µg/mL), its cytotoxicity was evaluated in comparison with DMSO 1% (100% viability). C) Hemolytic effects of methanolic extracts on human erythrocytes. Red blood cells treated with concentrations of extract and oleandrin to 50 µg/mL, its percentage hemolysis was evaluated in comparison with Triton X-100 (100% hemolysis) and PBS (0% hemolysis). Assays were performed in triplicate, and the results represent the mean ± standard error of mean (SEM).

Table 4. Chemical structures of cardenolides identified from the family *Apocynaceae with cytotoxic activity*



No	Name	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Biological Activity
1	Odorobioside G	Dil-Glc	CH_3	H	H	H	H	H	H	promyelocytic leukemia[64]
2	Odoroside H	Dil	CH_3	H	H	H	H	H	H	breast cancer lung cancer gastric cancer[4]
3	8β-Hydroxyodoroside A	Dil	CH_3	H	OH	H	H	H	H	ovarian cancer[4]
4	Deacetyloleandrin	L-ole	CH_3	H	H	OH	H	H	H	T cell leukemia [13]
5	7,8-dehydrocerberin	2-*O*-acetyl-L-thevetosyl	CH_3	H	-	H	H	H	Δ^7	epidermic carcinoma (KB), human breast cancer cell (BC) and human small cells lung cancer (NCI-H-187) [30]
6	16-desacetyl-16-anhydrocryptograndoside A	Sar	CH_3	H	H	H	H	H	H	carcinostatic [65]
7	Oleaside A	Din	CH_3	H	-	H	H	H	H	WI-38, VA-13, and HepG2 cells [66]
8	Oleandrin	L-ole	CH_3	H	H	OAc	H	H	H	Lung cancer, breast cancer gastric cancer, renal cancer, uterus carcinoma and melanoma [4]

9	Odoroside A	Din	CH_3	H	H	H	H	H	H	Breast cancer , lung cancer, gastric, renal cancer, uterus carcinoma and melanoma [4]
10	Beaumontoside	L-ole	CH_3	H	H	H	H	H	H	T cell leukemia [13]
11	Digitalin	Glc-Dil	CH_3	H	H	OH	H	H	H	enzymatic activity of the sodium-potassium ATPase pump
12	Opposide	Tal	CH_3	OH	H	H	OH	OH	H	activity against KB cell cultures [30]
13	Bipindoside	Tal	CH_3	OH	H	H	OH	H	H	Toxic, Mean lethal dose 0.1031-0.0042 mg/kg [30]
14	Lokundjoside	Rha	CH_3	OH	H	H	H	H	H	osteosarcoma [67]
15	Erysimosol	Glc-Dix	HOH_2C	OH	H	H	H	H	H	inotropic and negative chronotropic [30]
16	Ouabain	Rha	HOH_2C	OH	H	H	OH	OH	H	breast cancer [68]; prostate cancer[69] lung cancer [57]
17	Acolongifloroside K	Tal	HOH_2C	OH	H	H	OH	OH	H	activity against KB cell cultures[30]
18	Nerizoside	Dil	CH_3	H	H	OAc	H	H	H	Leukemic [70]
19	Obebioside D	Glc-the	CH_3	H	H	H	H	H	H	Antioxidant [71]
20	Thevetioside C	Glc-The	CH_3	H	H	O	H	H	H	cytotoxic activity in P15 SW1990, MGC-803, LO2 [5]
21	Affinoside T	Rha	CH_3	-	H	H	OH	O	H	HeLa, A-549 and HL-60 tumor cell [72]
22	Glucostrophanthidin	Glc	CO_H	OH	H	H	H	H	H	KB, HCT-116, SF-268, MCF-7, HL-60, PC-3 and MRC-5 cell lines. [73]
23	Monoacetylacoschimperoside P	AcAco	CH_3	H	H	$OCOCH_3$	H	H	H	Lethal dose 0.3017-0.0231 mg/kg [30]
24	Acovenoside A	meTam	CH_3	H	H	H	H	OH	H	KB cell cultures of human nasopharynx carcinoma [30]
25	Neriifolin	L-the	CH_3	OH	OH	H	H	H	H	hepatocellular carcinoma [74] Ovarian Cancer [75]

26	Neritaloside	Dil	CH_3	H	H	OAc	H	H	H	Brain cancer, pancreatic cancer, uterus cancer, melanoma, breast, prostate, lung and gastric cancer [4]
27	16-acetylstrospeside	Dil	CH_3	H	H	$OCOCH_3$	H	H	H	Cytotoxic against human epidermoid carcinoma [30]
28	Acoschimperoside P	Aco	CH_3	H	H	$OCOCH_3$	H	H	H	HaCaT-GLI1-Luc cellsN Pancreatic cancer (PANC-1) Prostate cancer (DU145) [76]
29	Cerberin	The	CH_3	H	H	H	H	H	H	Breast, lung, epidermoid [4]

Aco: acofrose, Glc: glucose, Dil: digitalose, Ole: oleandrose, The: thevetose, Tam: talomethylose, Rha: Rhamose, Cym: cymarose, Dix: digitoxose, Din: diginose, Sar: sarmentose

Table 5. Identification by UPLC-QTOF-MS of cardenolides in methanolic extract of *Nerium oleander* by comparing nominal mass of its precursor ion (H^+, Na^+, K^+), with ions produced by electrospray ionization (ESI-MS) considering peak intensity greater than 1×10^5

