International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2017.286.291

Research Article
Effects of Polyalthic Acid from *Croton reflexifolius* on Viability of Cancerous Cells

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Abstract

Background and Objective: *Croton reflexifolius* has been used in traditional medicine for treating cancer. Although one study reported cytotoxic activity, the active compounds have not been identified. Therefore, the purpose of this study was to isolate and identify at least one cytotoxic compound from *C. reflexifolius* through a bioassay-guided study using A549 cell line. **Methodology:** The cytotoxic effect was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **Results:** The current study indicates that the leaves of this plant contain more than one compound with the capacity of decreasing the viability of cancerous cells. From the hexane extract was isolated polyalthic acid, resulting in an IC_{50} value of 34.90±0.72 μg mL^{-1}. It also demonstrated cytotoxic activity on HCC827, MCF7, MDA-MB-231, HeLa and Colo 205 cancer cell lines (IC_{50} values = 15.9-45.9 μg mL^{-1}). Regarding A549 versus normal cells, polyalthic acid showed greater selectivity for growth inhibition but was less potent than cisplatin, the standard drug for chemotherapy. **Conclusion:** By demonstrating that polyalthic acid has the capacity to inhibit the viability of cancerous cells, these results support the traditional use of *C. reflexifolius* as an anticancer treatment.

Key words: *Croton reflexifolius*, polyalthic acid, anticancer treatment, non-small-cell lung cancer, cytotoxic activity, cell viability, bioassay-guided study, medicinal plants

Received: October 14, 2016 Accepted: November 26, 2016 Published: March 15, 2017

Citation: Marycruz García-Tapia, José Rubén García Sánchez, Jesús Arrieta, Ivonne María Olivares-Corichi, José Angel Santiago-Cruz, Ana Karen Ahumada-Anaya and María Elena Sánchez-Mendoza, 2017. Effects of polyalthic acid from *Croton reflexifolius* on viability of cancerous cells. Int. J. Pharmacol., 13: 286-291.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a leading cause of morbidity and mortality around the world. About 8.2 million cancer related deaths in 2012 are expected to rise by about 70% over the next 2 decades[1]. The greatest cause of cancer-related deaths worldwide is lung cancer[2], which is classified into two groups: Small cell and non-small-cell lung cancer (NSCLC). Of all lung cancer patients, NSCLC represents almost 85% of cases and has a low survival rate[3].

When lung cancer is diagnosed at an early stage, surgical resection can be effective. Nevertheless, most patients are diagnosed when they are in advanced stages and the conventional treatments such as chemotherapy have several adverse effects that reduce the quality of life[4]. This situation has led to an increased use of alternative and complementary medicine (including medicinal plants) in cancer management. Indeed, medicinal plants have played an important role in the development of drugs for the treatment of this disease[5,6].

In this context studies on the *Croton* genus have identified substances active against cancer, such as alkaloids, diterpenoids and essential oils[7-9]. In the case of *Croton reflexifolius* H.B.K., one study reported cytotoxic activity against the KB cell line (human nasopharyngeal carcinoma), but without identifying an active compound[10]. Herein, we report the isolation of one cytotoxic compound of *C. reflexifolius* H.B.K., through a bioassay-guided study using the A549 cell line (from NSCLC), in addition reported the activity of this compound in others cell cancer lines.

MATERIALS AND METHODS

Plant material: The leaves of *C. reflexifolius* H.B.K., were collected at Tehuetlan, in the state of Hidalgo, Mexico in September, 2014. A voucher specimen can be found in the Herbarium of the División de Ciencias Forestales at the Universidad Autónoma Chapingo (CHAP60955).

Preparation of extracts and isolation: After cleaning the leaves of *C. reflexifolius* and drying them in the shade, 3 kg were successively macerated for 3 days at ambient temperature (22±2°C), first with hexane (10 L), then dichloromethane (10 L) and finally methanol (10 L). The resulting liquid was filtered and concentrated using a rotary evaporator. The procedure was repeated 3 times with each dissolvent, finally yielding 83, 95 and 144 g of the hexane, dichloromethane and methanol extracts, respectively. The hexane extract showed the most cytotoxic activity (Fig. 1).

