

Cytotoxic activity of crude extracts and fractions from *Blepharocalyx cruckshanksii* against selected human cancer cell lines

[Actividad citotóxica de extractos crudos y fracciones de *Blepharocalyx cruckshanksii* contra líneas celulares de cáncer humano seleccionadas]

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Abstract: The present study aims to explore the potential applications of *Blepharocalyx cruckshanksii* bark and leaf extracts as a cytotoxic agent against *in vitro* cancer cell lines (MCF-7, PC-3 and HT-29) by using sulforhodamine B (SRB) assay. The cytotoxicity assay revealed that the ethyl acetate extract from the bark exhibited marked anticancer activity. The active extract was subjected to liquid-liquid partitioning by using hexane and ethyl acetate to obtain fractions based on their polarity. However, Fraction 4 (F4) was identified as the most effective of the series by displaying against all cancer cell lines a cytotoxicity close to antineoplastic agents assayed. Then, F4 was analyzed by gas chromatography–mass spectrometry (GC-MS) to identify their major components and to relate these components to the cytotoxic effect. The results obtained indicated that *B. cruckshanksii* bark have excellent cytotoxic activity and warrant further studies to isolate novel compounds for chemotherapeutic use.

Keywords: *Blepharocalyx cruckshanksii*; Bark; Cancer cell; Gas chromatography

Resumen: El presente estudio tiene como objetivo explorar las posibles aplicaciones de los extractos de corteza y hoja de *Blepharocalyx cruckshanksii* como agente citotóxico contra líneas celulares de cáncer *in vitro* (MCF-7, PC-3 y HT-29) mediante el uso de ensayo de sulforhodamine B (SRB). El ensayo de citotoxicidad reveló que el extracto de acetato de etilo de la corteza exhibía una actividad anticancerígena marcada. El extracto activo se sometió a un reparto líquido-líquido usando hexano y acetato de etilo para obtener fracciones basadas en su polaridad. Sin embargo, la Fracción 4 (F4) fue identificado como el más efectivo de la serie al mostrar contra todas las líneas celulares de cáncer una citotoxicidad cercana a los agentes antineoplásicos ensayados. Luego, F4 se analizó por cromatografía de gases-espectrometría de masas (GC-MS) para identificar sus componentes principales y relacionar estos componentes con el efecto citotóxico. Los resultados obtenidos indicaron que la corteza de *B. cruckshanksii* tiene una excelente actividad citotóxica y amerita estudios adicionales para aislar nuevos compuestos para quimioterapia.

Palabras clave: *Blepharocalyx cruckshanksii*; Corteza; Celulas de cáncer; Cromatografía de gases

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INTRODUCTION

Cancer is one of the most persistent health problems and is considered as a major threat to humankind (Tantengco & Jacinto, 2015). According to the World Health Organization, cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to cancer (WHO, 2019). Nowadays, chemotherapy is the main treatment with drugs that can destroy cancer cells, however, the drawback of these anticancer drugs is presented as they are highly toxic with low treatment effectiveness and significant reduction in the quality of life of the cancer patients (Nguyen & Scarlett, 2019). Currently, there is a considerable scientific and commercial interest in continuing the discovery of new anticancer agents from natural products with the strategy of chemotherapy protocols being killing the cancer cells with no toxic effect on the host (Elufioye *et al.*, 2017). Natural products are well recognized as source of cytotoxic molecules while several others can decrease the resistance of cancer cells to chemotherapeutic drugs, improve the efficiency of chemotherapeutic agents, and reduce the adverse side effects experienced due to chemotherapy (Elufioye *et al.*, 2017; Mbaveng *et al.*, 2019). However, the genus *Blepharocalyx* have been explored for various activities based on traditional uses. The active metabolites isolated from *Blepharocalyx salicifolius* (Kunth) O. Berg showed anti-cancer potential (Siqueira *et al.*, 2011). In other study the essential oil of *B. salicifolius* showed significant activity against breast cancer cell line MDA-MB-231 (46.60 µg/mL) and revealed to have induced cell death through apoptosis (Furtado *et al.*, 2018).

Blepharocalyx cruckshanksii (Hook. & Arn.) Nied. is native to the Chile and is locally known as temu. Leaves of *B. cruckshanksii* are used as astringent and vulnerary (Gusinde, 1936). Water decoction of its bark is used to treat diarrhea (Massardo & Rozzi, 1996). Compounds isolated from bioactive fractions of *B. cruckshanksii* were observed to have antimycobacterial activity (Massardo & Rozzi, 1996). Other ethnomedicinal uses of *B. cruckshanksii* are remedies for rheumatism and wounds (Gusinde, 1936; Massardo & Rozzi, 1996).

