Cytotoxicity and Antimicrobial Activity of the Methanol Extract and Compounds from *Polygonum limbatum*

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Key words
- *Polygonum limbatum*
- Polygonaceae
- extract
- compounds
- antimicrobial
- cytotoxicity

Abstract

The present study was designed to investigate the antimicrobial activity and the cytotoxicity of the methanol extract (PLA) as well as fractions (PLA1–4) and compounds [cardamomin (**1**), (±)-polygohomoisoflavanone (**2**), (S)-(−)-pinostrobin (**3**), 2′,4′-dihydroxy-3′,6′-dimethoxychalcone (**4**), (2S)-(−)-5-hydroxy-6,7-dimethoxyflavanone (**5**), and (2S)-(−)-5,7-dimethoxyflavanone (**6**)] obtained from leaves of *Polygonum limbatum*. The microbroth dilution was used to determine the minimal inhibitory concentration (MIC) of the samples against 11 microbial strains including *Candida albicans, C. krusei, C. tropicalis, Aspergillus fumigatus, Pseudomonas aeruginosa, Escherichia coli*, vancomycin-resistant *Enterococcus faecalis* (VRE), *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *S.epidermidis*, and *Mycobacterium tuberculosis* H37Rv. The sulphorhodamine B cell growth inhibition assay was used to assess the cytotoxicity of the above samples on lung A549 adenocarcinoma, breast carcinoma MCF-7, prostate carcinoma PC-3, cervical carcinoma HeLa, and the acute monocytic leukemia cell line THP-1. The results of the MIC determination indicated that, apart from fraction PLA3, all other fractions as well as PLA and compound **3** were selectively active. MIC values were noted on 100% of the 11 tested microorganisms for fraction PLA3, 72.7% for PLA, fraction PLA2, and compound **4**, 63.6% for PLA1, and 54.5% for fraction PLA4. The results of the cytotoxicity assay revealed that, except for A459 cells, more than 50% inhibition of the proliferation was obtained with each of the tested samples on at least one of the four other cell lines. IC_{50} values below 4 µg/mL were obtained with **1** and **4** on THP-1 cells. The overall results of the present study provided baseline information for the possible use of *Polygonum limbatum* as well as some of the isolated compounds for the control of cancer diseases and mostly leukemia.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

Polygonum is a genus of the Polygonaceae family with several species being edible or bearing medicinal virtues [1]. In Chinese medicine, a *Polygonum* extract called Rèlínqīng Kēlì is used to treat urinary tract infections [1]. Several studies have documented the pharmacological potential of plants and compounds of the genus *Polygonum.* They were reported to have antimollucidal [2], antibacterial [3], antifungal [4], antiviral [4], anticancer [4,5], and anti-inflammatory [3] activities. Compounds isolated from *P. hypoleucum* Nakai ex Ohwi such as emodin, emodin 1-*O*-*β*-*D*-glucoside, physcion, and physcion 1-*O*-*β*-*D*-glucoside showed suppressing activity on several tumor cells proliferation including K562, Raji, HeLa, Calu-1, and Wish cell lines [6]. Resveratrol was also identified as an anticancer compound from *P. cuspidatum* Siebold & Zucc., with action against adriamycin-resistant MCF-7 cells [6]. As part of our continuous search for antimicrobial and anticancer products from medicinal plants, *Polygonum limbatum* Meisn. was evaluated in the present work. In Cameroon folk medicine, the leaves of this plant are extracted with raffia wine to treat venereal diseases and gastrointestinal disorders. The aqueous extract of the leaves is also used to treat skin infections (personal communication). The molluscicidal activity of *P. limbatum* was previously reported [2]. The present study was then undertaken to evaluate the antibacteri-

al, antifungal, and antimycobacterial activities as well as the cytotoxicity of the leaves extract and compounds of *P. limbatum*.

Materials and Methods

Plant material

The leaves of *P. limbatum* were collected in Balatchi village (Metap swampy area), near the city of Mbouda, Western Region of Cameroon in March 2010. The plant was identified at the Cameroon National Herbarium, Yaoundé where a voucher specimen was deposited under the reference number 38 852/HNC.

