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CYTOTOXIC ACTIVITY OF PRIMULASAPONIN ISOLATED FROM *Jacquinia macrocarpa* Cav. *spp. pungens* (A. Gray) B. Ståhl

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Abstract

Primulasaponin was isolated from the methanolic extract of the fruit shells of *Jacquinia macrocarpa* Cav. *spp. pungens*, and characterized by nuclear magnetic resonance (NMR) and mass spectrometry techniques. The evaluation of the cytotoxic activity was performed by the MTT cell proliferation assay using the cancer cell lines A549, LS180 and HeLa, and the non-cancerous cell line ARPE-19, obtaining the IC_{50} of 13.6 µM, 9.3 µM, 23.5 µM, and 22.2 µM respectively. This is the first report of primulasaponin in the genus *Jacquinia* and its cytotoxic activity in cancer cell lines.

Keywords: *Jacquinia macrocarpa, Saponin, Cytotoxic activity, Primulasaponin*

Introduction

Cancer is one of the main health problems worldwide; the prognosis for 2030 is about 13 million deaths due to this disease. Although there have been great advances in modern medicine, it is essential to continue researching substances with anticancer potential (1). Natural products were our first source of medicines; there are records between 3000 and 6000 years old, which describe the use of natural products for pharmaceutical preparation. At present, despite the development of science and technology, natural products continue to be an important source of bioactive compounds for the development of phytopharmaceutical products for different fields of medicine (2,3). *Jacquinia macrocarpa* Cav. *spp. pungens* (A. Gray) B. Ståhl (*J. macrocarpa*) of the family Theophrastaceae, order of the Ericales, is distributed between Mexico and Panama (4). In the northwest of Mexico, this plant is used as a medicinal remedy, mainly by the Mayo ethnic group, for different types of ailments and the strengthening of the heart (5). In exploratory studies conducted by our research team, cytotoxic activity was observed in the methanolic extract of the fruit shells from *J. macrocarpa* in different cell lines (6) , consequently, a bioassay-guided study was carried out to investigate the compounds responsible for the cytotoxic activity in *J. macrocarpa.*

Methods

Plant Material

The vegetal sample was collected at San Carlos Nuevo Guaymas region in the state of Sonora, Mexico. On a beach known as Los Algodones (27˚ 57'47.15 N, 111˚ 6'3.574 W), the sample was identified by Ing. José Jesús Sánchez Escalante, president of the herbarium of the University of Sonora, with a number of Voucher USON22681.

Isolation of Primulasaponin

Only the shells of the green fruit were used, the sample was dried at room temperature and then grounded in wiley 200 mesh type mill. The pulverized sample was macerated in 100% methanol (MeOH) (1:10 w/v) for 10 days and subsequently filtered, the resulting filtrate was concentrated at 40 ˚C and reduced pressure. The MeOH extract was dissolved in MeOH:H_2O (6:4) for the solvent partition at increasing polarity, where hexane (Hx), dichloromethane (DCM) and ethyl acetate (EtOAc) were used respectively. From the polar partition, a chromatographic column (4.5 cm × 45 cm) was made with 200 – 400 mesh silica gel (Sigma – Aldrich ™), and eluted with a gradient of EtOAc:MeOH from 100:0 to 5:95. Primulasaponin was acquired in the gradient of 30:70. The monitoring of the samples, fractions and sub-fractions were carried out by thin layer chromatography (TLC) (Sigma – Aldrich ™) 5 cm × 10 cm using the mobile phase of EtOAc:MeOH (4:6) and phosphoric vanillin to reveal the TLC.

Identification of Primulasaponin

For the determination of molecular weight, high-performance liquid chromatography equipment (Agilent Technologies) equipped with a Synergi 4µ Polar-RP 80Å 150 × 2.0 mm column and an Ion Trap mass spectrometer (HPLC-MS) (Bruker Esquire 6000) with electrospray ionization source was used. The analysis was carried out in positive mode (ESI^+) drying temperature at 300 ˚C, and a nebulization flow of 7 L/min. For the structural elucidation, using CD_3OD as solvent of the sample, NMR techniques of ^1H, ^{13}C, HSQC, HMBC and COSY were implemented, the experiments were carried out in Bruker ascend console Avance III of 700MHz.