Therefore, the present study was carried out to explore the anticancer activity of extracts and fractions from *B. cruckshanksii* bark and leaves on a

panel of three human cancer cell lines: MCF-7 (breast), PC-3 (prostate) and HT-29 (colon), and two human non-tumoral cell lines, HDF (Human dermal fibroblasts) and CoN (colon epithelial).

MATERIALS AND METHODS

Plant Material

Leaves and bark of *B. cruckshanksii* were collected in the locality Laguna Verde, Valparaíso, Chile (33°03'04" S, 71°39'34" O), in the morning (10 am) of October 17, 2018. A voucher specimen was deposited in the Natural Products Laboratory, University of Playa Ancha (Valparaíso, Chile), under the BC2018-17.10. The identity of the species was validated by Patricio Novoa, Forestal engineer and expert in botany.

Extraction of crude extracts

The bark (1.00 Kg) and leaves (1.00 kg) were air dried at room temperature after collection. The dried samples were crushed by using a blender. Dried and crushed samples were subjected to successive extractions using different solvents of increasing polarity (n-hexane, dichloromethane, ethyl acetate and ethanol) like a procedure reported in a previous study (Mellado *et al.*, 2019). All the obtained extracts were concentrated in a rotary evaporator at 40 °C, and then each extract was stored at room temperature in the dark.

Fractionation of active extract

The bark ethyl acetate extract (4.00 g) was fractionated by column chromatography on silica gel using n-hexane–ethyl acetate (100:0 to 0:100, v/v) to obtain one hundred and fifty fractions of 50 mL each were collected and combined on the basis of their thin layer chromatography (TLC) profiles to afford seven main fractions. Fractions 1–22, 23–45, 46–66, 67–90, 91–118, 119–138, and 139–150 were referred to as F1, F2, F3, F4, F5, F6 and F7 respectively. These fractions were tested for their cytotoxicity activity and the most active fraction was subjected to gas chromatography–mass spectrometry (GC-MS) in order to identify the active principles.

Identification of active fraction

F4 was diluted with ethyl acetate, and analysis was performed by GC/MS using the instrumentation described elsewhere (Canales *et al.*, 2016).

Cell Viability Assay

The sulforhodamine B assay was assessed by the procedure previously described in reference (Madrid *et al.*, 2011). Daunorubicin and dunnione were used as positive controls. Results were analyzed using MS Excel and Sigmaplot 12.5 software.

Statistical analysis

Determinations of IC₅₀ were performed in triplicate

and the results are expressed as mean values \pm SD. The results were analyzed by the standard method (Madrid *et al.*, 2011).

RESULTS AND DISCUSSION**Yield of crude extracts**

The yield of the crude n-hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and ethanol (EtOH) extracts from leaves and bark of *B. cruckshanksii* are summarized in Table No. 1.

Table No. 1
Extraction yield of crude extracts

	Hex	DCM	EtOAc	EtOH
	Yield (% w/w)			
Leaves	1.51	1.33	6.04	4.7
Bark	1.17	1.03	1.50	1.32

The highest yields were achieved with ethanol 12.62% (w/w), followed by hexane 5.49% (w/w), and dichloromethane 4.76% (w/w), while the lowest was ethyl acetate 3.55% (w/w).

Chemical Composition of F4 from bark AcOEt extract

Results of the gas chromatographic analysis of F4 from bark ethyl acetate extract of *B. cruckshanksii* are summarized in Table No. 2.

Table No. 2
Main components of F4

No.	Main Components	% Area ^a	RI ^b	RIref ^c	Identification
1	α -pinene	0.67	941	941	RI, MS
2	camphene	0.81	953	952	RI, MS
3	1-octen-3-ol	0.57	979	978	RI, MS
4	β -pinene	0.84	985	985	RI, MS
5	myrcene	0.91	994	994	RI, MS
6	limonene	2.01	1038	1039	RI, MS
7	<i>E</i> - β -ocimene	0.34	1049	1050	RI, MS
8	methyl eugenol	4.85	1400	1401	RI, MS
9	aromadendrene	29.05	1439	1439	RI, MS
10	cis-calamenene	1.35	1531	1531	RI, MS
11	Caryophyllene oxide	0.33	1581	1581	RI, MS
12	syringaldehyde	9.93	1655	1656	RI, MS, Co-I
13	Patchouli alcohol	3.28	1632	1663	RI, MS, Co-I
14	Loliolide	2.36	1698	1698	RI, MS
15	neophytadiene	0.68	1837	1839	RI, MS
16	curcumenone	5.45	1845	1844	RI, MS
17	cyclopentadecanol	0.57	1987	1988	RI, MS
18	hexacosane	1.58	2600	2600	RI, MS
19	octacosane	0.72	2800	2800	RI, MS
20	nonacosane	1.09	2900	2900	RI, MS
21	quercetin	6.55	3174	3176	RI, MS, Co-I

^a Surface area of GC peak; ^b RI: Retention indices relative to C₈-C₃₆ *n*-alkanes on the Rtx-5MS capillary column

^c RIref: Retention indices reported in literature.