Extraction and isolation of the compounds

The air-dried and powdered leaves of *P. limbatum* (3.7 kg) were extracted three times with MeOH (3 × 3 L) at room temperature. The filtrate obtained was evaporated under reduced pressure to give a dark residue (182 g).

A portion of the MeOH extract (PLA, 170 g) was suspended in water and successively partitioned with EtOAc (fraction PLA2) and *n*-BuOH (fraction PLA1). Active fractions were subjected to purification. Hence, fraction PLA2 (120 g) was subjected to silica gel (240 g) column chromatography, eluted with gradients of *n*-hexane-EtOAc. Fourty fractions of 600 mL each were collected and combined on the basis of TLC analysis to afford three main subfractions (A–C). Subfraction B (PLA3, 37 mg) was submitted to a silica gel (74 g) column chromatography eluted with *n*-hexane-EtOAc to afford further subfractions (PLA_{31}-PLA_{35}). Subfraction PLA_{32} (13.2 g) was then purified by Sephadex LH-20 (30 g) using CH_2Cl_2-MeOH (1 : 1) to give compounds **1** (yellow powder; m: 14 mg; m.p. 194–196; Rf: 0.53 in *n*-hexane-EtOAc 6 : 4; *m/z* 270; purity: 96.76%) and **2** (whitish cristals; m: 13.4 mg; m.p. 133–134; $[\alpha]_D$: 0°, *c* 1, MeOH; Rf: 0.4 in *n*-Hex-EtOAc 6 : 4; *m/z* 316; purity: 97.73%). Repeated column chromatography of subfraction PLA_{33} (11 g) on silica gel (22 g) using *n*-hexane-EtOAc (6 : 4) yielded compounds **3** (yellowish powder; m: 16 mg; m.p. 103–105; $[\alpha]_D$: − 31.9°, *c* 1, MeOH; Rf: 0.73 in *n*-Hex-EtOAc 6 : 4; *m/z* 270; purity: 98.6%) and **4** (orange powder; m: 17 mg; m.p. 124–126; Rf: 0.53 in *n*-Hex-EtOAc 6 : 4; *m/z* 300; purity: 100%). Subfraction PLA_{35} (9 g) eluted with *n*-hexane-EtOAc (8 : 2) was further purified on Sephadex LH-20 (25 g) using CH_2Cl_2-MeOH (1 : 1) to give compound **5** (yellow powder; m: 14 mg; m.p. 148–150; $[\alpha]_D$: − 32.8°, *c* 1, MeOH; Rf: 0.70 in *n*-Hex-EtOAc 6 : 4; *m/z* 300; purity: 96.4%).

Subfraction C (PLA4, 34 g) was submitted to a silica gel (68 g) column chromatography eluted with *n*-hexane-EtOAc gradient to afford four other subfractions (PLA_{41}-PLA_{44}). Subfraction PLA_{41} (12 g) was chromatographed on a silica gel (24 g) column with increasing mixtures of *n*-hexane-EtOAc. Subfractions eluted with *n*-hexane-EtOAc (7.5 : 2.5) were further purified on Sephadex LH-20 (30 g) using CH_2Cl_2-MeOH [250 mL; (1 : 1)] to give compound **6** (white powder; m: 18 mg; m.p. 282–284; $[\alpha]_D$: − 7.6°, *c* 1, MeOH; Rf: 0.4 in *n*-Hex-EtOAc 6 : 4; *m/z* 284; purity: 98.4%). (More details of isolation procedure are available as Supporting Information.)