Entry	Proposed metabolites	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z (theoretical)	m /z (observed)	peak intensity (u.a.)	Apocynaceae species reported
1	Adynerin; 16-Desacetyl-16-Anhydrocryptograndoside A***; Oleaside A***	1.15	$C_{30}H_{44}O_7$	516.3	$[M+Na]^+$	539.3	539.2	9246440	*Nerium olenader* [77,78]
2	Oleaside C	3.86	$C_{36}H_{54}O_{12}$	678.4	$[M+Na]^+$	701.4	701.5	461506	*Nerium olenader* [79]
3	Oleaside D	3.86	$C_{36}H_{54}O_{13}$**	694.4	$[M+Na]^+$	717.3	717.5	110935	*Nerium olenader* [30,78]
4	Gentiobiosyloleandrin	5.26	$C_{44}H_{68}O_{19}$	900.4	$[M+Na]^+$	923.4	923.4	466465	*Nerium olenader* [80]
5	Neogitostin	5.26	$C_{44}H_{68}O_{20}$	916.4	$[M+Na]^+$	939.4	939.4	228731	*Nerium olenader* [81]
6	Glucodigitoxigenin	6.11	$C_{29}H_{44}O_9$	536.3	$[M+Na]^+$	559.3	559.2	235712	*Nerium olenader* [82]
7	Gentiobiosylodoroside A; Odorotrioside G; Odoroside K	6.16	$C_{42}H_{66}O_{17}$	842.4	$[M+Na]^+$	865.4	865.5	580864	*Nerium olenader* [4,80,83]
8	Neriantin;	6.31	$C_{29}H_{42}O_9$	534.3	$[M+Na]^+$	557.3	557.3	294677	*Nerium olenader*[83]
9	Digistroside; Odoroside A***; Beaumontoside***	6.31	$C_{30}H_{46}O_7$	518.3	$[M+K]^+$	557.3	557.3	294677	*Strophanthus vanderijstii Staner* [84]*; Nerium oleander*[13,78]

10	Odorobioside G***	6.43	$C_{36}H_{56}O_{13}$	696.4	$[M+H]^+$	697.4	697.3	187451	*Nerium olenader* [4,30]
11	Uzarigenin 3-O-β-D-Glucopyranosyl-(1→4)-β-D-digitalopyranoside	6.43	$C_{36}H_{56}O_{13}$	696.4	$[M+H]^+$	697.4	697.3	187451	*Nerium oleander* [30]
12	Neriumoside B-2	6.43	$C_{36}H_{50}O_{12}$	674.3	$[M+Na]^+$	697.3	697.3	187451	*Nerium olenader*[79]
13	Desacetylnerigoside; Desacetylcryptograndoside A; Desacetyloleandrin; Odoroside H***; 8β-Hydroxyodoroside A***; Deacetyloleandrin***; Neriifolin***	7.39	$C_{30}H_{46}O_8$	534.3	$[M+H]^+$	535.3	535.3	890755	*Nerium olenader* [4,13]
14	Oleandrin***	7.58	$C_{32}H_{48}O_9$*	576.3	$[M+Na]^+$	599.3	599.3	255709	*Nerium oleander*[13,85]*; Nerium odorum*[30]*; Beaumontia grandiflora*[30]*; Plumeria obtusa*[86]
15	Thevetin B; Odoroside G	11.54	$C_{42}H_{66}O_{18}$	858.4	$[M+H]^+$	859.4	859.5	317786	*Nerium olenader* [4]
16	Oleaside E	12.14	$C_{42}H_{64}O_{17}$	840.4	$[M+H]^+$	841.4	841.5	520113	*Nerium olenader* [87]
17	Adigoside	12.72	$C_{35}H_{54}O_9$	618.4	$[M+H]^+$	619.4	619.4	111803	*Nerium olenader* [78]
18	7,8-Dehydrocerberin***	12.77	$C_{32}H_{46}O_9$	574.3	$[M+H]^+$	575.3	575.4	174510	*Nerium olenader* [4]
19	Digitalin***	12.87	$C_{36}H_{56}O_{14}$	712.4	$[M+K]^+$	751.3	751.5	104622	*Adenium honghel*[84]*; Adenium bohemianum* [84]*; Nerium oleander*[84]

*** Oleandrin, **compounds with more than one adduct, *** biological activity**

Table 6. Identification by UPLC-QTOF-MS of cardenolides in methanolic extract of *cf. Aspidosperma excelsum* by comparing nominal mass of its precursor ion (H^+, Na^+, K^+) with ions produced by electrospray ionization (ESI-MS) considering peak intensity greater than 1×10^5

Entry	Proposed metabolites	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z (theoretical)	m /z (observed)	peak intensity (u.a.)	Apocynaceae species reported
1	16β-Hydroxyaffinoside A	3.3	$C_{30}H_{38}O_{12}$	590.2	$[M+H]^+$	591.2	591.2	144638	*Anderdron affine*[88]
2	Musaroside; Affinoside S-I; Apocynum Cannabinum Saponin 6; Apocynum Cannabinum Saponin 7	4.7	$C_{30}H_{44}O_{10}$	564.3	$[M+Na]^+$	587.3	587.3	161259	*Strophanthus sarmentosus*[30]*; Anodendron affine*[89]*; Apocynum cannabinum*[89]