Twenty-one components were identified, representing 73.94% of the total F4 and 26.06% were unknown compounds. The F4 was mainly characterized by aromadendrene (29.05%), syringaldehyde (9.93%), quercetin (6.55%), curcumenone (5.45%), methyl eugenol (4.85%), and Patchouli alcohol (3.28%). In addition, this is the first report made on the composition of bark from *B. cruckshanksii* in which terpenes were the predominant portion.

Cytotoxic activity

The cytotoxic activity of *B. cruckshanksii* extracts was evaluated using a colorimetric assay, in vitro against three different cancer cell lines and two non-tumour cell line of human cells. The half-maximal inhibitory concentration (IC₅₀) values of the most active extract and their respective fractions are summarized in Table No. 3. The IC₅₀ values of the remaining extracts they were not shown because they were not significantly active.

Table No. 3
Cytotoxic activity of the bark ethyl acetate extract and fractions from *B. cruckshanksii*.

Cell	IC ₅₀ (µg/mL)									
	Crude*	F1	F2	F3	F4	F5	F6	F7	Dau ^a	Dox ^b
MCF-7	12.4±0.12	>50.0	44.2±0.3	>50.0	18.6±0.4	48.2±0.5	>50.0	>50.0	0.89±0.21	1.19±0.51
HT-29	10.0±0.01	>50.0	39.5±0.1	>50.0	13.9±0.1	37.2±0.1	>50.0	>50.0	14.9 ± 0.3	0.69±0.33
PC-3	9.8±0.11	>50.0	38.4±0.3	41.9±0.2	7.4±0.1	38.7±0.3	>50.0	>50.0	2.75±0.20	2.78±0.11
CON	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	5.5±0.30	5.61±0.31
HDF	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	14.5±0.11	10.3±0.23

*Crude: bark ethyl acetate extract; ^a: Daunorubicin; ^b: Doxorubicin

Among the extracts tested, the results revealed that the ethyl acetate extract and fraction F4 showed the highest cytotoxic activity against all cancer cell lines, without affecting non-tumoral cells. However, due to the activity shown by the F4, it was subjected to GC-MS to identify the components in the fraction responsible for the cytotoxic activity observed in the study. Aromadendrene and patchouli alcohol (PA) were the main compounds in the fraction; these terpenes are found in many essential oils, and these compounds show a wide spectrum of action involving antitumor, antiviral, antibacterial and cytotoxic activity (Oliveira *et al.*, 2015; Pavithra *et al.*, 2018). In addition, PA exert an anti-cancer activity via a decrease of cell growth and an increase of apoptosis in human cancer cells (Jeong *et al.*, 2013). Moreover, syringaldehyde has been described for the treatment of cancer from a palliative point of view as a preventive one, due to its mass presence of fruits, vegetables and cereals of mass consumption (Colaric *et al.*, 2005). Furthermore, there is quercetin in the fraction, which is an important member of flavonoids and is considered as attractive candidate for cancer treatment and prevention (Srivastava *et al.*, 2016). It has also quercetin has been studied extensively as a chemoprevention agent in several cancer models (Jeong *et al.*, 2009). On the other hand, curcumenone isolated from the rhizomes of *Curcuma zedoaria* has been reported to induce

apoptosis in MCF-7 cells by inhibiting the proliferation of the cancer cells (Ahmed-Hamdi *et al.*, 2014). In addition, the presence in the extract of methyleugenol (ME) is a substituted alkenylbenzene found in a variety of foods, products, and essential oils, and ME is a potent anticancer agent in RB355 human retinoblastoma cells (Ahmed-Hamdi *et al.*, 2014), justifies the potential antiproliferative activity of this extract or fraction on different cancer cell lines. Interestingly, F4 presented a cytotoxicity close to the positive controls used in the study, with an activity superior to daunorubicin in HT-29, see Table No. 2.

CONCLUSIONS

The ethyl acetate extract from the bark of *B. cruckshanksii* exhibited promising anticancer activity and is therefore and can be a potential source of molecules to be exploited in medicine or by the pharmaceutical industry for the fight against cancer.

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