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Original Article

CYTOTOXICITY, ANTI-*POLIOVIRUS* ACTIVITY AND *IN SILICO* BIOLOGICAL EVALUATION OF CONSTITUENTS FROM *MAYTENUS GONOCLADA* (CELASTRACEAE)

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ABSTRACT

Objective: The *in silico* free access web tools PASS online and ChemMapper were used to predict potential biological activities of compounds **1** to **8** isolated from *Maytenus gonoclada* (Celastraceae). The constituents 4'-*O*-methylepigalocatequin (**6**), tingenone (**7**) and proanthocyanidin A (**8**), and ethanolic extracts were subjected to *in vitro* cytotoxicity using VERO cells and anti-*Poliovirus* assays.

Methods: QSAR and molecular superposition, correlating the average number of pharmacophores were used in the prediction studies. Cellular line VERO ATCC CCL-81 was used to determine anti-*Poliovirus* effect, observed by colorimetric (MTT) method**.** The annexing V/propidium iodide assay was used to determine the occurrence of apoptosis in the cytotoxicity assays.

Results: The experimental results found for constituents **6**-**8** were in accordance with observed data obtained through PASS online and ChemMapper simulation.

Conclusion: Compound **7** showed higher cytotoxic and apoptosis induction properties, and **6** and **8** presented anti-*Poliovirus* activity.

Keywords: *Maytenus gonoclada*, VERO cells Cytotoxicity, Anti-*Poliovirus*, Apoptosis, ChemMapper, PASS online.

INTRODUCTION

The use of natural products is widespread, mainly by people of less developed countries, to treat gastrointestinal problems, including ulcers, hepatic disorders, bacterial infections, and in the treatment of patients with viral infections [1-3]. In function of this antiviral potential, various natural compounds already registered focusing viral diseases such as influenza, herpes, hepatite and AIDS and others [4]. The main challenge of natural products researches involves high costs and a long time needed for larger scale evaluation of the pharmacological activity of isolated compounds. The majority of academic researches involving evaluation of biological effects is realized in small scale and in general is abandoned after work, without complete knowledgement about the real pharmacological potentials of the analyzed compounds. The large scale biological assays [High-throughput *screening* (HTS)] adopted by great pharmaceutical industries does not represent a feasible alternative in the university research centers due to the number of pure compounds needed, high cost and low yield of the process [5]. For enhance the efficacy of academic research and to provide an effective direction to the biological assays, in recent years, virtual screening has been applied to predict pharmacological effects of chemical compounds. These approaches performed on a computer (*in silico*) represent a new paradigm in the discovery and development of new bioactive compounds, both in academic as industrial level [6]. Furthermore, when studies of natural products are associated within *silico* evaluations a more appropriate targeting of biological tests are allowed, contributing to the process of drug discovery and making it more suitable for application of public financial support.

The processes used in virtual screening can be complex and difficult to analyze. However, there are some accessible computational tools freely available through web servers that make more rapid and easy to do predictions about potential biological effects of chemical compounds. In general, the results are produced by methods that compare the structure of target compound with those available in databases of active compounds in biological assays realized *in vitro* and/or *in vivo*, new compounds in drug development pipeline, and drugs used in medicine and veterinary. The computer-aided predictions of several types of biological activity over the past decades have accelerated the early-stage pharmaceutical research [7]. Among the *in silico* programs used to predict potential biological activities PASS online and ChemMapper represent alternative tools. Through PASS (Prediction Activity Spectra of Substances) online tool are possible made predictions involving more than 3500 types of biological activities which includes adverse or toxical effects and action against virus, bacteria, fungi, protozoans, aquatic and terrestrial organisms related with environmental impacts, and other information. The results are produced based on quantitative structure–activity relationship (QSAR) models [8,9]. The ChemMapper tool [10] is based on SHAFTS (shape-feature **s**imilarity) software that was validating with the base data DUD-E (Directory of Useful Decoys) [7,11]. In ChemMapper tool is used the superposition of tridimensional (3D) chemical structures of compounds, together with the identification of a set of potential pharmacophores, to predict the potential biological effects of target compound [10,12,13].

Biological potentials of constituents isolated from *M. gonoclada*

Until now, different biological properties have been associated to constituents isolated from species of the Celastraceae family, including some of the genera *Maytenus*. Species of this genus are distributed in tropical and sub-tropical regions, being Brazil considered as having the largest number of species. Among these species the most distinguished is *M. ilicifolia*, popularly known as "espinheira santa", that is officially used as phytotherapeutic in Brazil for the treatment of gastrointestinal disorders [14].

According with Haslam and coworkers [15] are attributed to species of *Maytenus* several pharmacological properties such as anti-inflammatory, antiviral, bactericidal, anthelmintic, antiseptic and protector of the gastric mucosa. Furthermore, anti-protozoan [16] and antitumor [17,18] properties of some compounds isolated from *Maytenus* species reinforce the necessity of more detailed

pharmacological studies to establish the real potential of these compounds.

The species *Maytenus gonoclada* Mart. (Celastraceae), popularly known as "tiuzinho" is naturally found in the state of Bahia, Minas Gerais and Rio de Janeiro, Brazil [19]. The pentacyclic triterpenes (TTPC) represent the principal group of secondary metabolites isolated from *M. gonoclada*, predominating compounds of the friedelane series [19] and a quinonemethide tingenone that has been found in more quantity in roots [20,21].

Properties such as antitumor, antimicrobial, anti-malaria, cytotoxic and antiviral effects [22] were attributed to various TTPC. These properties make these terpenes candidates for new drugs [23]. To tingenone are attributed several biological activities, mainly associated to its cellular toxicity observed *in vitro* [24].

The 4'-*O*-methylepigalocatequin and proanthocyanidins have received special attention as protective agents in the prevention of urinary tract infections and, due to its anti-oxidant property, also in the prevention of cardiovascular diseases [25]. Flavonoid 4'-*O*-methylepigalocatequin was also observed to present anti-inflammatory, immune-stimulant, anti-allergy and antiviral activities [26]. The triterpenes lupeol, α-amirin and β-amirin, commonly found in species of the Celastraceae family, present great potential for targeting cancer through various mechanisms such as the down regulation of transcription factors (*e. g*., nuclear factor-kappaB [NF-κB]), anti-apoptotic proteins (*e. g*., bcl-2, bcl-xL) and promoters of cell proliferation (*e. g*., prostaglandin/cyclooxygenase-2 [COX-2]) [18].