3	Ouabain ***; Acolongifloroside K***	5.6	$C_{29}H_{44}O_{12}$	584.3	$[M+Na]^+$	607.3	607.2	155610	*Acocanthera oppositifolia*[90]*; Acokanthera longiflora*[30]
4	Basikuloside; Cerbera Odollam Saponin 2	5.9	$C_{36}H_{52}O_{14}$**	708.3	$[M+Na]^+$	731.3	731.4	319989	*Apocynum venetum*[91]*; Cerbera odollam*[92]*; Cerbera manghas*[92]
5	Nerizoside***	6.4	$C_{31}H_{46}O_8$	546.3	$[M+Na]^+$	569.3	569.3	387454	*Nerium oleander*[93]
6	Affinoside S-Xi	6.6	$C_{29}H_{38}O_{11}$	562.2	$[M+Na]^+$	585.2	585.3	162484	*Anodendron affine* [94]
7	Anhydrodigitalin; Apobioside; Obebioside D***; Cerleaside B; Thevetioside C***	7.2	$C_{36}H_{54}O_{13}$	694.4	$[M+Na]^+$	717.3	717.4	114951	*Adenium honghe*[30]*; Nerium odorum*[30]*; Adenium obesum*[95]*; Apocynum androsaemifolium L*[30]*; Cerbera odallam*[96]*; Cerbera manghas*[96]*; Thevetia neriifolia*[97]
8	Opposide***; Sarhamnoloside; Sarmentoloside	7.4	$C_{29}H_{44}O_{11}$	568.3	$[M+H]^+$	569.3	569.3	829052	*Acokanthera longifloraf*[30,98]*; Strophanthus tholloni*[30]
9	Bipindoside; Lokundjoside***	7.4	$C_{29}H_{44}O_{10}$	552.3	$[M+H]^+$	553.3	553.3	1619792	*Strophanthus thollonii* [30]
10	Affinoside T***;	7.4	$C_{29}H_{38}O_{10}$	546.2	$[M+Na]^+$	569.2	569.3	829052	*Anodendron affine*[94]
11	Δ^{16}-Dehydroadynerigenin-3-O-β-D-Digitalopyranoside; Kaneroside	7.4	$C_{30}H_{42}O_8$**	530.3	$[M+Na]^+$	553.3	553.3	1619792	*Nerium odorum*[99]*; Nerium oleander*[93]
12	Oleandrin***	7.5	$C_{32}H_{48}O_9$*	576.3	$[M+Na]^+$	599.3	599.4	102247	*Nerium oleander*[13,85]*; Nerium odorum*[30]*; Beaumontia grandiflora*[30]*; Plumeria obtusa*[86]
13	Erysimosol***	8.9	$C_{35}H_{54}O_{14}$	698.4	$[M+H]^+$	699.4	699.4	8250200	*Strophanthus kombé*[100]

*** Oleandrin, **compounds with more than one adduct, *** biological activity**

Table 7. Identification by UPLC-QTOF-MS of cardenolides in methanolic extract of *Himatathantus bracteatus* by comparing nominal mass of its precursor ion (H^+, Na^+, K^+) with ions produced by electrospray ionization (ESI-MS) considering peak intensity greater than 1×10^5

Entry	Proposed metabolites	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z (theoretical)	m /z (observed)	peak intensity (u.a.)	Apocynaceae species reported
1	16β-Hydroxyaffinoside A	3.6	$C_{30}H_{38}O_{12}$**	590.2	$[M+Na]^+$	613.3G	613.2	285882	*Anderdron affine*[88]
2	2'-O-Acetylcerleaside A; 7,8-Dehydrocerberin***	3.6	$C_{32}H_{46}O_9$	574.3	$[M+K]^+$	613.3	613.3	285882	*Cerbera manghas*[101]*; Cerbera odollam*[101]

3	Beaumontia Brevituba Saponin 3	3.8	$C_{36}H_{54}O_{12}$**	678.4	$[M+Na]^+$	701.3	701.4	153394	*Beaumontia brevituba*[95]*; Beaumontia murtonii*[95]
4	Anhydrodigitalin; Apobioside; Obebioside D***; Cerleaside B; Thevetioside C***	3.8	$C_{36}H_{54}O_{13}$	694.4	$[M+Na]^+$	717.3	717.3	396382	*Adenium honghe*[30]*; Nerium odorum*[30]*; Adenium obesum*[95]*; Apocynum androsaemifolium*[30]*; Cerbera odallam*[96]*; Cerbera manghas*[96]*; Thevetia neriifolia*[97]
5	Acetylobebioside A	3.9	$C_{38}H_{58}O_{14}$	738.4	$[M+Na]^+$	761.3	761.4	259888	*Adenium obesum*[95]
6	Affinoside S-IV; Affinoside S-V; Affinoside S-VI	3.9	$C_{29}H_{40}O_{11}$	564.3	$[M+K]^+$	603.2	603.2	604384	*Strophanthus sarmentosus*[30]*; Anodendron affine*[89]*; Apocynum cannabinum*[89]
7	Δ^{16}-Dehydroadynerigenin 3-O-β-D-Diginopyranoside	5.2	$C_{30}H_{42}O_7$	514.3	$[M+H]^+$	515.2	515.3	139715	*Nerium odorum*[99]
8	Neriumoside B-2	6.0	$C_{36}H_{50}O_{12}$	674.3	$[M+Na]^+$	697.4	697.3	94159	*Nerium odorum*[30]
9	Odorobioside G***	6.3	$C_{36}H_{56}O_{13}$	696.4	$[M+H]^+$	697.3	697.4	187451	*Nerium oleander*[4]
10	Erysimosol***	6.3	$C_{35}H_{54}O_{14}$	698.4	$[M+H]^+$	699.4	699.4	805186	*Strophanthus kombé*[100]
11	Opposide***; Sarhamnoloside; Sarmentoloside	6.4	$C_{29}H_{44}O_{11}$	568.3	$[M+H]^+$	569.2	569.3	240312	*Acokanthera longifloraf*[30,98]*; Strophanthus tholloni*[30]
12	Nerizoside***	6.4	$C_{31}H_{46}O_8$**	546.3	$[M+Na]^+$	569.2	569.3	240312	*Nerium oleander*[102]
13	Affinoside S-Xi	6.5	$C_{29}H_{38}O_{11}$	562.2	$[M+Na]^+$	585.2	585.2	243962	*Anodendron affine*[94]
14	Basikuloside; Cerbera Odollam Saponin 2	6.9	$C_{36}H_{52}O_{14}$	708.3	$[M+Na]^+$	731.3	731.3	214318	*Apocynum venetum*[91]*; Cerbera odollam*[92]*; Cerbera manghas*[92]
15	Bipindoside; Lokundjoside***; Periplogenin 3-O-β-D-Glucopyranoside; Strophanthus Gratus Saponin 1;	7.5	$C_{29}H_{44}O_{10}$	552.3	$[M+H]^+$	553.3	553.3	2443614	*Strophanthus thollonii*[30]
16	Δ^{16}-Dehydroadynerigenin 3-O-β-D-Digitalopyranoside; Kaneroside	7.5	$C_{30}H_{42}O_8$	530.3	$[M+Na]^+$	553.3	553.3	2443614	*Nerium odorum*[99]*; Nerium oleander*[85]
17	Oleandrin***	7.5	$C_{32}H_{48}O_9$*	576.3	$[M+Na]^+$	599.3	599.2	3239976	*Nerium oleander*[13,93]*; Nerium odorum*[30]*; Beaumontia grandiflora*[30]*; Plumeria obtusa*[86]