General experimental procedure

Aluminum sheet precoated with silica gel 60 F_{254} (Merck) was used for thin-layer chromatography (TLC). The spots were visualized using both ultraviolet light (254 and 366 nm) and 5% H_2SO_4 spray reagent and 1% vanillin followed by heating. ^1H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a JEOL JNM-ECA 600 spectrometer with tetramethylsilane as an internal standard. NMR spectra were recorded on a Bruker Avance 300 at 300 MHz (^1H) and 75 MHz, and Bruker Avance 600 at 600 MHz (^1H) and 150 MHz (^{13}C), with the residual solvent peaks as internal references. Optical rotations were measured with a Jasco-DIP-370 digital polarimeter. The melting point (m.p.) was determined using a Kofler microhot stage apparatus. Mass spectra were recorded with an API QSTAR pulsar mass spectrometer. The structures of the isolated compound were confirmed by comparing their data with those from available literature. The purity of the isolated compounds was monitored by HPLC (Shimadzu HPLC system).

Chemicals for biological assays

Ciprofloxacin ≥ 98.0%, amphotericin B ~ 80%, and rifampicin ≥ 97.0% (Sigma-Aldrich) were used as reference antibiotics (RA) against bacteria, fungi, and *M. tuberculosis*, respectively. *p*-Iodonitrotetrazolium chloride (INT; Sigma-Aldrich) was used as a microbial growth indicator [4]. Paclitaxel ≥ 95.0% (Sigma-Aldrich) was used as a positive (cytotoxic) control.

Antimicrobial assays

A total of 11 microbial strains obtained from the American Type Culture Collection (ATCC) and Microbial Type Culture Collection (MTCC) were tested for their susceptibility to extracts, fractions, and compounds. These strains included three yeasts: *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258), and *Candida tropicalis* (ATCC 750); one filamentous fungi: *Aspergillus fumigatus* (MTCC 1811); two Gram-negative bacteria: *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25292; four gram-positive bacteria: vancomycin-resistant *Enterococcus faecalis* (VRE, ATCC 51299), *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591), and *Staphylococcus epidermidis* (ATCC 12228); and one strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294) (see Supporting Information for microbial treatment, activation, and inoculum preparation).

The MIC on fungi, gram-negative and gram-positive bacteria was performed by the broth microdilution method, with RPMI 1640 medium (containing *L*-glutamine, without sodium bicarbonate and buffered to pH 7.0 with 0.165 M morpholine propanesufonic acid) for fungi and MHB for bacteria. The assay was performed following the rapid *p*-iodonitrotetrazolium chloride (INT) method as previously described [7, 8] [see Supporting Information for more details]. The final concentration of samples ranged from 0.78 to 100 µg/mL for compounds and from 7.80 to 1000 µg/mL for extracts. The reference antibiotics (RA) were tested at the concentration range from 0.12–100 µg/mL. The RA ciprofloxacin and amphotericin B served as positive controls, respectively, on bacteria and fungi. The MIC of samples was detected following the addition (40 µL) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride and incubation at 37 °C for 30 min [7, 8]. MIC was defined as the lowest sample concentration that prevented the color change of the culture medium and exhibited complete inhibition of bacterial growth [9].

The MIC determination of *M. tuberculosis* H37Rv was performed using the broth microdilution assay [10, 11] (see Supporting Information for more details). Stock solutions of extracts were prepared at 2000 mg/mL in 100% dimethylsulfoxide (DMSO), and twofold serial dilutions were prepared in media in amounts of 100 µL per well in a 96-well plate. One hundred microliters of inoculum was added to the appropriate wells of microplates to