A large number of evidences confirm the role of inflammation in cancer development through mediators such as reactive oxygen species (ROS), free radicals, and inflammatory cytokines like tumor necrosis factor-α (TNFα), lymphotoxins, and angiogenic factors [18]. It was also described a stimulation of the apoptosis mechanism in cancer cells, that can be triggered by the activation of proteases such as caspase-3 and caspase-9, leading to the cells' destruction [18].

In this work the constituents 3,7-dioxofriedelane (**1**), 3,16-dioxo-12α-hydroxyfriedelane (**2**), 3,12-dioxofriedelane (**3**), friedelin (**4**), 3-oxo-12α-hydroxyfriedelane (**5**), 4'-*O*-methylepigalocatechin (**6**), tingenone (**7**) and proanthocyanidin A (**8**) (Figure 1), isolated from *M. gonoclada*, were subjected to web tools PASS online and ChemMapper aiming to the prediction of their potential biological properties. Additionally, compounds **6**, **7** and **8** and the ethanolic extracts from leaves, stems and roots of *M. gonoclada* were submitted to *in vitro* assays to determine the VERO cells cytotoxicity, apoptosis *versus* necrosis occurrence and anti-*Poliovirus* activity.

MATERIAL AND METHODS

Exploratory studies of potential biological activities

The following i*n silico* tools were used to predict potential biological activities for compounds **1** to **8** isolated from *M. gonoclada*.

PASS online

PASS online provides simultaneous predictions of several types of biological activities based on the chemical structures furnishing both the probabilities of each compound to be active (Pa) and inactive (Pi)**.** Pa estimates the chance that the studied compound to belong to the sub-class of active compounds (resembles the structure of the molecules, which are the most typical in a sub-set of "actives" in PASS training set). Pi estimates the chance that the studied compound to belong to the sub-class of inactive compounds (resembles the structure of the molecules, which are the most typical in a sub-set of "inactives" in PASS training set). (http: //www. pharmaexpert. ru/passonline/index. php).

To develop the study using PASS online firstly is necessary upload the chemical structure, saved in sdf file. The tool carries on the decomposition of the structure in the form of descriptors and compare with descriptors of biologically active compounds available in the database. Through QSAR models, each constituent of *M. gonoclada* was compared with more than 250,000 compounds, including drugs, drug candidates, compounds under register processes, toxic substances, oncogenes and other biologically active compounds [8,9]. At the end of analytical process the results were established by difference (Pa – Pi), and the potential biological activities generated for compounds **1** to **8** were organized in Table 1.

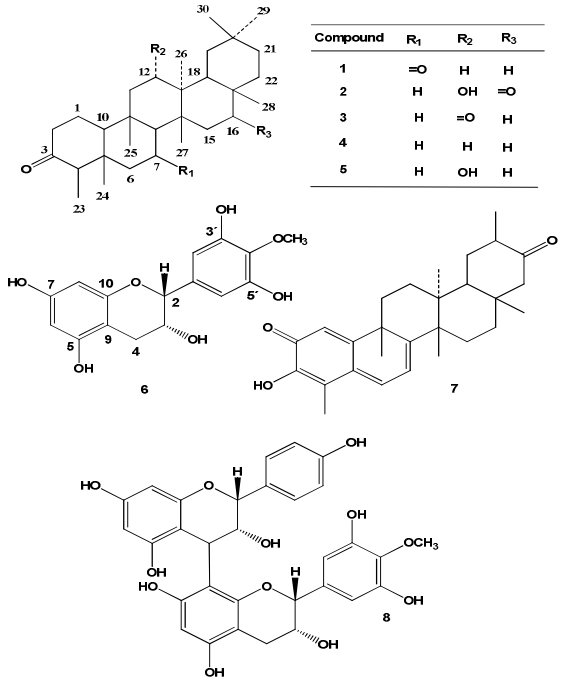


Fig. 1: Chemical structures of compounds 1 to 8 isolated from *M. gonoclada* and subjected to *in silico* PASS online and Chem Mapper tools, to determine its potential biological effect. Compounds 6, 7 and 8 were submitted to *in vitro* anti-*Poliovirus* assays using VERO cells.

Chem Mapper

The ChemMapper tool (http: //lilab. ecust. edu. cn/chemmapper/ index. html) produces a number of possible tautomers together with protonation states to increase the extension of the search [10]. For most cases, this increased search extent, improves the accuracy of the final result [10,12,13]. To establish the potential biological activities of target compound is essential the acknowledgment of better chemical structure *versus* biological activity relationships profiles and the similarity of conformational superposition degrees. The similarity score suggested by this tool were converted in perceptual of similarity with the compounds of the database (1.2 similarity score = 0.6 % similarity). The normalized values (score) presented by the tools were not used. The maximum values selected for the similarity score for the same target were considered only when > 60 % similarity. The profile of constituents **1** to **8** was analyzed both in terms of chemical structure, such as in relation to the type of molecule superposition, correlating the average number of pharmacophores found in each one, as well as the same function in terms of similarity of the spatial conformation. At the end of analytical process applied to compounds **1** to **8**, the potential biological targets generated by this tool were organized in Table 2.

Plant material

M. gonoclada was collected in Serra da Piedade, Caeté, Minas Gerais, Brazil and identified by Dr. Rita Maria Carvalho-Okano, botanist of Universidade Federal de Viçosa, MG, Brazil. The voucher specimen (HBCB 60280) was deposited in the Herbarium of the Universidade Federal de Minas Gerais, BH, Minas Gerais, Brazil. Leaves, stems and roots were dried at room temperature and pulverized on a knife mill. Then, the powdered leaves and stems were respectively subjected to extraction in a Soxhlet apparatus using hexane, chloroform, ethyl acetate and ethanol. The powder of roots was extracted with hexane-ethyl ether (1:1), ethyl acetate and ethanol.

The extracting solvent was recovered using rotary evaporator at temperature < 55 °C. Through phytochemical methods, compounds **1** to **8** (Figure 1) were previously isolated by [19,21]. In function of the water solubility, for the cytotoxicity and anti-*Poliovirus in vitro* assays, were selected the crude ethanolic extract from leaves (MGEEF), stems (MGEG) and roots (MGR) and constituents **6**, **7** and **8** (Figure 1).