Cell Culture

The cancer cell lines A549 (human alveolar carcinoma), HeLa (human cervical carcinoma) and LS180 (human colon cancer) and non-cancerous cell line ARPE-19 (human retina) were used. Cell culture was carried out in Dulbecco's Modified Eagle's Medium (DMEM) culture medium (Sigma – Aldrich™) supplemented with 5% bovine fetal serum (Gibco®), at 37 ˚C in an incubator (Thermo Fisher Scientific, USA) with a 5% CO_2 atmosphere. The manipulation of the cell cultures was carried out in a type II biosafety cabinet (LABCONCO®) equipped with HEPA filters.

Cytotoxic Activity

For the evaluation of the cytotoxic activity, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma – Aldrich™) was used. Primulasaponin was dissolved in dimethylsulfoxide (DMSO) (Sigma – Aldrich™) and

subsequently resuspended in DMEM, obtaining a final maximum concentration of 0.25% DMSO. In a 96 well ELISA plate (Costar, Corning, N.Y. USA), 50 µL per well of a cell suspension of 200,000 cells/mL was distributed and incubated for 24 h. Later, 50 µL of the primulasaponin solutions prepared previously was added to each well. Subsequently, at the end of 48 h of incubation, the plate was washed with a phosphate buffer solution 1X (PBS 1X), and then 100 µL of DMEM and 10 µL of MTT solution at a concentration of 5 mg/mL in PBS 1X were added per well. The plate was left for 4 h under culture conditions. Once the incubation time was over, the formazan crystals were dissolved with 100 µL acidified isopropanol. The analysis was carried out in a microplate reader (Benchmark microplate reader, Bio-Rad, Hercules CA. USA) at a wavelength of 570 nm and another of 630 nm as a reference. DMSO concentrations of 0.01 – 0.25 % were used as vehicle control (2).

Results and Discussion

In exploratory tests, it was found that the MeOH extract from the fruit shells of *J. macrocarpa* has cytotoxic activity in the A549 cell line (6). In this investigation, extractions were first carried out with Hx, DCM, EtOAc and MeOH from the aerial parts and fruit shells of *J. macrocarpa*. It is the MeOH extract of the fruit shells that has the highest cytotoxic activity of the extracts with an IC_{50} of 33.1 µg/mL. Proceeding to perform a bioassay-guided study choosing the fractions with the highest cytotoxic activity of the MeOH extract until the isolation of any compound. Once the cytotoxic activity of the MeOH extract from the fruit shells was confirmed, a solvent partition was performed as described in the materials and methods section, together with MTT tests to determine the partition with the highest cytotoxic activity, where the polar partition was chosen for its IC_{50} of 28.5 µg/mL. Subsequently, this partition was fractionated on a chromatographic column, where the fraction obtained at 30:70 (EtOAC:MeOH) has the highest cytotoxic activity with an IC_{50} of 15 µg/mL, this fraction corresponds to primulasaponin ($C_{54}H_{88}O_{23}$) ((3β, 13α, 16α, 17α)-16-hydroxy-13,28-epoxyoleanan-3-yl 6-deoxy-α-L-mannopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranosiduronic acid), with molecular weight of 1104 g/mol (Figure 1) identified by NMR (Table 1). This compound was previously described in the species *Primula sp.*(7), belonging, like *J. macrocarpa*, to the order of the Ericales. There are small differences between some chemical shifts described by *Siems et al*. (7), this can be attributed mainly to the fact that in this investigation a frequency of 700 MHz was used in the NMR, obtaining a higher resolution that described by Siems et al., where the reported data corresponds to an experiment with a frequency of 400 MHz. However, there are no previous reports about its presence in the genus *Jacquinia* and neither about its cytotoxic activity in cancer cell lines.