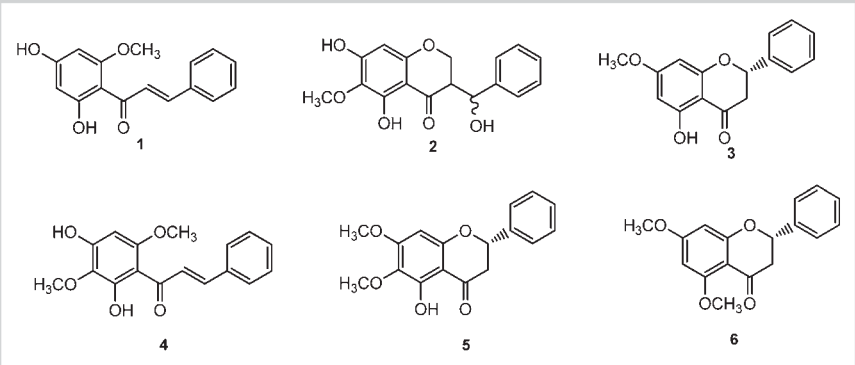

Fig. 1 Chemical structures of compounds isolated from *Polygonum limbatum*. **1**: cardamomin; **2**: (±)-polygohomoisoflavanone; **3**: (S)-(−)-pinostrobin; **4**: 2′,4′-dihydroxy-3′,6′dimethoxychalcone; **5**: (2S)-(−)-5-hydroxy-6,7-dimethoxyflavanone; **6**: (2S)-(−)-5,7-dimethoxyflavanone.

achieve a final volume of 200 µL per well. The medium without the tested samples was used as a growth control, and the blank control used contained only the medium. Rifampicin served as the standard drug control. Microtiter plates were incubated for 3–4 weeks at 37 °C in a CO_2 incubator and read visually for the absence of growth turbidity.

Cytotoxicity activity

Cell lines and treatment: The effect of the extract, fractions, and compounds on cell growth was determined on five human tumor cells including lung A549 adenocarcinoma, breast carcinoma MCF-7, prostate carcinoma PC-3, cervical carcinoma HeLa, and acute monocytic leukemia THP-1 cell lines, obtained from the National Cancer Institute, USA. THP-1, A-549, and PC-3 were maintained in RPMI medium while MCF-7 and HeLa were cultured in MEM medium. All media used were supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humidified environment.

The cytotoxicity of the samples against the five studied human cell lines was determined using the sulphorhodamine B (SRB) assay as previously described [12] (see Supporting Information for more details). The cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity in a CO_2 incubator. Paclitaxel 0.1, 1, and 10 µM was used as a positive reference. Suitable controls with equivalent concentration of DMSO were also included. The optical density (OD) was recorded using a 96-well plate reader, and the growth inhibition was calculated [12]. IC_{50} value is the concentration of sample required to inhibit 50% of the cell proliferation and was calculated by plotting the percentage survival versus the concentrations, using Microsoft Excel. For all samples, each compound concentration was tested thrice in triplicates.

Statistics

The one-way ANOVA at 95% confidence level was used for statistical analysis.

Supporting information

The extraction and isolation procedure as well as protocols for antimicrobial and cytotoxic assays are detailed in the Supporting Information. In addition, a summary of the MIC values (minimal inhibitory concentrations) of the extract, fractions, and compounds from *Polygonum limbatum* and reference antibiotics against the studied microorganisms is available.

Results and Discussion

The structures of the isolated compounds from *Polygonum limbatum* (**Fig. 1**) were established by spectroscopic methods. The six isolated compounds included two chalcones, cardamomin $C_{16}H_{14}O_4$ (**1**) [13] and (±)polygohomoisoflavanone $C_{17}H_{18}O_6$ (**2**) [14], (S)-(−)-pinostrobin or (2S)-(−)-5-hydroxy-7-methoxyflavanone $C_{16}H_{14}O_4$ (**3**) [15]; 2′,4′-dihydroxy-3′,6′-dimethoxychalcone $C_{17}H_{16}O_5$ (**4**) [16]; (S)-(−)-onysilin or (2S)-(−)-5-hydroxy-6,7-dimethoxyflavanone; $C_{17}H_{16}O_5$ (**5**) [17]; (S)-(−)-chrysin or (2S)-(−)-5,7-dimethoxyflavanone $C_{17}H_{16}O_4$ (**6**) [18].