Preparation of chemical solutions

Crude extracts MGEEF, MGEG and MGR, and constituents **6**, **7** and **8** were weighed into Eppendorf tubes and then dissolved in dimethylsulfoxide (DMSO) Merck, by means of ultrasound, for 10 minutes, to obtain stock solution of 10.0 ± 2.0 mg/mL. Solutions of extracts MGEEF (12.4 mg/mL), MGEG (10.4 mg/mL) and MGR (10.4 mg/mL) and of constituents **6** (12.5 mg/mL), **7** (10.5 mg/mL) and **8** (11.2 mg/mL) were prepared.

Cells, virus and stock solution

Five passage adherent epithelial cells cultures of green monkey *Cercopithecus aethiops* (Cellular line VERO ATCC CCL-81), *Poliovirus* strain were kindly donated by Erna Geessien Kroon of Universidade Federal de Minas Gerais, Brazil and used for cytotoxicity and antiviral assays. Cells are prepared from healthy, confluent VERO cells that are maintained by passing every 5 ± 2 days, in Dulbecco modified eagle medium (DMEM Sigma-Aldrich®) supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich®), 50 mg/mL gentamicine, 100 U/mL penicillin and 5.0 µg/mL of amphotericin B. The culture was maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. Prior to the beginning of the trials, VERO cells were tested negative for mycoplasma [27]. The virus multiplication was conducted in monolayers of VERO cells. Stock viruses were prepared from supernatants of infected cells and stored at -70 °C. This cell line was chosen due to its known susceptibility to *Poliovirus*.

Cytotoxicity evaluation

Extracts and constituents of *M. gonoclada* were subjected to cytotoxicity assays aiming to establish a safe range concentration for further evaluation of anti-*Poliovirus* effect. VERO cells were treated with different concentrations of the ethanolic extract MGEEF, MGEG or MGR, and of constituents **6**, **7** or **8** for 72h. After this incubation period, the cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction method [28]. Each sample was treated (in 3 replicas) with concentration varying from 0.30 to 125 µg/mL. The cytotoxicity was expressed as CC_{50} values (Concentration of sample that induces 50 % inhibition of the cell growth).

Viral titer

The infectious titer of *Poliovirus* stock was determined through direct plate counting that is a method used to count the number of viable cells described by Burleson and coworkers [29]. VERO cells monolayers, previously growth in 6-well plate, were seeded at a density to yield 90-100 % confluence, about 2 days incubation in DMEM. Then the medium was discarded and replaced with DMEM with 200.0 µL of trypsin 10 µg/mL, without FBS and containing various 10-fold dilutions of *Poliovirus* in different well. A well containing the mock-infected cells was used as a control cell. After one hour of incubation, the inoculum was replaced with DMEM overlay containing 0.7 % agarose and 1.0 µg/mL of trypsin, followed by an incubation period of 3 days at 37 °C in a humidified atmosphere containing 5 % CO_2. Then, the cells were fixed with 10 % formaldehyde (v/v) for 60 minutes, washed with distilled water and stained with crystal violet an 10 % (m/v). Dilutions which showed 30-300 plaques were chosen for the calculation of virus titer using the formula: plaque forming units (PFU)/mL = n x 10^i x CF, in which n = number of plates, CF = correction factor (1000/volume used for infect with *Poliovirus*), i = reciprocal of the dilution factor [30].

Anti-*Poliovirus* assay

VERO cells were treated with trypsin 10 µg/mL and transferred to a 96 well microplate ($5x10^4$ cells/well). For each ethanolic extract or compound **6**, **7** or **8**, were used four serial dilution (in 3 replicas). The *Poliovirus* suspensions were added to extracts, in four concentrations from CC_{50}. During each assay, in parallels the following controls were used for cells (Not infected and untreated cells), cytotoxicity (Not infected cells treated with samples) and for *Poliovirus* (Infected untreated cells). The anti-*Poliovirus* activity was evaluated by colorimetric (MTT) method [31].

The inhibition of *Poliovirus* replication was determined by the formula: **%** viral replication inhibition = **(CVE-CV)/(C-CV).** In this formula, V, CV, CC is equal to the average value of optical density at 570 nm (DO_{570}) observed for the well containing: cell + virus + extract (CVE), cell + virus (CV) and for the well with only cells (C). The concentration of sample effective for inhibit 50 % death of cells (EC_{50}), observed in the assay CVE was established by linear regression considering adequate the values for $R^2 \geq 0.90$.

Annexin V/propidium iodide apoptosis assay

The annexin V/propidium iodide assay was used to determine the occurrence of apoptosis in the cytotoxicity assays. Initially, the annexin-V binding buffer 10x was diluted (1:10) in Milli-Q water. The VERO cells were washed 2x with FBS and re-suspended aiming to obtain 3 x 10^6 cells/mL in annexin binding buffer 1x (containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$).

In each assay tube were added 100.0 µL of the cell suspension, 5.0 µL of anexin V FITC and 10.0 µL of propidium buffer. The tubes were incubated for 15 minutes at 25 °C, in the dark. Then, 400.0 µL anexin V binding buffer 1x/tube were added and analyzed, in at most 60 minutes, using a Becton Dickinson FACSCalibur Flow Cytometer, with fluorescence FL1 at 414 nm and FL2 at 451 nm.

Table 1: *In silico* test prediction of potential biologic effect using PASS online*tool

Potential biological effect	Constituents of *M. gonoclada*							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Anti-inflammatory	0.82	0.68	0.68	0.77	0.76	0.53	0.79	0.33
Antiulcerative	0.54	0.18	0.39	0.61	0.51	0.62	0.48	0.36
Apoptosis agonist	0.87	0.72	0.82	0.87	0.81	0.72	0.87	0.62
Bcl-xLinhibitor	0.06	----	0.06	0.08	0.05	----	0.10	----
Bcl2 antagonist	0.02	----	0.02	0.09	0.02	----	0.23	----
Topoisomerase II inhibitor	----	----	----	----	----	0.29	----	0.26
Transcription factor NF kappa B inhibitor	0.46	0.51	0.32	0.34	0.49	0.50	0.11	0.46
Cytostatic	----	----	----	----	----	0.56	0.04	0.41
DNA polymerase I inhibitor	----	0.12	----	0.02	0.18	0.31	0.11	0.24
Antiviral	----	----	----	----	----	0.32	----	0.25
Antiviral (Hepatitis B)	----	----	----	----	----	0.30	----	0.20
Antiviral (Herpes)	0.31	0.41	0.36	0.39	0.42	0.43	0.30	0.38
Antiviral (HIV)	----	----	----	----	----	0.24	----	0.17
Antiviral (Influenza)	0.58	0.44	0.58	0.70	0.67	0.65	0.64	0.52
Antiviral (Rhinovirus)	----	0.27	----	----	0.32	0.48	0.07	0.40
Viral entryinhibitor	----	----	----	0.12	----	0.18	----	----

*Values of difference Pa – Pi. (http: //www. pharmaexpert. ru/passonline), --- Not indicated or unsatisfactory.