One of the most known biological activities of saponins is about their ability to alter the permeability of membranes (8). However, saponins have been attributed different types of biological activities, such as they are; hypoglycemic activity, anti-hypercholesterolemic, antiviral, antibacterial, antifungal, anti-inflammatory, antiproliferative, antitumor, antineoplastic, among others; exercising their activities, not only through the modification in the permeability of cell membranes but also through a variety of different types of mechanisms of action (9–17).

In addition to primulasaponin, another saponin with biological activity isolated from the genus *Jacquinia* is sakurasaponin. The only structural difference between these two saponins in that sakurasaponin has an additional rhamnose to primulasaponin in its carbohydrate moieties chain. However, sakurasaponin was isolated through bioassay-guided of antifungal activity, leaving primulasaponin as the only isolated saponin of the genus *Jacquinia* with cytotoxic activity in cancer cell lines (18).

Firstly, cytotoxic activity assays were evaluated in the cancer cell lines A549, LS180 and HeLa as *in-vitro* representatives of some of the most common cancer in humans (1), and non-cancerous cell line ARPE-19 as a reference and to work on an experimental panel of only human cell lines. The results of cytotoxic activity were satisfactory, with a statistically significant difference to the IC_{50} of ARPE-19, primulasaponin shows a higher cytotoxic activity in LS180 and then in A549 (Table 2), for HeLa and ARPE-19 there is no statistically significant

difference in their IC_{50}, it can be suggested the presence of some signaling pathway, which is more active first on LS180 and then on A549, or perhaps totally different between them, where primulasaponin produces its effect. Microscopic observation at 24 h in LS180 and A549 show membrane blebs, apoptotic bodies and cell shrinkage and rounding (Figure 2) (2), probably induced through a mechanism of apoptosis, making it a molecule with anticancer potential. However, this evidence is not enough to determine the type of cell death induced by primulasaponin**.**

Another saponin with an oleanolic triterpenic structure having cytotoxic activity in cancer cells *in-vitro* is; 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl oleanolic acid, which has an IC_{50} of 9.9 µM and 15.5 µM for A549 and HeLa respectively (19). In this investigation, primulasaponin showed the same tendency of cytotoxic activity in A549 and HeLa, with 13.6 µM and 23.5 µM respectively. *Wang et al.*(19) attribute the cytotoxic activity of saponins to the presence of the free carboxyl group in C-28. However, the difference between primulasaponin and 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl oleanolic acid (19), is that primulasaponin lacks the carboxyl group in C-28 and the double bond between C-12 and C-13, forming in turn an ether group between the carbonyl of C-28 and C-13 and an additional hidroxil group in C-16 (figure 1). It has also been observed that saponins with a carbohydrate residue in the carboxyl group of C-28 also have cytotoxic activity, but to a lesser extent (19). This suggests that, the functional group of C-28 is a pharmacophore in the cytotoxic activity of saponins with oleanolic triterpenic structure.

Conclusion

Using the bioassay-guided method, it was isolated from *J. macrocarpa* and identified by spectrometric techniques, primulasaponin, a saponin with cytotoxic activity in human cancer cells *in-vitro* by MTT cell proliferation assay, mainly in the cell line LS180. It is recommended to continue with the investigation of primulasaponin to determine the type of cell death that induces and its mechanism action in LS180 and other colorectal cancer cell lines. As well as the influence of the functional groups of C-28 on the cytotoxic activity of saponins.