The results of the MIC determination (see Supporting Information, **Table 1S**) indicated that, apart from fraction PLA3, all other fractions as well as PLA and compound **3** were selectively active. Other compounds did not show any antimicrobial activity. The recorded MIC values were noted on 100% of the tested microorganisms for fraction PLA3, 8 of the 11 (72.7%) tested organisms for the crude extract, fraction PLA2, and compound **4**, 7/11 (63.6%) for fraction PLA1, and 6/11 (54.5%) for fraction PLA4. However, even for samples with observed activity, the effects obtained are either moderate or weak. In fact, phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce MIC in the range of 100 to 1000 mg/mL [19]. Activity is considered to be significant if MIC values are below 100 µg/mL (or < 10 µg/mL) for crude extracts (or compounds) and moderate when 100 < MIC < 625 µg/mL (10 < MIC < 100 µg/mL for compounds) [20]. Therefore, the activity recorded could mostly be considered as moderate for compound **4** on 7/11 tested microorganisms, 4/11 for fractions PLA2 and PLA3, and 3/11 for the crude extract. However, none of the tested samples was as active as the reference antimicrobial drug used, confirming their moderate activity on the studied microbial species. Nonetheless, some of the tested species such as MRSA, VRE, and *P. aeruginosa* are resistant phenotypes, giving some interest to the possible use of the fraction PLA3 and compound **4** in the treatment of drug resistant microbial infections. It is noteworthy that other *Polygonum* species, such as *P. spectabile* Mart. ex Meisn., were also found to have antimicrobial activities against *Staphylococcus aureus, Bacillus subtillis, Micrococcus luteus, M. canis, Tricophyton mentagrophytes*, and *T. rubrum* [4], highlighting the anti-infective potential of the taxon studied herein. *P. multiflorum* Thunb. was also reported to have antibacterial activity against MRSA [21].

It has been recommended that ethnopharmacological usages such as in the treatment of immune and skin disorders, inflammatory, infectious, parasitic, and viral diseases should be taken into account when selecting plants used to treat cancer, since