Thus 80 g of this extract was separated by silica gel column (400 g) and eluted with hexane (1.5 L, fraction 1 (F1)), hexane/EtOAc (7:3, 1.5 L, F2) and hexane/ EtOAc (1:1, 1.5 L, F3). Although all of these fractions were found to be active (Fig. 2), we decided to work with F2 due to the greater yield. Consequently, 40 g was chromatographed on a silica gel column (360 g) by using a step gradient of hexane, involving mixtures of hexane/EtOAc, EtOAc and MeOH, to give 60 fractions of 20 mL each. Fractions 24-36 (hexane/EtOAc, 9:1) yielded a white solid (1.0 g, mp 98-100°C) that was identified as polyalthic acid (Fig. 3) by comparing ^{1}H and ^{13}C NMR spectroscopic data reported in the literature[11].

Cell cultures: The cancer cell lines used in this study were obtained from the American Type Culture Collection (ATCC), (Manassas, Virginia, USA). They consisted of A549 and HCC827 (human non-small-cell lung cancer), MCF7 and MDA-MB-231 (breast cancer cells), HeLa (cervical cancer cells) and Colo 205 (colon cancer cells). Also, it was included the Human Umbilical Vein Endothelial Cells (HUVECs) as normal cells.

The A549 and HCC827 cells were grown in DMEM/F12 media. The HeLa, MCF7 and MDA-MB-231 in DMEM high glucose. While, Colo 205 cell line in RPMI 1640 media. All the media (Gibco Lab, USA) were supplemented with 5% fetal bovine serum (FBS, Atlanta Biological), 2 mM glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Human Umbilical Vein Endothelial Cells (HUVEC) were from primary culture. The HUVEC were obtained from umbilical cord according to a previously described procedure[12]. Cells were grown in M199 media with 1% endothelial cell growth supplement, heparin and 15% fetal bovine serum. The donor of umbilical cord was recruited at the Hospital Juárez de México. The Ethics and Research Committee of the Hospital Juárez de México approved the participation of the donor and a written informed consent was obtained.

Cell viability assays: The seven cell lines were seeded at 1×10^4 cells in 96-well tissue culture plates, allowed to attach and incubated for 24 h before the test. The cells were then treated with different concentrations of the *C. reflexifolius* extract (10-200 µg mL^{-1}), fractions (20-120 µg mL^{-1}), polyalthic acid (5-120 µg mL^{-1}) or cisplatin (10-70 µg mL^{-1}). The extracts, fractions and polyalthic acid were dissolved in DMSO and cisplatin was dissolved in DMEM F12. A negative control contained DMSO was also evaluated. All culture on microplates were incubated at 37°C under humid atmosphere with 5% CO_2 for 72 h, then the MTT (final concentration

1 mg mL^{-1}) was added and incubated for 2.5 h[13]. After the medium had been discarded, 150 µL of DMSO was applied to each well to dissolve the dark-blue formazan crystals in intact cells and the resulting solution was measured by spectrophotometry with the microplate reader (The synergy HT, BioTek) at a wavelength of 550 nm. Thus, the quantity of formazan produced is directly proportional to the number of living cells. The results are expressed as the percentage of viability cells in relation to the negative control, whose viability was designated as 100%.

Statistical analysis: All experiments were carried out in triplicate, in three different experiments and the results are expressed as the Mean±SEM. We utilized one-way analysis of variance (ANOVA) followed by the Tukey's test. Differences were considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

In the bioassay-guided study of *C. reflexifolius*, the first step was to evaluate the effect on cell viability of the hexane, dichloromethane and methanol extracts. The hexane extract showed the highest cytotoxic activity (Fig. 1). The maximum inhibition (almost 93%) was reached with the hexane and dichloromethane extracts at 80 and 140 µg mL^{-1}, respectively. Meanwhile, the methanol extract was only capable of obtaining 27.9% inhibition and this required 200 µg mL^{-1}. These results demonstrate that *C. reflexifolius* has cytotoxic activity on the A549 cell line and it has more than one active compound.