Table 2: *In silico* test prediction of potential biologic targets using Chem Mapper tool**

Potential biological target	Constituents of *M. gonoclada*							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Androgen receptor	0.65	0.65	0.65	0.64	0.62	0.65	0.67	----
Estrogen receptor	0.66	0.64	----	0.69	0.63	0.66	0.71	----
Estrogen receptor β	----	----	----	0.63	----	0.66	0.63	----
Progesterone receptor	0.69	0.66	0.67	0.70	0.65	----	0.61	----
Proto-oncogene serine/threonine-protein kinase Pim-1	----	----	----	----	----	0.74	----	----
Estradiol 17β-dehydrogenase 1	0.63	0.61	0.61	0.66	0.61	0.76	0.64	----
Retinoic acid receptor RXR-α	0.63	0.61	0.62	0.65	0.61	----	0.62	----
Phospholipase A2	----	----	----	----	----	0.78	----	----
Glutathione S-transferase a2	----	----	----	0.66	----	----	0.66	----
Glutathione S-transferase a3	----	----	----	0.66	----	----	0.67	----
Cell division protein kinase 6	----	----	----	----	----	0.62	----	----
Cell division protein kinase 2	----	----	----	----	----	0.65	----	----
Thymidylate synthase	----	----	----	----	----	0.62	----	----

** http: //lilab. ecust. edu. cn/chemmapper/index. html., --- Not indicated or unsatisfactory.

Cytotoxic effect based on percent cell viability of VERO cells in function of the treatment with ethanol extract from leaves (MGEEF), stems (MGEG) and roots (MGR) of *M. gonoclada* is showed in Figure 2. The *Poliovirus* effects based on percent cell viability of VERO cells in function of the treatment with ethanol extracts are showed in Figure 3. The histograms obtained through the annexin V/propidium iodide assay related to apoptosis induced in VERO cells treated with compound **6**, **7** and **8** are presented in Figure 4. The progression of apoptosis process of VERO cells associated to the concentration of compound **7**, in Figure 5.

Statistical analysis

The median values ± standard deviation was representative of four independent *in vitro* assays. To establish CC_{50} and EC_{50} were used values of linear regression of concentrations associated to dose/response. The statistical analysis of data was realized using the software Prism 5.0, GraphPad Software Inc., San Diego, CA.

RESULTS

Through the analysis of data generated by the tool PASS online were found potential biological activities (Table 1) and by ChemMapper, the potential biological targets (Table 2) attributed for compounds **1** to **8** (Figure 1).

DISCUSSION

Eight compounds isolated from *M. gonoclada* were subjected to the free web tools PASS online and ChemMapper to make predictions of their potential biological properties. The use of these tools is important as being a previously study aiming to establish adequate choice for *in vitro* or *in vivo* assays.

The PASS online and ChemMapper tools were used *in silico* to make a prediction of potential biological activities (Table 1 and 2) of compounds **1** to **8** (Figure 1) isolated from *M. gonoclada*. The direct indication of potential biological activities, without mentioning the species of target virus was realized by comparison with the database of compounds with antiviral properties. A similar approach was adopted in the prediction of other biological effects (Table 1).

For compound **6** and **7** was possible to infer the apoptosis inductor effect, observed by the prediction made by PASS online (Table 1). The suggestion of possible activities is important, because it opens perspectives for conducting studies aiming to identify the mechanisms of pro-apoptotic action involving the compounds **6** and **7**. The PASS online tool indicated potential antiviral activity against species of the virus influenza and Herpes (Table 1). Also indicate potential activity against DNA topoisomerase II, transcription factor NF kappa B inhibitor and DNA polymerase I (Table 1) that are important targets for researches of new substances with antiviral activity. DNA polymerase is considered as being a potential target associated to anti-*Poliovirus* activity [32]. The ChemMapper tool indicates potential inhibition properties of important antiviral targets, such as cell division protein kinase 2 and thymidylate synthase, attributed to compound **7** (Table 2). This tool does not provide potential biological targets for compound **8**. According to Mitsuishi and coworkers [33] there are a relationship between phospholipase A2 activity and infection prevention with adenovirus. This can be suggestive of a potential anti-virus activity of compound **6**.

Assenco and coworkers [24] reported the *in vitro* anti-*Rotavirus* (RV-SA11) effect induced by compounds **6**, **7** and **8** (Figure 1), isolated from *M. gonoclada*. Thus, to continue the research related to pharmacological potential of substances isolated from *M. gonoclada*, in this work the ethanolic extracts and constituents **6**, **7** and **8** were subjected to anti-*Poliovirus in vitro* assays.

In general, the *in vitro* assays are based on the inhibition of viral cytopathic effect (CPE) in the cell cultures. In recent years, in the most widely used method the compound MTT is reduced to formazan by mitochondrial enzymes of cells that not suffer the CPE caused by viral infection. The assay is rapid, requires small amount of test sample and enables a direct assessment of cell viability [31]. Through the MTT method was quantified the cell viability of extracts MGEEF, MGEG and MGR, and of constituents **6**, **7** and **8**, using VERO cells and *Poliovirus*.

Poliovirus is a human enterovirus of RNA genome, single positive strain, waterborne, transmitted from person to person through fecal excretion and oropharyngeal secretions, mainly oral fecal route. The receptor for *Poliovirus* is only expressed in human cells and some species of primates [34,35]. Part of the infection process occurs with the replication of virus in the gastrointestinal tract, causing viremia [36,37]. When the virus reaches the central nervous system it destroys motor nerves causing distinctive flaccid paralysis with permanent sensory loss [38]. The average incubation period of paralysis is approximately 10 days [39,40]. Only one in 150 primary polio infection causes paralysis, since most of them are subclinical infections [39]. Data inserted in Tables 1 and 2 indicate that compound **6** could be an antiviral candidate. None have been detected in PASS online and ChemMapper associated specifically to *Poliovirus*. Nevertheless, the relationship with phospholipase A2 [41,42] is important for future studies related to anti-*Poliovirus* activity of compound **6**.