18	Affinoside P; Affinoside R; Affinoside S; Affinoside B; Affinoside F; Anodendroside G	7.5	$C_{30}H_{40}O_{10}$	560.3	$[M+K]^+$	599.3	599.2	3239976	*Anodendron affine*[103]*; Anodendron paniculatum*[104]
19	Neriumogenin A 3-O-β-D-Digitalopyranoside	8.0	$C_{30}H_{40}O_8$**	528.3	$[M+Na]^+$	551.2	551.3	180951	*Nerium odorum*[94]*; Nerium oleander*[105]

*** Oleandrin, **compounds with more than one adduct, *** biological activity**

Table 8. Identification by UPLC-QTOF-MS of cardenolides in methanolic extract of *Himatathantus tarapotensis* by comparing nominal mass of its precursor ion (H^+, Na^+, K^+) with ions produced by electrospray ionization (ESI-MS) considering peak intensity greater than 1×10^5

Entry	Proposed metabolites	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z (theoretical)	m /z (observed)	peak intensity (u.a.)	Apocynaceae species reported
1	16β-Hydroxyaffinoside A	3.6	$C_{30}H_{38}O_{12}$	590.2	$[M+Na]^+$	613.2	613.3	119320	*Anderdron affine*[88]
2	2'-O-Acetylcerleaside A; 7,8-Dehydrocerberin***	3.6	$C_{32}H_{46}O_9$	574.3	$[M+K]^+$	613.3	613.3	119320	*Cerbera manghas*[101]*; Cerbera odollam*[101]
3	Anhydrodigitalin; Apobioside; Obebioside D***; Cerleaside B; Thevetioside C***	3.8	$C_{36}H_{54}O_{13}$	694.4	$[M+Na]^+$	717.3	717.3	218582	*Adenium honghe*[30]*; Nerium odorum*[30]*; Adenium obesum*[95]*; Apocynum androsaemifolium*[30]*; Cerbera odallam*[96]*; Cerbera manghas*[96]*; Thevetia neriifolia*[97]
4	Beaumontia Brevituba Saponin 3	3.8	$C_{36}H_{54}O_{12}$	678.4	$[M+K]^+$	717.3	717.3	218582	*Beaumontia brevituba*[95]*; Beaumontia murtonii*[95]
5	Acetylobebioside A	3.9	$C_{38}H_{58}O_{14}$	738.4	$[M+Na]^+$	761.4	761.3	150805	*Adenium obesum*[106]
6	Affinoside S-IV; Affinoside S-V; Affinoside S-VI	3.9	$C_{29}H_{40}O_{11}$	564.3	$[M+K]^+$	603.2	603.1	153095	*Strophanthus sarmentosus*[30]*; Anodendron affine*[89]*; Apocynum cannabinum*[89]
7	Acetylobeside B; Monoacetylacoschimperoside P***	5.8	$C_{34}H_{50}O_{11}$	634.3	$[M+K]^+$	673.3	673.3	143784	*Adenium obesum*[106]*; Vallaris solanaceae*[30]*; Vallaris heynei*[30]
8	Basikuloside; Cerbera Odollam Saponin 2	6.7	$C_{36}H_{52}O_{14}$	708.3	$[M+Na]^+$	731.3	731.3	357378	*Apocynum venetum*[91]*; Cerbera odollam*[92]*; Cerbera manghas*[92]

9	Opposide***; Sarhamnoloside; Sarmentoloside	7.4	$C_{29}H_{44}O_{11}$	568.3	$[M+H]^+$	569.3	569.2	159575	*Acokanthera longifloraf*[30,98]*;* *Strophanthus tholloni*[30]
10	Bipindoside; Lokundjoside***; Periplogenin 3-O-β-D-Glucopyranoside; Strophanthus Gratus Saponin 1;	7.4	$C_{29}H_{44}O_{10}$	552.3	$[M+H]^+$	553.3	553.3	263908	*Strophanthus thollonii*[30]
11	Nerizoside***	7.4	$C_{29}H_{38}O_{10}$	546.2	$[M+Na]^+$	569.2	569.2	159575	*Nerium oleander*[102]
12	Δ^{16}-Dehydroadynerigenin 3-O-β-D-Diginopyranoside; Kaneroside	7.4	$C_{30}H_{42}O_8$**	530.3	$[M+Na]^+$	553.3	553.3	263908	*Nerium odorum*[99]*; Nerium oleander*[85]
13	Adynerin	7.5	$C_{30}H_{44}O_7$	516.3	$[M+H]^+$	517.3	517.3	86615	*Nerium olenader*[77,78]
14	Oleandrigenin 3-O-β-D-Glucopyranoside***	7.5	$C_{31}H_{46}O_{11}$	594.3	$[M+Na]^+$	617.3	617.2	111677	*Nerium odorum*[80]*; Nerium oleander*[82]
15	Oleandrin***	7.5	$C_{32}H_{48}O_9$*	576.3	$[M+Na]^+$	599.3	599.3	3080844	*Nerium oleander*[13,93]*; Nerium odorum*[30]*; Beaumontia grandiflora*[30]*; Plumeria obtusa*[86]
16	Affinoside P; Affinoside R; Affinoside S; Affinoside B; Affinoside F; Anodendroside G	7.5	$C_{30}H_{40}O_{10}$	560.3	$[M+K]^+$	599.2	599.3	3080844	*Anodendron affine*[103]*; Anodendron paniculatum*[104]
17	Affinoside Lb; Affinoside G; Affinoside Lc; Affinoside Ld;	7.5	$C_{30}H_{42}O_{11}$	578.3	$[M+K]^+$	617.2	617.2	111677	*Anodendron affine*[89]
18	Glucostrophanthidin***; Sarmentoside C; Thollosid	8.0	$C_{29}H_{42}O_{11}$	566.3	$[M+Na]^+$	589.3	589.2	132886	*Apocynum venetum*[91]*; Strophanthus sarmentosus*[30]*; Strophanthus thollonii*[30]
19	Beaumontia Brevituba Saponin 3	8.0	$C_{36}H_{54}O_{12}$	678.4	$[M+Na]^+$	701.4	701.3	132886	*Beaumontia brevituba*[95]*; Beaumontia murtonii*[95]