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References

1. Cronin KA, Lake AJ, Scott S, Sherman RL, Noone AM, Howlader N, et al. Annual Report to the Nation on the Status of Cancer, part I: National cancer statistics. Cancer. 2018;
2. Torres-Moreno H, Velázquez CA, Garibay-Escobar A, Curini M, Marcotullio MC, Robles-Zepeda RE. Antiproliferative and apoptosis induction of cucurbitacin-type triterpenes from Ibervillea sonorae. Ind Crops Prod [Internet]. 2015;77:895–900. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0926669015304210
3. Halberstein R a. Medicinal plants: Historical and cross-cultural usage patterns. Ann Epidemiol. 2005;15(9):686–99.
4. Källersjö M, Ståhl B. Phylogeny of Theophrastaceae (Ericales s. lat.). Int J Plant Sci. 2003;
5. YETMAN D, VAN DEVENDER TR. Mayo Ethnobotany [Internet]. 1st ed. University of California Press; 2002. Available from: http://www.jstor.org/stable/10.1525/j.ctt1pntcs
6. Vidal Gutiérrez M. Actividad Antioxidante y Antiproliferativa de Seis Plantas Medicinales Sonorenses. Universidad de Sonora; 2014.
7. Siems K, Jaensch M, Jakupovic J. Structures of the two saponins isolated from commercially available root extract of Primula sp. Planta Med. 1998;
8. Augustin JM, Kuzina V, Andersen SB, Bak S. Molecular activities, biosynthesis and evolution of triterpenoid saponins. Phytochemistry. 2011.
9. Zhao YC, Xue CH, Zhang TT, Wang YM.

Saponins from Sea Cucumber and Their Biological Activities. Journal of Agricultural and Food Chemistry. 2018.

10. Cui C, Zong J, Sun Y, Zhang L, Ho CT, Wan X, et al. Triterpenoid saponins from the genus Camellia: Structures, biological activities, and molecular simulation for structure-activity relationship. Food and Function. 2018.

11. Yu J, Li G, Mu Y, Zhou H, Wang X, Yang P. Anti-breast cancer triterpenoid saponins from the thorns of Gleditsia sinensis. Nat Prod Res. 2019;

12. Tam DNH, Truong DH, Nguyen TTH, Quynh LN, Tran L, Nguyen HD, et al. Ginsenoside Rh1: A Systematic Review of Its Pharmacological Properties. Planta Medica. 2018.

13. Hu Q, Chen YY, Jiao QY, Khan A, Li F, Han DF, et al. Triterpenoid saponins from the pulp of Sapindus mukorossi and their antifungal activities. Phytochemistry. 2018;

14. Ur Rahman S, Ismail M, Khurram M, Ullah I, Rabbi F, Iriti M. Bioactive Steroids and Saponins of the Genus Trillium. Molecules (Basel, Switzerland). 2017.

15. Wu HB, Liu TT, Wang WS, Feng JC, Tian HM. Oleanane-type saponins from the roots of ligulariopsis shichuana and their α-glucosidase inhibitory activities. Molecules. 2017;

16. Osbourn A, Goss RJM, Field RA. The saponins-polar isoprenoids with important and diverse biological activities. Natural Product Reports. 2011.

17. Lan X, Deng K, Zhao J, Chen Y, Xin X, Liu Y, et al. New Triterpenoid Saponins from Green Vegetable Soya Beans and Their Anti-Inflammatory Activities. J Agric Food Chem. 2017;

18. García-Sosa K, Sánchez-Medina a, Álvarez SL, Zacchino S, Veitch NC, Simá-Polanco P, et al. Antifungal activity of sakurasosaponin from the root extract of Jacquinia flammea. Nat Prod Res. 2011;25(May 2014):1185–9.

19. Wang X, Wang M, Xu M, Wang Y, Tang H, Sun X. Cytotoxic oleanane-type triterpenoid saponins from the rhizomes of Anemone rivularis var. flore-minore. Molecules. 2014

Table 1. Spectroscopic data of primulasaponin [a, b]