Apoptosis or programmed cell death is a physiological process which is essential for the elimination of modified cells, damaged or infected by a virus, for the removal of self-reactive lymphocytes, and to organize in developing tissues [43]. Apoptosis is morphologically characterized by condensation of nuclear chromatin, reduction in cell size, cytoplasm condensation, and loss of intercellular linkages and detachment of cells from the growth surface [44].

The evaluation of apoptosis is important, since one of the main objectives of the current chemotherapy is the destruction of cells by inducing death through apoptosis and not by necrosis [45]. In the

case of cell death by necrosis, no programming occurs and the process is morphologically characterized by distinct mode of apoptosis. The necrosis is characterized by enlargement of the endoplasmic reticulum, mitochondria and other organelles, and consequently the cell. Subsequently, the cell membrane becomes permeable to molecules of high molecular weight, the chromatin suffer fragmentation, occur a dissolution of the nuclear membrane, resulting in cell disruption [46]. All compounds subjected to PASS online and ChemMapper *in silico* tools presented potential properties such as apoptosis agonist (Tables 1 and 2). The values of (Pa-Pi) ≥ 0.5 established by through PASS online indicate compounds **1** to **8** as being potential apoptosis agonists (Table 1) were herein considered important data associated to apoptosis. However, the values ≤ 0.5 attributed shows low probability of compounds **1** to **8** as regulator Bcl-xL inhibitor and Bcl-2 antagonist. All compounds presented potential activity as transcription factor NF kappa B inhibitors (Table 1). Through ChemMapper tool were predicted for compounds **1** to **8** potential targets associated to apoptosis (Table 2) such as proto-oncogene serine/threonine-protein kinase Pim-1 [47,48], Glutathione s-transferase [49], Androgen receptor [50], cytoplasmic phospholipase A2 [43], retinoic acid receptor RXR-alpha [51] and estrogen receptors [52,53]. According to Wong and coworkers [54], the induction of apoptosis represents good strategy in the cancer treatment. In the *in vitro* labeling by annexin-v, this compound binds to the epithelial membrane of apoptotic cells that are in apoptosis process induced by treatment of cytotoxic substance. By the annexin V/propidium iodide assay is possible to quantify the initial and end populations of cells submitted to apoptosis process.

The ethanolic extract MGEEF, MGEG and MGR, and constituents **6**, **7** and **8** (Figure 1) were subjected to cytotoxic assays using VERO cells and subsequent assay against *Poliovirus*. The value of CC_{50}, with 95 % confidence interval was determined for MGEEF (87.74 to 119.30 µg/mL), MGR (71.71 to 95.01 µg/mL) and MGEG (158.9 to 212.9 µg/mL). MGEG was the safest of extracts subjected to tests (Figure 2).

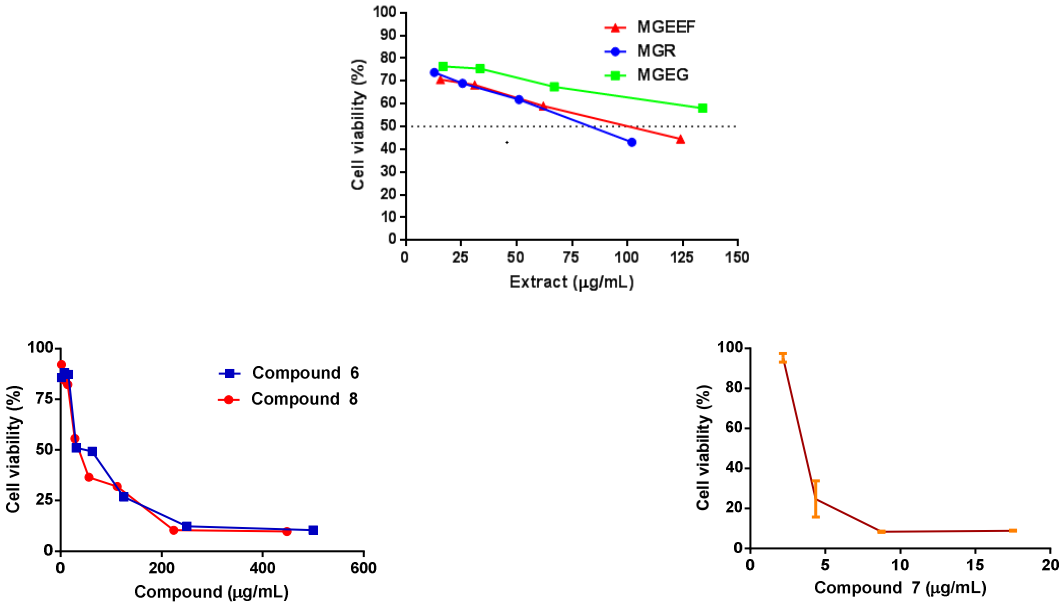


Fig. 2: Cytotoxic effect based on percent cell viability of VERO cells in function of the treatment with ethanol extract from leaves (MGEEF), stems (MGEG) and roots (MGR) of *M. gonoclada* determined through optical density at 570 nm (DO_{570}). (DO_{570} of treated cells ÷ DO_{570} of control cells) × 100. Each point corresponds to the median of each value of serial dilution.

In the assays conditions, the extracts showed no anti-*Poliovirus* effect, or if it is present, it was herein considered insufficient to offset the cytotoxic effects generated (Figure 3). Compounds **6** and **8** showed anti-*Poliovirus* properties.