The three fractions obtained from hexane extract were active and their effect was concentration-dependent. They had similar efficacy (Fig. 2), but the F3 fraction was more potent (IC_{50} = 42.7±3.1 µg mL^{-1}) followed by F2 (IC_{50} = 57.1±3.8 µg mL^{-1}) and F1 (IC_{50} = 75.1±1.7 µg mL^{-1}). As can be appreciated, the hexane extract also had more than one active compound. From F2 with the highest yield, the active compound obtained was identified as polyalthic acid (Fig. 3). With this compound, there was a concentration-dependent cytotoxic effect against the A549 cell line (Fig. 4). It reached the maximum inhibitory effect (94.58%) at a concentration of 60 µg mL^{-1} (IC_{50} = 34.90±0.72 µg mL^{-1}). This shows for the first time that polyalthic acid is one of the compounds responsible for the cytotoxic activity of *C. reflexifolius* on the A549 cell line.

In a previous study, polyalthic acid was isolated from Fructus Viticis Negundo and its antiproliferative activity against the same cell line was determined by SRB method, reporting an IC_{50} of 20.3 µM[14]. This value was lower in comparison with the value obtained in the present study (IC_{50} = 110.3 µM), which was probably due to variations in experimental conditions, especially by use of the SRB method (versus the MTT assay) to assess antiproliferative activity. With adriamycin as the positive control, these researchers obtained a IC_{50} of 0.05 µM[14]. In others studies, the IC_{50} values of this drug were 1.61 and 9.20 µM, assessed by the SRB method and the MTT assay, respectively[15,16]. Thus, there have been great differences between the IC_{50} values obtained on the same cell line with distinct experimental conditions, which justifies the IC_{50} value found for polyalthic acid.

Regarding the evaluation of polyalthic acid on normal human cells (HUVEC), inhibition of survival was only 20.3% at 70 µg mL^{-1} (Fig. 5), compared to around 95% (Fig. 4) at this same concentration on the A549 cell line. This demonstrates that polyalthic acid has a selectivity for growth inhibition of the A549 than HUVEC. A previous study reported that polyalthic acid has antimutagenic activity in the Ames and the umu tests when using *Salmonella typhimurium* TA100 and TA1535/pSK1002 strains, respectively[17]. The fact that polyalthic

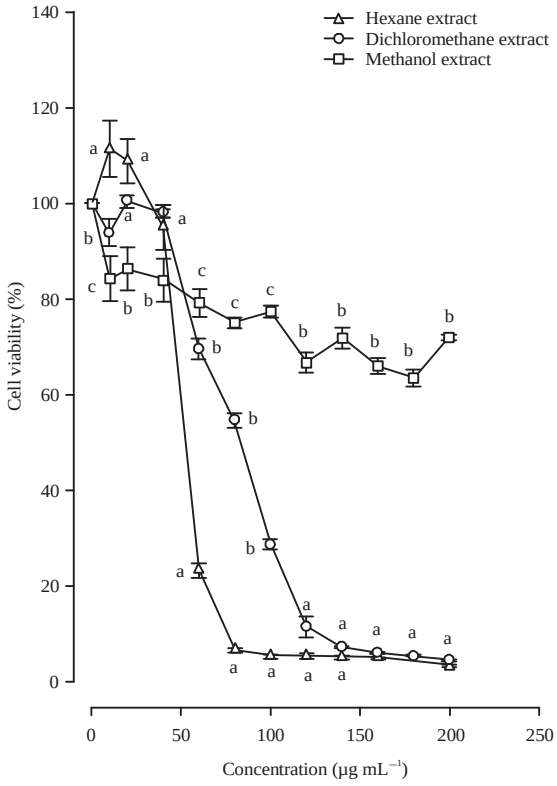

Fig. 1: Cytotoxic activity of the extracts of *Croton reflexifolius* on the A549 cell line. The results are expressed as the Mean±SEM (n = 3) and were analyzed by ANOVA followed by Tukey's test $p<0.05$. Values with different letters above each symbol are significantly different between doses

acid does not produce mutagenic effects on the strains tested, supports the safety of using this compound. It has been reported that polyalthic acid produces other activities, such as an anti-ulcer effect in Wistar rats[11], a relaxant activity in guinea pig tracheal rings [18] and the moderation of leishmanicidal and trypanocidal activities[19].