20	Acovenoside A***; Alloemicymarin**;** Cerdollaside; 17α-Cerdollaside***	8.0	$C_{30}H_{46}O_9$	550.3	$[M+K]^+$	589.3	589.2	132886	*Acokanthera venenata*[30]*; Strophanthus eminii*[30]*; Cerbera odollam*[107]*; Cerbera manghas*[107]
21	Erysimosol***	8.9	$C_{35}H_{54}O_{14}$	698.4	$[M+H]^+$	699.4	699.3	3909612	*Strophanthus kombé*[100]

*** Oleandrin, **compounds with more than one adduct, *** biological activity**

Table 9. Identification by UPLC-QTOF-MS of cardenolides in methanolic extract of *Rauvolfia praecox* by comparing nominal mass of its precursor ion (H^+, Na^+, K^+) with ions produced by electrospray ionization (ESI-MS) considering peak intensity greater than 1×10^5

Entry	Proposed metabolites	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z (theoretical)	m /z (observed)	peak intensity (u.a.)	Apocynaceae species reported
1	Nerizoside***	3.5	$C_{31}H_{46}O_8$	546.3	$[M+H]^+$	547.3	547.3	101200	*Nerium oleander*[102]
2	16β-Hydroxyaffinoside A	3.6	$C_{32}H_{46}O_{10}$	590.3	$[M+Na]^+$	613.3	613.3	192638	*Anderdron affine*[88]
3	Beaumontia Brevituba Saponin 3	3.8	$C_{36}H_{54}O_{12}$**	678.4	$[M+Na]^+$	701.4	701.4	90476	*Beaumontia brevituba*[95]*; Beaumontia murtonii*[95]
4	Anhydrodigitalin; Apobioside; Obebioside D***; Cerleaside B; Thevetioside C***	3.8	$C_{36}H_{54}O_{13}$	694.4	$[M+Na]^+$	717.3	717.4	388940	*Adenium honghe*[30]*; Nerium odorum*[30]*; Adenium obesum*[95]*; Apocynum androsaemifolium*[30]*; Cerbera odallam*[96]*; Cerbera manghas*[96]*; Thevetia neriifolia*[97]
5	Acetylobebioside A	3.9	$C_{38}H_{58}O_{14}$	738.4	$[M+Na]^+$	761.4	761.4	245227	*Adenium obesum*[95]
6	Mandevilla Pentlandiana Saponin 3	4.1	$C_{42}H_{66}O_{16}$	826.4	$[M+Na]^+$	849.4	849.5	85730	*Mandevilla pentlandiana*[108]
7	Neriumogenin A 3-O-β-D-Digitalopyranoside	6.0	$C_{30}H_{40}O_8$	528.3	$[M+H]^+$	529.3	529.2	553305	*Nerium odorum*[94]*; Nerium oleander*[105]
8	17α-Helveticoside***	6.1	$C_{29}H_{42}O_9$**	534.3	$[M+Na]^+$	557.3	557.3	139794	*Strophanthus komb*[30]
9	Desacetylnerigoside; Desacetylcryptograndoside A; Desacetyloleandrin; Odoroside H***; 8β-Hydroxyodoroside A***; Deacetyloleandrin***; Neriifolin***	6.1	$C_{30}H_{46}O_8$**	534.3	$[M+Na]^+$	557.3	557.3	139794	*Nerium olenader*[4,13]

10	Affinoside Lb; Affinoside Lc; Affinoside Ld;	6.1	$C_{30}H_{42}O_{11}$**	578.3	$[M+Na]^+$	601.3	601.3	96191	*Anodendron affine*[89]
11	Oleandrigenin 3-O-β-D-Glucopyranoside***	6.1	$C_{31}H_{46}O_{11}$	594.3	$[M+Na]^+$	617.3	617.3	356950	*Nerium odorum*[80]*; Nerium oleander* [82]
12	Odoroside A***; Digistroside; Beaumontoside***	6.1	$C_{30}H_{46}O_7$	518.3	$[M+K]^+$	557.3	557.3	139794	*Strophanthus vanderijstii Staner* [84]*; Nerium oleander*[13,78]*; Mandevilla pentlandiana*[108]
13	Acovenoside A***; Alloemicymarin; Cerdollaside; 17β-Cerdollaside***	6.5	$C_{30}H_{46}O_9$	550.3	$[M+Na]^+$	573.3	573.3	395315	*Acokanthera venenata*[30]*; Strophanthus eminii*[30]*; Cerbera odollam*[107]*; Cerbera manghas*[107]