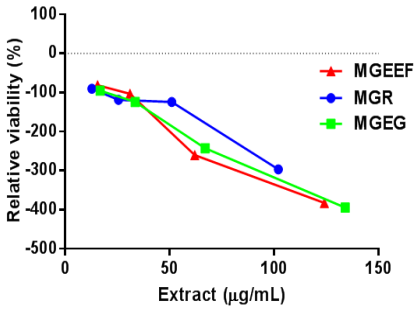
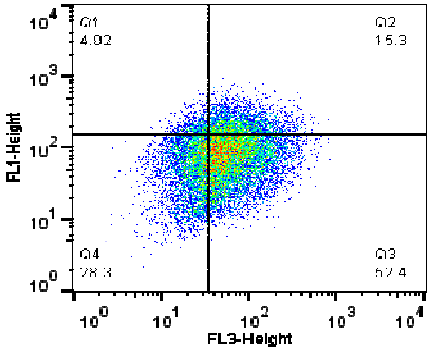


Fig. 3: ***Poliovirus* effect based on percent cell viability of VERO cells in function of the treatment with ethanol extract from leaves (MGEEF), stems (MGEG) and roots (MGR) of *M. gonoclada* against determined through optical density at 570 nm (DO_{570}). [(DO_{570} of treated cells - DO_{570} of virus control) ÷ DO_{570} of control cells) × 100. Each point corresponds to the median of each value of serial dilution.**

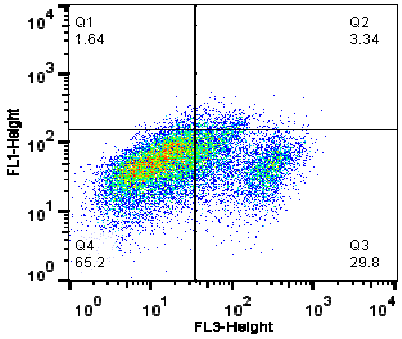
Cytotoxicity *in vivo* is a complex event, whose expression is manifested through a wide spectrum of effects, ranging from functional or metabolic aberrations and complex morphological changes, depending on which no cell death or cell death is observed. Based on the study, the definitions of cytotoxicity vary considering if cells are death or simply have altered metabolism [55]. The *in vitro* assays using cell culture are useful and necessary to define the basal cytotoxicity, *i. e.*, a compound that has the property of inducing damage to essential functions of the cell or even cell death. Cytotoxic assays are important to establish the maximum concentration used, for subsequent antiviral activity evaluation [56]. Smaller percentage of surviving cells indicates greater cytotoxic potential of the tested sample. If the cell viability was reduced to 50 % in wells containing only cells, the sample has a median cytotoxic potential CC_{50} [57]. According to Rieser and coworkers [58], extracts and compounds that shown values of CC_{50} ≤ 20.0 µg/mL or 4.0 µg/mL, respectively, are considered cytotoxic.

According to Vasilevsky and coworkers [59] plant-derived of pentacyclic triterpenes provide a versatile structural platform for the discovery of new biologically active compounds. In the present study tingenone (**7**) (Figure 1) was considered the more cytotoxic

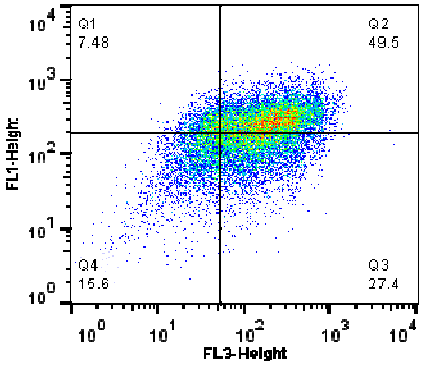
constituent evaluated (Figure 2). For this reason, terpene **7** was submitted to annexin V/propidium iodide assay to verify if the cell death was induced by apoptosis process, or not. The percent of viable cells and apoptotic cells was determined by flux cytometry.



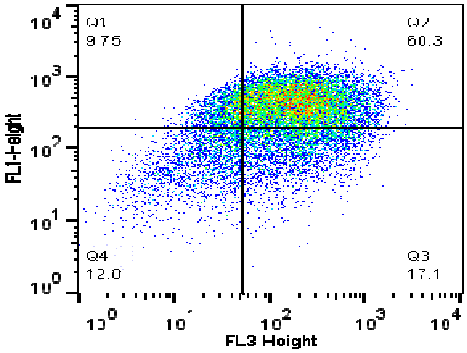
Compound 6: 125.0 µg/mL
Necrosis (Q1): 4.0%
Apoptosis (Q2+Q3): 67.7%
Viable cells: 28.3%



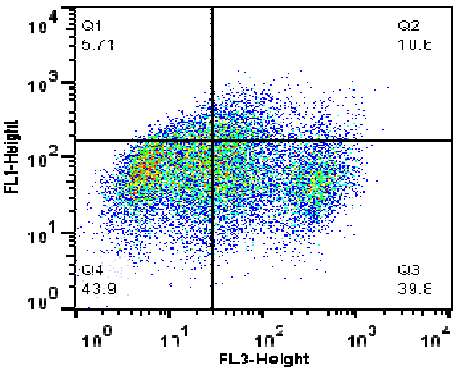
Compound 6: 15.6 µg/mL
Necrosis (Q1): 1.6%
Apoptosis (Q2+Q3): 33.1%
Viable cells: 65.2%



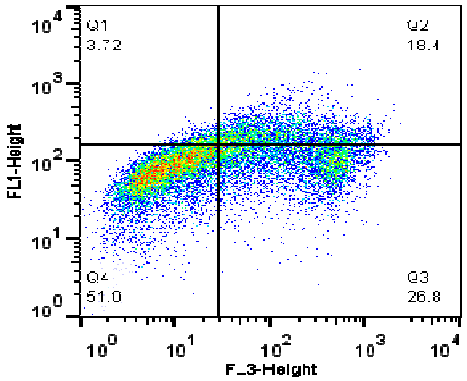
Compound 7: 17.5 µg/mL
Necrosis (Q1): 7.5%
Apoptosis (Q2+Q3): 76.9%
Viable cells (Q4): 15.6%



Compound 7: 4.3 µg/mL
Necrosis (Q1): 9.8%
Apoptosis (Q2+Q3): 77.1%
Viable cells (Q4): 12.8%



Compound 8: 15.0 µg/mL
Necrosis (Q1): 5.7%
Apoptosis (Q2+Q3): 50.4%
Viable cells (Q4): 43.9%



Compound 8: 15.0 µg/mL
Necrosis (Q1): 3.7%
Apoptosis (Q2+Q3): 45.2%
Viable cells (Q4): 51.0%

Fig. 4: Histograms obtained from the annexin V/propidium iodide assay used to determine apoptosis induced in VERO cells treated with 4'-*O*-methylepigalocatequin (6), tingenone (7) and proanthocyanidin A (8). Fluorescence FL1 at 414 nm and FL2 at 451 nm. In the lower left quadrant of hystogram is shown the incidence of viable cells (annexin V-/PI-), in the lower right quadrant, the incidence of early or initial apoptosis (annexin V+/PI-), in the upper right quadrant, cells in late apoptosis or secondary necrosis (annexin V+/PI+), and in the upper left quadrant necrotic cells (annexin V-/PI+).