We also explored the activity of polyalthic acid against other human cancer cells: HCC827 (human non-small-cell lung cancer cells), MCF7 and MDA-MB-231 (breast cancer cells), HeLa (cervical cancer cells) and Colo 205 (colon cancer cells). Whereas polyalthic acid showed cytotoxic activity with all tumors cell lines tested (IC_{50} values in Table 1), it was more active against the HCC827 and MDA-MB-231 cell lines. These results indicate that the cytotoxic effect of polyalthic acid could be important for treating patients with an unfavorable prognosis for cancer without validating molecular targets[20,21].

Since the current standard treatment for NSCLC is with cisplatin[3], we compared its cytotoxic effect versus polyalthic acid on the A549 cell line. Cisplatin exhibited greater potency on this cell line (IC_{50} = 15.0±0.6 μg mL^{-1}) than polyalthic acid (IC_{50} = 34.90±0.72 μg mL^{-1}). However, cisplatin also induced

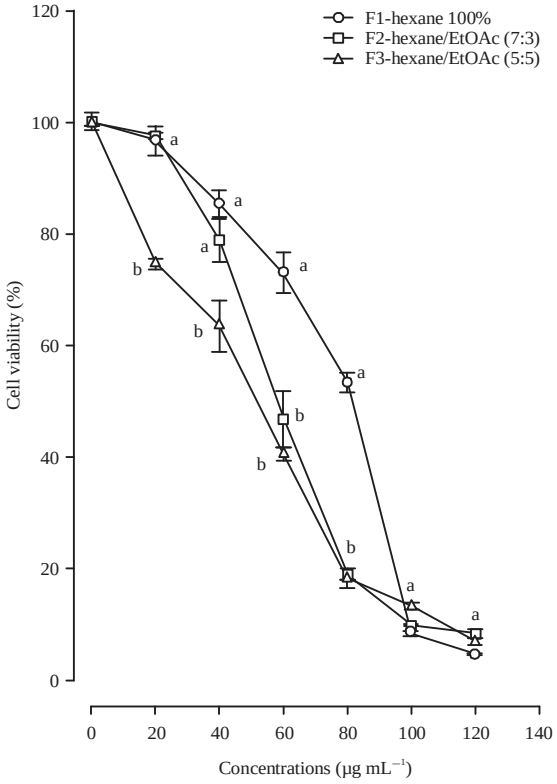

Fig. 2: Cytotoxic activity of the fractions of the hexane extract on the A549 cell line. The results are expressed as the Mean±SEM (n = 3) and were analyzed by ANOVA followed by the Tukey's test p<0.05. Values with different letters above each symbol are significantly different between doses

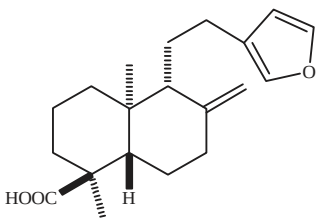

Fig. 3: Polyalthic acid

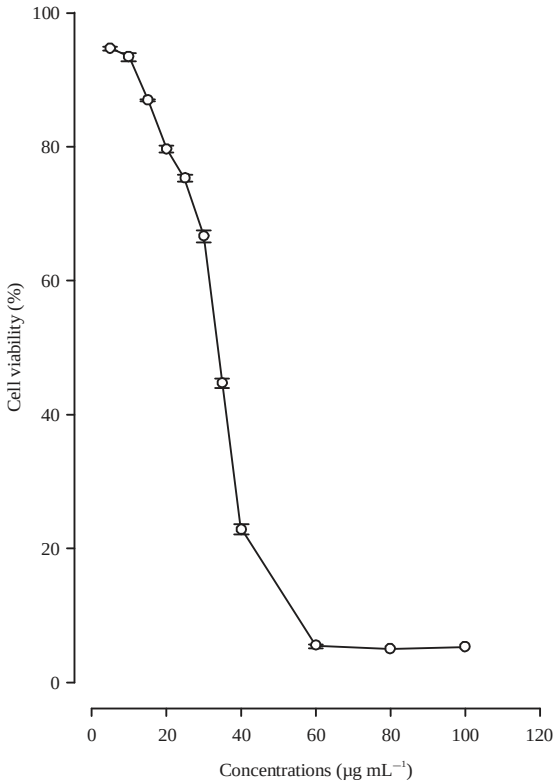

Fig. 4: Cytotoxic activity of polyalthic acid on the A549 cell line. Data represent the Mean±SEM (n = 3)