C n°	^{13}C	^{1}H (m, *J* in Hz)	C n°	^{13}C	^{1}H (m, *J* in Hz)
1	40.2	1.72(m) 0.98(m)	28	78.68	3.48(d, 7.5) 3.11(d, 7.5)
2	27.0	2.01(m) 1.74(m)	29	33.93	0.94 (s)
3	92.0	3.19(dd, 4.65, 11.5)	30	24.98	0.9(s)
4	40.7		GlcA-1	105.44	4.44(d, 7.9)
5	56.8	0.73(dd, 2.3, 11.9)	GlcA-2	79.27	3.9 (dd, 7.9, 8.9)
6	18.73	1.48(m) 1.43(m)	GlcA-3	81.08	4.05 (t, 9.9)
7	35.18	1.53(m) 1.22(m)	GlcA-4	72.1	3.58 (d, 9.3)
8	43.2		GlcA-5	76.87	3.63 (d, 9.3)
9	51.4	1.26(m)	GlcA-6	175.93	
10	37.8		Gal-1	100.79	5.18 (d, 7.7)
11	19.81	1.6(qd, 12.9, 12.8, 12.8, 4.6) 1.45(m)	Gal-2	75.88	3.78 (m)
12	33.3	2.03(m) 1.28(dd, 3.8, 6.3)	Gal-3	76.1	3.72 (m)
13	88.3		Gal-4	72.98	3.7 (m)
14	45.2		Gal-5	76.87	3.52 (m)
15	37.3	2.09(m) 1.23(m)	Gal-6	62.74	3.8 (d, 2.2) 3.65 (dd, 7.3, 4.7)
16	78.0	3.87(m)	Rha-1	101.99	5.27 (d, 1.6)
17	45.3		Rha-2	72.63	3.94 (dd, 1.61, 3.4)
18	52.4	1.5(m)	Rha-3	72.25	3.7 (m)
19	39.7	2.37(dd, 11.9, 14.5) 1.18(d, 5.3)	Rha-4	73.72	3.4 (d, 9.5)
20	32.4		Rha-5	70.2	4.1 (dq, 5.8, 6, 6, 9.5)
21	37.0	2.08(m) 1.88(m)	Rha-6	17.93	1.25 (d, 2.1)
22	32.14	1.77(m) 1.5(m)	Glu-1	102.5	4.86 (d, 8.3)
23	28.2	1.05(s)	Glu-2	76.15	3.22 (dd, 7.6, 9.5)
24	16.7	0.86(s)	Glu-3	77.88	3.33 (t, 9.2, 9.2)
25	16.81	0.88(s)	Glu-4	72.63	3.05 (dd, 8.9, 9.8)
26	18.8	1.14(s)	Glu-5	78.13	3.38 (m)
27	19.9	1.22(s)	Glu-6	63.51	3.61 (dd, 2.7, 4.1) 3.53 (dd, 3.7, 7.7)

[a] The chemical shifts are expressed in δ values (ppm). TMS was used as internal reference
[b] Spectra recorded in CD_3OD

Table 2. Cytotoxic activity of primulasaponin.

Compound	Cell lines*			
	A549	HeLa	LS180	ARPE19
Primulasaponin	13.6 ± 1.5[b]	23.5 ± 1.0[c]	9.3 ± 0.8[a]	22.2 ± 2.1[c]
Doxorubicin**	8.3 ± 0.2	5.6 ± 0.2	4.7 ± 0.3	7.8 ± 0.3

* IC_{50} values represent a mean and standard derivation (μM ± SD; *n*=3) of three independent experiments.
**Doxorubicin was used as a positive control.
[a, b, c] Statically significant differences ($p < 0.05$); one-way ANOVA with post hoc Tukey test.