In the lower left quadrant of the cytogram obtained (Figure 4) is shown the incidence of viable cells (annexin V-/PI-), in the lower right quadrant, the incidence of early or initial apoptosis (annexin V+/PI-), in the upper right quadrant, cells in late apoptosis or secondary necrosis (Annexin V+/PI+), and in the upper left quadrant necrotic cells (annexin V-/PI+). This assay was useful to determine that terpene **7** induces cellular death by apoptosis, confirming the predictions made using PASS online and ChemMapper. The late

apoptose represent the principal event observed in progression of apoptosis process of VERO cells induced by compound **7**. Even in low concentration (4.34 µg/mL) the apoptose rate was considered as important when compared to viable cells (Figure 5).

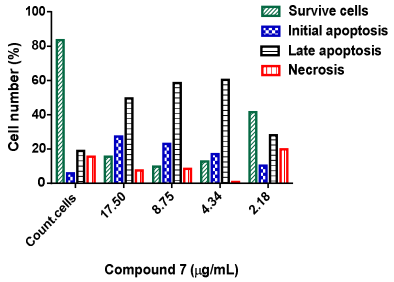


Fig. 5: Progression of apoptosis process of VERO cells associated to the concentration of tingenone (7).

CONCLUSION

By the results was possible to conclude for compound **7** a higher cytotoxic and apoptosis inductor properties in VERO cells, and constituent **6** and **8** showed anti-*Poliovirus* activity. These results were observed by the prediction made through PASS online and ChemMapper and by the *in vitro* assays.

CONFLICT OF INTERESTS

Declared None **s**

ACKNOWLEDGMENT

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REFERENCES

1. Schulz PV, Hansel R, Tyler VE. Rational Phytotherapy, A Physicians Guide for Physicians and Pharmacists. Berlin, Springer-Verlag, 5th ed. 2004. p. 417.
2. Veiga VFJ, Pinto AC, Maciel MAM. Plantas medicinais: cura segura? Quím Nova 2005;28(3):519-28.
3. Newman DJ, Cragg GM. Natural products as sources of new drugs over last 25 years. J Nat Prod 2007;70:461-77.
4. Jassim SAA, Naji MA. Novel antiviral agents: a medicinal plant perspective. J Appl Microb 2003;95:412-27.
5. Harvey AL. Natural products in drug discovery. Drug Discov Today 2008;13(19):894–901.
6. Schmidt BJ, Papin JA, Musante CJ. Mechanistic systems modeling to guide drug discovery and development. Drug Discov Today 2013;18:13-4.
7. Wang X, Chen H, Yang F, Gong J, Li S, Pei J, *et al*. iDrug: a web-accessible and interactive drug discovery and design platform. J Cheminform 2014;6:28.
8. Lagunin A, Stepanchikova A, Filimonov D, Poroikov V. PASS: prediction of activity spectra for biologically active substances. Bioinformatics 2000;16(8):747-8.
9. Lagunin A, Filimonov D, Poroikov V. Multi-targeted natural products evaluation based on biological activity prediction with PASS. Current Pharm Design 2010;16(15):1703-17.
10. Gong J, Cai C, Liu X, Ku X, Jiang H, Gao D, *et al*. Chem mapper: a versatile web server for exploring pharmacology and chemical structure association based on molecular 3d similarity method. Bioinformatics 2013;29(14):1827-9.
11. Mysinger MM, Carchia M, Irwin JJ, Shoichet BK. Directory of useful decoys, enhanced (DUD-E): better ligands and decoys for better benchmarking. J Med Chem 2012;55:6582-94.
12. Lu W, Liu X, Cao X, Xue M, Liu K, Zhao Z, *et al*. SHAFTS: a hybrid approach for 3d molecular similarity calculation. 2. prospective case study in the discovery of diverse p90 ribosomal s6 protein kinase 2 inhibitors to suppress cell migration. J Med Chem 2011;54:3564-74.
13. Liu X, Jiang H, Li H. SHAFTS: a hybrid approach for 3d molecular similarity calculation. 1. method and assessment of virtual screening. J Chem Inform Modell 2011;51(9):2372-85.
14. Wilker MMJ, Tarcísio NC. Study of the pharmacological properties of espinheira-santa (*Maytenus ilicifolia* Mart. ex Reissek) and of two adulterating species. Rev Saúde Desenv 2012;1:20-46.
15. Haslam E, Lilley TH, Martin R, Magnolato D. Traditional herbal medicines, the role of polyphenols. Pl Med 1989;55:1-8.
16. Leite JPV, Lombardi JA, Chiari E, Oliveira AB. Isolamento biomonitorado de uma substância tripanossomicida de *Arrabidaea triplinervia* (Bignoniaceae), o ácido ursólico. Rev Bras Farmacogn 2001;11:77-87.
17. Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, *et al*. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med 1995;1:1046-51.
18. Vivek R, Yadav S, Prasad B, Ramaswamy K, Bharat BA. Targeting inflammatory pathways by triterpenoids for prevention and treatment of cancer. Toxins 2010;2:2428-66.
19. Oliveira MLG, Duarte LP, Silva GDF, Vieira Filho SA, Knupp VF, Alves FG. 3-Oxo-12α-hydroxyfriedelane from *Maytenus gonoclada*: structure elucidation by 1H and [13]C chemical shift assignments and 2D-NMR spectroscopy. Mag Res Chem 2007;45:895-8.
20. Silva FC, Guedes FAF, Franco MW, Barbosa FAR, Marra CA, Duarte LP, *et al*. Algistatic effect of a quinonamethide triterpene on *Microcystis novacekii*. J Appl Phycol 2013;25(6):1723-8.
21. Silva FC, Oliveira MLG, Rodrigues VG, Carvalho SM, Duarte LP, Silva GDF, *et al*. Attractive effects of hexane extract and triterpenes from *maytenus gonoclada* on *tenebrio molitor*. Chem Nat Comp 2013;49(3):571-4.
22. Gong Y, Raj KM, Luscombe CA, Gadawski I, Tam T, Chuj GC, Sacks SL. The synergistic effects of betulin with acyclovir against *Herpes simplex* viruses. Antivir Res 2004;64:127-30.
23. Alvarenga E, Ferro EA. Bioactive triterpenes and related compounds from Celastraceae. Stud Nat Prod Chem 2006;33:239-307.
24. Assenco RAG, Silva FC, Lanna MCS, Magalhaes CB, Duarte LP, Silva GDF, *et al*. Anti-*Rotavirus* (RV, SA-11) property of polar substances isolated from *maytenus gonoclada* (celastraceae). Eur J Sci Res 2013;114:256-68.
25. Marles MAS, Ray H, Gruber MY. New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry 2003;64:367-83.
26. Vayalil PK, Elmets CA, Katiyar SK. Treatment of green tea polyphenols in hydrophilic cream prevents UVB-induced oxidation of lipids and proteins, depletion of antioxidant enzymes and phosphorylation of MAPK proteins in SKH-1 hairless mouse skin. Carcinogenesis 2003;24:927-36.
27. Dobrovolný PL, Bess D. Optimized PCR-based detection of mycoplasma. J Vis Exp 2011;52:4-7.
28. Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 1987;56:279–85.
29. Burleson FG, Chambers TM, Wiedbrauk DL. Virology: a laboratory manual. London: Academic Press; 1992. p. 250.
30. Chattopadhyay D, Naik TN. Antivirals of ethnomedicines and ethnomedicinal origin, structure-activity relationship and scope. Mini-Rev Med Chem 2007;7(3):275-301.
31. Betancur-Galvis LA, Morales GE, Forero JE, Roldan J. Citotoxic and antiviral activitie of Colombian medicinal plant extracts of the *Euphorbia* genus. Mem do Inst Oswaldo Cruz 2002;97:541-6.
32. Berdis AJ. DNA polymerases as therapeutic targets. Biochem 2008;47(32): 8253-60.
33. Mitsuishi M, Masuda S, Kudo I, Murakami M. Group V and X secretory phospholipase A2 prevents adenoviral infection in mammalian cells. Biochem J 2006;393(1):97-106.
34. Mueller S, Wimmer E, Cello J. *Poliovirus* and poliomyelitis: a tale of guts, brains, and an accidental event. Virus Res 2005;111(2):175-93.
35. Racaniello VR. One hundred years of poliovirus pathogenesis. Virology 2006;344(1):9-16.