****compounds with more than one adduct, *** biological activity**

Table 10. Identification by UPLC-QTOF-MS of cardenolides in methanolic extract of *Tabernaemontana sananho* by comparing nominal mass of its precursor ion (H^+, Na^+, K^+) with ions produced by electrospray ionization (ESI-MS) considering peak intensity greater than 1×10^5

Entry	Proposed metabolites	Retention time (min)	Formula	Molecular weight	Precursor Ion	m/z (theoretical)	m /z (observed)	peak intensity (u.a.)	Apocynaceae species reported
1	Sarmentoside A Acid; Thollosidic Acid	3.3	$C_{29}H_{42}O_{12}$**	582.2	$[M+Na]^+$	605.3	605.2	342993	*Strophanthus tholloni;* *Strophanthus sarmentosus*
2	Glucostrophanthidin***; Sarmentoside E; Thollosid	3.3	$C_{29}H_{42}O_{11}$	566.2	$[M+K]^+$	605.2	605.2	342993	*Apocynum venetum*[91]*; Strophanthus sarmentosus*[30]*; Strophanthus thollonii*[30]
3	Neritaloside***; 16-Acetylstrospeside***; Acoschimperoside P***	4.3	$C_{32}H_{48}O_{10}$**	592.3	$[M+Na]^+$	615.3	615.3	180248	*Nerium oleander*[78]*; Adenium obesum*[30]*; Acokanthera schimperi*[30]*; Vallaris solanaceae*[30]
4	Acetylobebioside; Beauwalloside***; Cerberin***; Cryptograndoside A***;	4.5	$C_{32}H_{48}O_9$**	576.3	$[M+Na]^+$	599.3	599.3	190397	*Adenium obesum*[106]*; Beaumontia grandiflora*[30]*; Cerbera odollam*[30]*; Tanghinia venenifera*[30]*; Thevetia neriifolia*[30]*; Nerium oleander*[78]*;*

	Honghelosid A***; Nerigoside***								*Adenium honghel*[30]*;Adenium lugardii*[30]*; Mandevilla pentlandiana*[108]
5	Affinoside P; Affinoside R; Affinoside S; Affinoside O; Affinoside B; Affinoside F; Anodendroside G	4.5	$C_{31}H_{44}O_9$	560.2	$[M+K]^+$	599.3	599.3	190397	*Anodendron affine*[103]*; Anodendron paniculatum*[104]
6	16β-Hydroxyaffinoside A	5.6	$C_{32}H_{46}O_{10}$	590.3	$[M+Na]^+$	613.3	613.3	534787	*Anderdron affine*[88]
7	2'-O-Acetylcerleaside A; 7,8-Dehydrocerberin***	5.6	$C_{32}H_{46}O_9$	574.3	$[M+K]^+$	613.3	613.3	534787	*Cerbera manghas*[101]*; Cerbera odollam*[101]

**** Compounds with more than one adduct, *** biological activity**

4. Conclusion

Nature is the greatest source of medicinal agents capable of fighting intractable and drug-resistant diseases. In the case of cardenolides present in plants of the Apocynaceae family there is still much to research. The presence of oleandrin is reported in *H. tarapotensis, H. bracteatus*, and *cf. A. Excelsum*. Additionally, the species of the genus *Himatanthus* and *cf. A. excelsum* in this study show high antitumor activity against Hela and D-384 cell lines, even higher than *N. oleander*. Likewise, the methanolic extracts obtained from these species show minimal damage to normal cells due to specificity by $\alpha 3$ subunit of Na^+/K^+-ATPase typical of cardenolides. For this reason, the methanolic extracts must be fractionated and purified in a biodirected way to find out which cardenolides or non-cardenolides compounds are responsible for the antitumor activity. In other words, a phytochemical screening must be carried out to know if other families of bioactive compounds (alkaloids, triterpenoids, or iridoids) are present in the extracts of this family of plants and they could be contributing to anticancer activity. Finally, cell death mechanism assays should be performed to establish the cell death pathway by which senescence occurs in the D-384 cell line.

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Supporting information

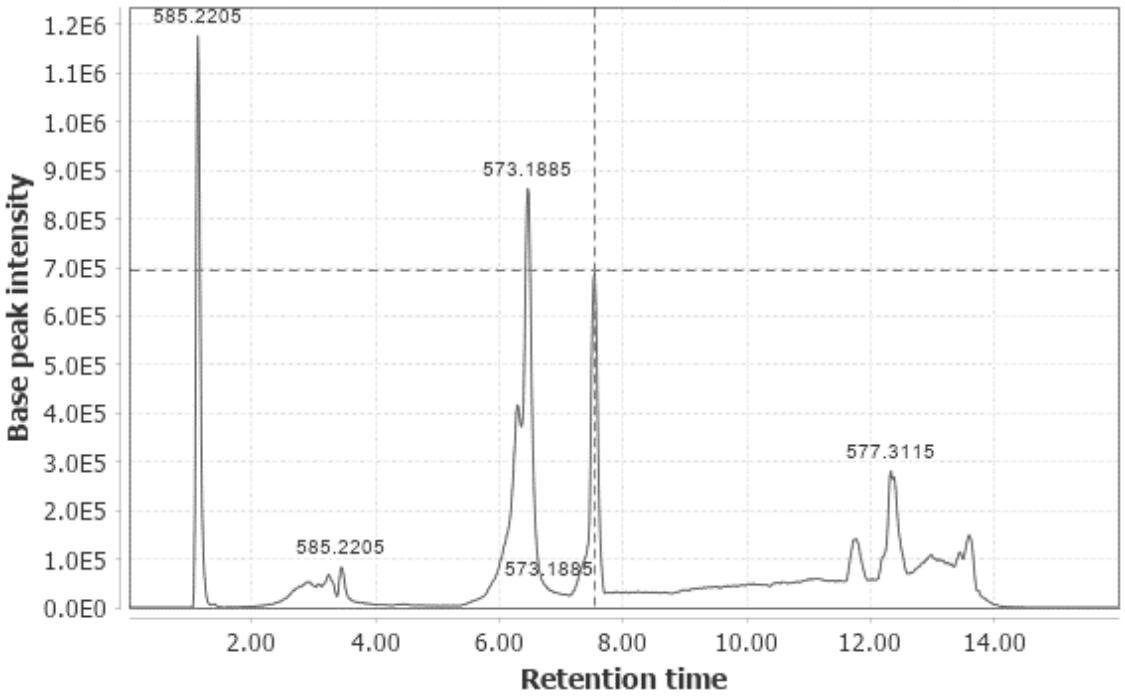

Figure S1. LC spectrum from HPLC-QTOF-MS analysis for oleandrin standard at retention time: 7.54 min.