Table 1: Cytotoxic activity of polyalthic acid (IC_{50} μg mL^{-1}) on several cancer cell lines

	Cell				Lines[a]	
Treatment	A549	HCC827	MCF7	MDA-MB-231	HeLa	Colo 205
Polyalthic acid	34.9±0.7	18.0±0.6	21.9±0.4	15.9±0.3	35.7±0.2	45.9±0.8

[a]A549 and HCC827: Human non-small-cell lung cancer cells, MCF7 and MDA-MB-231: Breast cancer cells, HeLa: Cervical cancer cells, Colo 205: Colon cancer cells

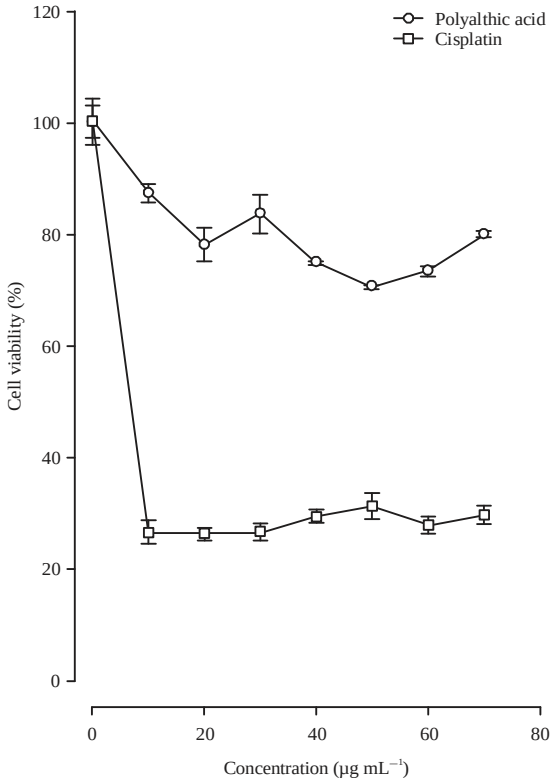

Fig. 5: Effect of polyalthic acid or cisplatin on the HUVEC cells. Data represent the Mean±SEM (n = 3)

a stronger inhibition of growth than polyalthic acid on HUVECs (Fig. 5). Cisplatin reached inhibition values of 73.5 and 70.4% at dose of 10 and 70 µg mL^{-1}, respectively, meaning that its inhibition remained constant at this dose range. Thus, HUVECs were more resistant to growth inhibition by polyalthic acid than cisplatin. Moreover, cisplatin has a long response time and is prone to drug resistance[20,22].

There is an urgent necessity to find novel drugs or combination regimens with low toxicity that are effective for the treatment of NSCLC[3]. In this context, it has been proposed that the combination of phytochemicals with chemotherapeutic agents would enhance efficacy while reducing toxicity to normal tissues[23]. Although polyalthic acid is less potent than cisplatin, herein was proved less toxic to normal cells. The greater selectivity of this compound (versus cisplatin) may make it beneficial as adjuvant drug in cancer treatment, suggesting that it merits further study.

CONCLUSION

The bioassay-guided study of *C. reflexifolius* validates of the traditional use of this plant for cancer treatment. It was found that the leaves of this plant contain more than one cytotoxic compound. Polyalthic acid, isolated from the hexane extract showed cytotoxic activity against the A549, HCC827, MCF7, MDA-MB-231, HeLa and Colo 205. It proved to be more selective than the reference drug, cisplatin, in regard to its activity against A549 versus normal cells. Although polyalthic acid proved to be less potent that cisplatin against this NSCLC cell line, it was also less cytotoxic to HUVEC cells.

ACKNOWLEDGMENTS

This study was supported by two grants from the Escuela Superior de Medicina, Instituto Politécnico Nacional, México (SIP 20160919 and SIP 20161601).

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