36. Bodian D. Emerging concept of poliomyelitis infection. Sci 1955;122(3159):105-8.
37. Sabin AB. Pathogenesis of poliomyelitis, reappraisal in the light of new data. Sci 1956;123(3209):1151-7.
38. Nathanson N. The pathogenesis of poliomyelitis: What we dont't know. Adv Virus Res 2008;71:1-50.
39. Horstmann DM, Paul JR. The incubation period in human poliomyelitis and its implications. J Amer Med Assoc 1947;135(1):11-1.
40. Sartwell PE. The incubation period of poliomyelitis. Am J Public Health 1952;42(11):1403-8.
41. Guinea R, López-Rivas A, Carrasco L. Modification of phospholipase C and phospholipase A2 activities during poliovirus infection. J Biol Chem 1990;264(36):21923-7.
42. Irurzun A, Pérez L, Carrasco L. Enhancement of phospholipase activity during *Poliovirus* infection. J Gen Virol 1993;74(6):1063-71.
43. Adam-Klages S, Schwandner R, Lüschen S, Ussat S, Kreder D, Krönke M. Caspase-mediated inhibition of human cytosolic phospholipase a2 during apoptosis. J Immunol 1998;161:5687-94.
44. Darnell JE. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2001;2:740-9.
45. Wyllie AH. Apoptosis: cell death under homeostatic control. Mechanisms Models Toxicol 1987;11:3-10.
46. McDermott CM, Nho CW, Holton BH. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types Cover image. Toxicon 1998;36(12):1981–96.
47. Bachmann M, Möröy T. The serine/threonine kinase Pim-1. Int J Biochem Cell Biol 2005;37(4):726-30.
48. Brault L, Gasser C, Bracher F, Huber K, Knapp S, Schwaller J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. Haematologica 2010;95:1004-15.
49. Yabuno T, Konishi N, Nakamura M, Tsuzuki T, Tsunoda S, Sakaki T, *et al*. Drug resistance and apoptosis in ENU-induced rat brain tumor treated with anti-cancer drugs. J Neuro-Oncol 1998;36(2):105-12.
50. Li RJ, Qiu SD, Wang HX, Tian H, Wang LR, Huo YW. Androgen receptor: a new player associated with apoptosis and proliferation of pancreatic beta-cell in type 1 *Diabetes mellitus*. Apoptosis 2008;13(8):959-71.
51. Liu R, Takayama S, Zheng Y, Froesch B, Chen G, Zhang X, *et al*. Acid-induced apoptosis in cancer cells receptor and its inhibition of retinoic interaction of bag-1 with retinoic acid. J Biol Chem 1998;273:16985-92.
52. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 2009;11(3):1-12.
53. Bailey ST, Shin H, Westerling T, Liu XS, Brown M. Estrogen receptor prevents p53-dependent apoptosis in breast cancer. Proc Natl Acad Sci 2012;109(44):18060-05.
54. Wong RSY. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 2011;30(87):1-14.
55. Freshney RI. Culture of Animal Cells, a Manual of Basic Technique. 4th ed. New York: Wiley; 2000. p. 215–7.
56. Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blaauboer BJ, Boobis A, *et al*. Methods of *in vitro* Toxicology. Food Chem Toxicol 2002;40:193-236.
57. ISO 10993-5. International Standard for Biological Evaluation of Medical Devices-Part 5. Tests for *in vitro* cytotoxicity; 2009.
58. Rieser MJ, Gu ZN, Fang X, Zeng L, Wood KV, Mclaughlin JL. Five novel mono-tetrahydrofuran ring acetogenins from the seeds of *Annona muricata*. J Nat Prod 1996:59(2):100-8.
59. Vasilevsky SF, Govdi AI, Sorokina IV, Tolstikova TG, Baev DS, Tolstikov GA, *et al*. Rapid access to new bioconjugates of betulonic acid via click chemistry. Bioorg Med Chem Lett 2011;21(1):62-5.