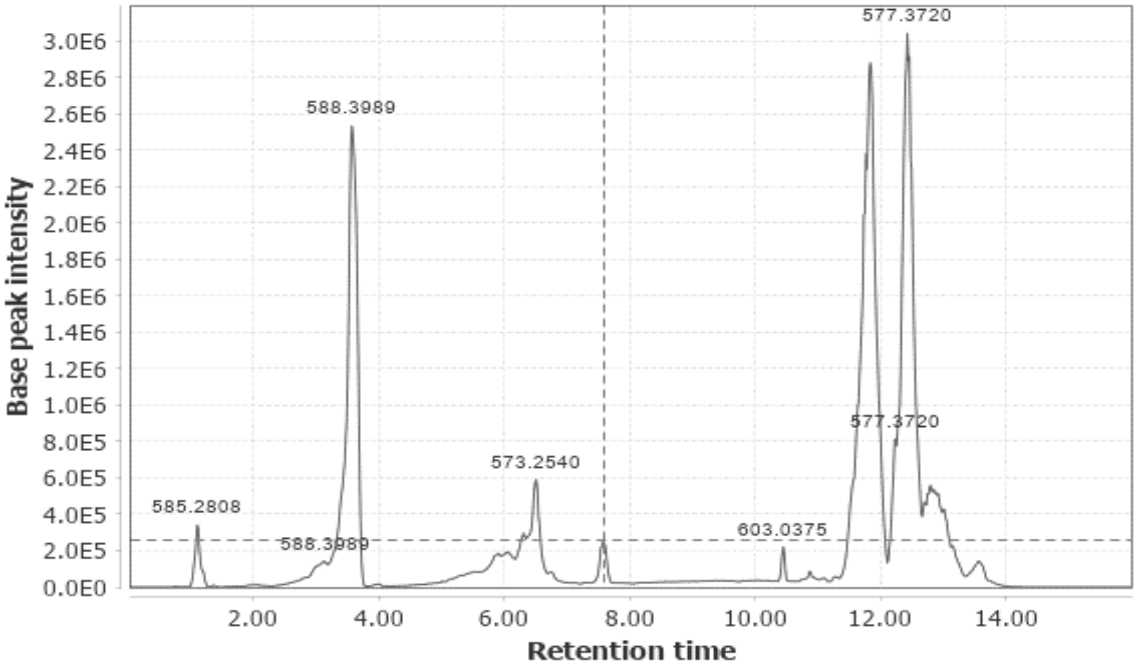

Figure S2. LC spectrum from HPLC-QTOF-MS analysis for *N. oleande* at retention time: 7.57 min.

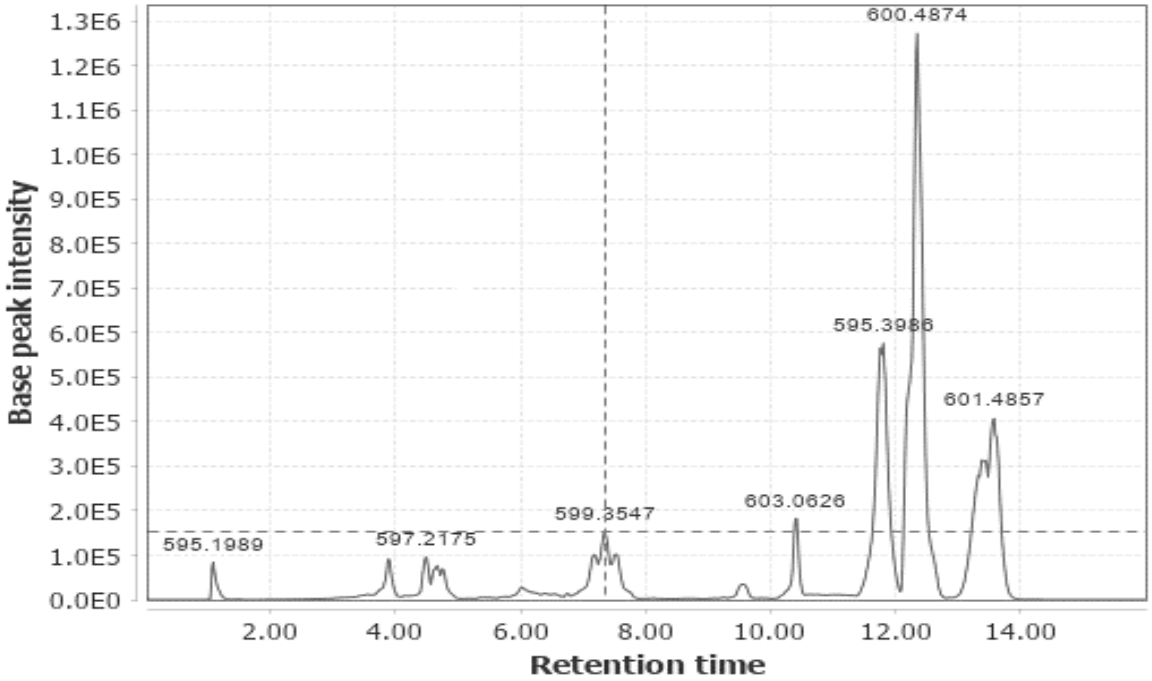

Figure S3. LC spectrum from HPLC-QTOF-MS analysis for *cf. A. excelsum* at retention time: 7.52 min.