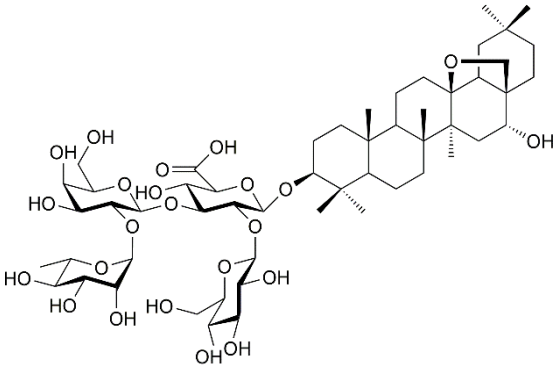

Figure 1. Structure of primulasaponin

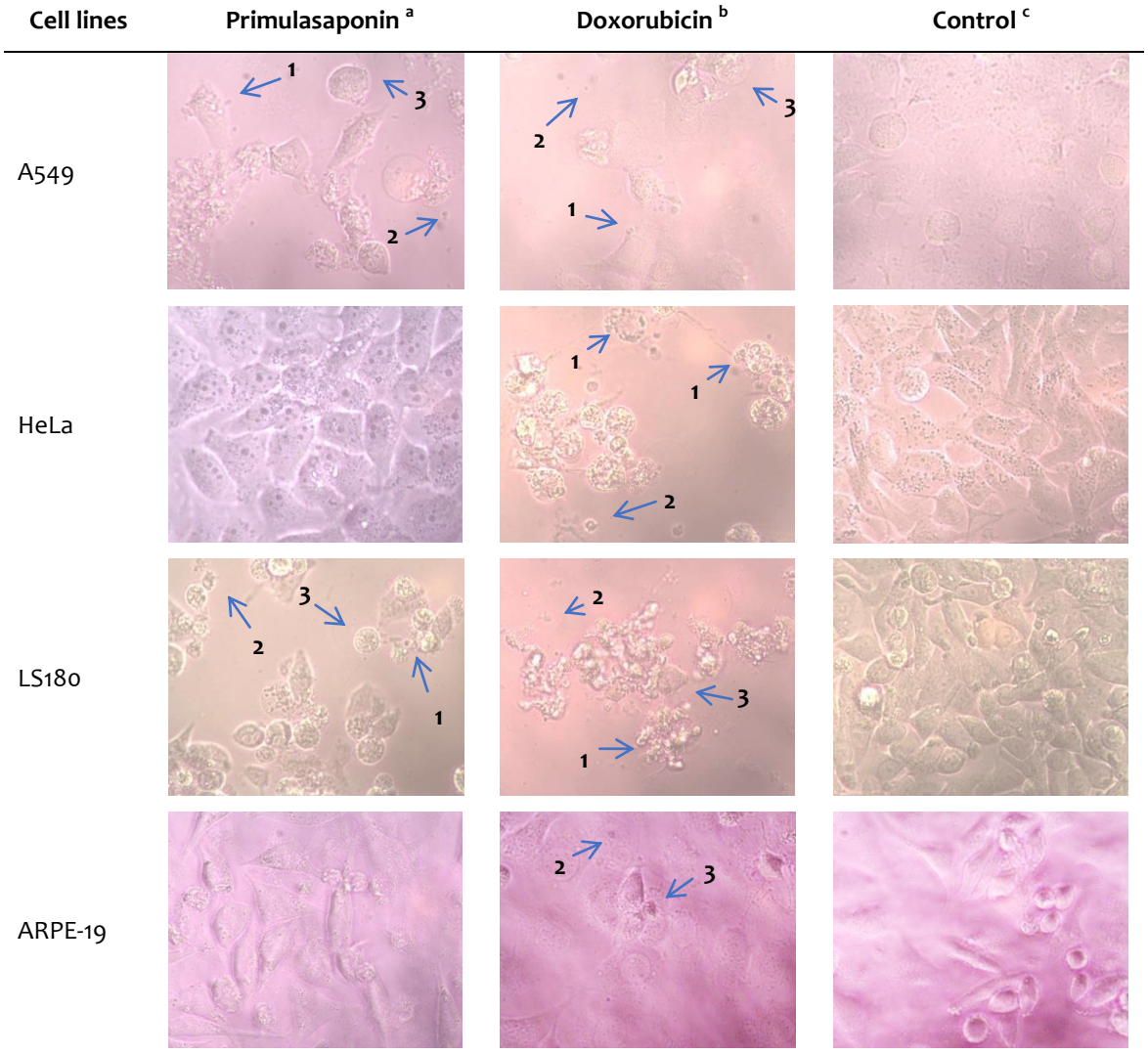

Figure 2. Morphological changes of primulasaponin in cell lines at 24 h.* 40X magnification; (a) Primulasaponin at 11.3 µM; (b) Doxorubicin at 23.9 µM for A549 and ARPE-19, and 11.9 µM for HeLa and LS180; (c) DMSO at 0.25%; (1) Membrane blebs; (2) Apoptotic bodies; (3) Shrinkage and rounding.