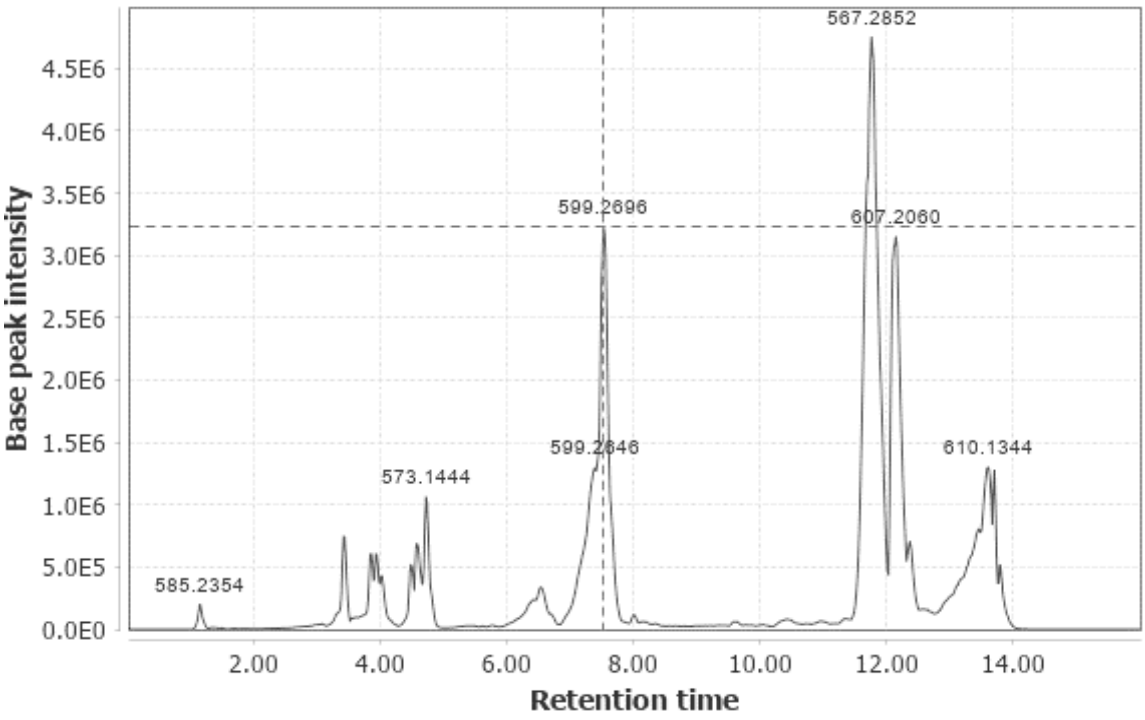

Figure S4. LC spectrum from HPLC-QTOF-MS analysis for *H. Bracteatus* at a retention time: 7.52 min.

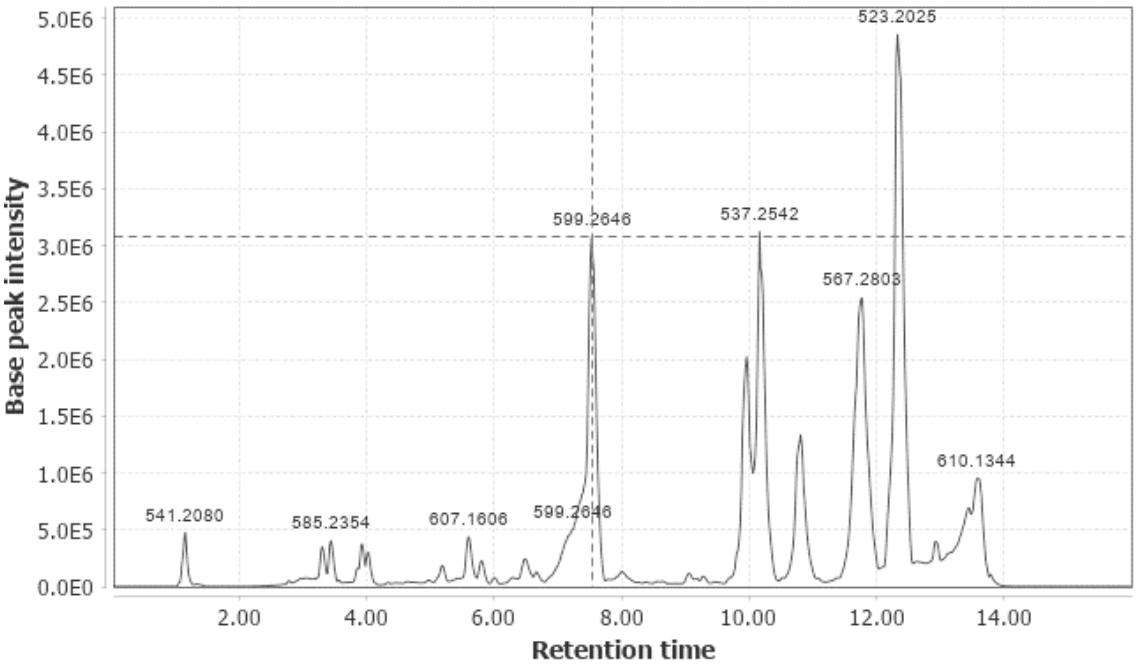

Figure S5. LC spectrum from HPLC-QTOF-MS analysis for *H. Tarapotensis* at a retention time: 7.52 min.