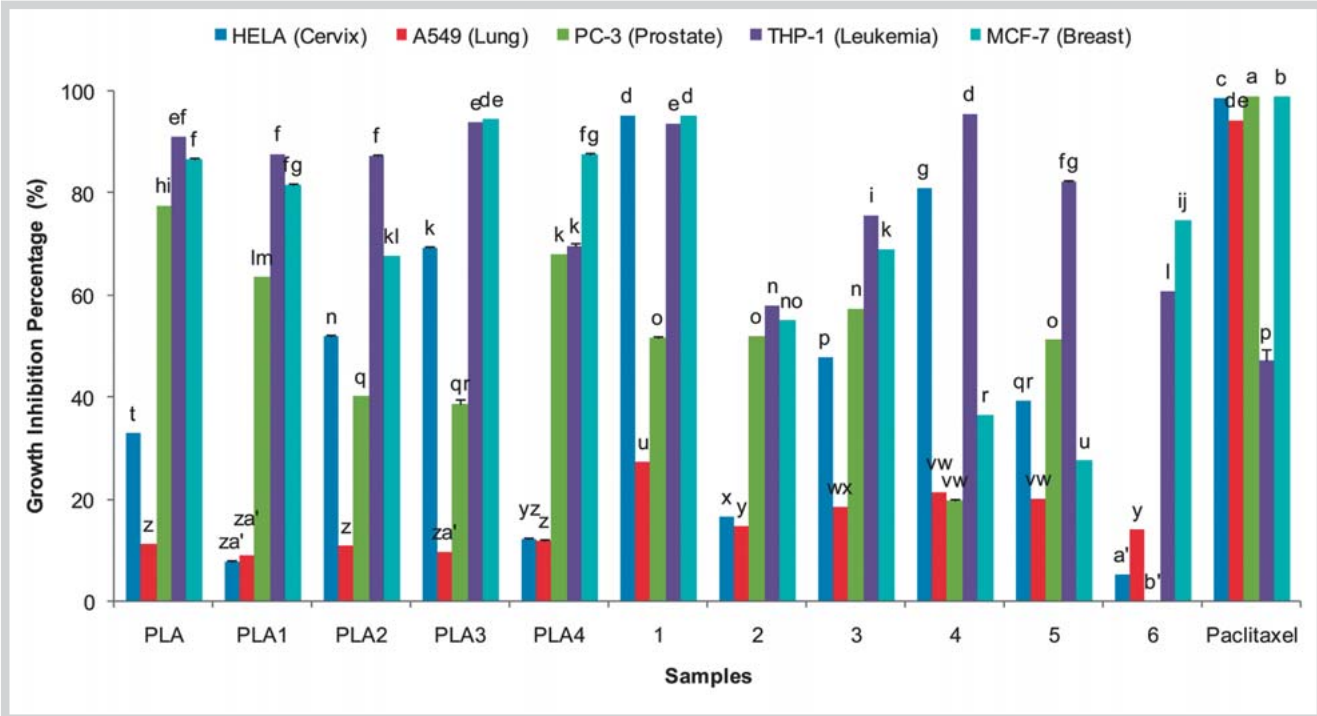

Fig. 2 Cytotoxic activity of the extracts, compounds from *Polygonum limbatum*, and paclitaxel against human cancer cell lines. Samples were tested at 100 µg/mL (for crude extract and fractions) and 50 µg/mL (for compounds and paclitaxel). Tested samples were crude methanol extract (PLA), *n*-BuOH fraction (PLA1), EtOAc fraction (PLA2), subfraction B from EtOAc extract (PLA3), subfraction C from EtOAc extract (PLA4); **1**: cardamomin; **2**: (±)-polygohomoisoflavanone; **3**: (S)-(−)-pinostrobin; **4**: 2′,4′-dihydroxy-3′,6′dimethoxychalcone; **5**: (2S)-(−)-5-hydroxy-6,7-dimethoxyflavanone; **6**: (2S)-(−)-5,7-dimethoxyflavanone. Data with different alphabetic letters are significantly different ($p < 0.05$).

Table 1 IC_{50} values of the extracts, compounds from *Polygonum limbatum*, and paclitaxel against human cancer cell lines.

Samples*	Cell lines and IC_{50} (µg/mL)				
	THP-1 (leukemia)	HeLa (cervix)	A549 (lung)	PC-3 (prostate)	MCF-7 (breast)
Extract and fractions					
PLA	**10**	–	–	28	**20**
PLA1	**9**	–	–	**9**	**18**
PLA2	**8.5**	–	–	–	23
PLA3	**18**	78	–	–	**8**
PLA4	36.5	–	–	13	16
Compounds					
1	**1.8**	17	–	49	32
2	32.5	–	–	49	44
3	9	–	–	40	36
4	**3.5**	22	–	–	–
5	25.5	–	–	47	–
6	10	–	–	–	37
Paclitaxel	**4**	12	**1**	–	**3**

* Samples [*Polygonum limbatum* crude extract from aerial part (PLA); *n*-BuOH fraction (PLA1), EtOAc fraction (PLA2), subfraction B from EtOAc extract (PLA3), subfraction C from EtOAc extract (PLA4); **1**: cardamomin; **2**: (±)-polygohomoisoflavanone; **3**: (S)-(−)-pinostrobin; **4**: 2′,4′-dihydroxy-3′,6′dimethoxychalcone; **5**: (2S)-(−)-5-hydroxy-6,7-dimethoxyflavanone; **6**: (2S)-(−)-5,7-dimethoxyflavanone]; (−) value above 100 µg/mL for extract or fractions and 50 µg/mL for compounds and paclitaxel; in **bold** are values for significant activity [29]

these reflect disease states bearing relevance to cancer or canceliker symptoms [22–24]. Consequently, all the studied samples including extracts, fractions, and compounds were tested for their ability to inhibit the proliferation of five cancer cell lines. In the preliminary step to select samples for a dose-response study, extracts and fractions were tested at a single concentration of 100 µg/mL; meanwhile the isolated compounds and paclitaxel were tested at 50 µg/mL. The results summarized in **Fig. 2** revealed that, except for A459 cells, more than 50% inhibition of the proliferation was obtained with each of the tested samples on at least one of the four other cell lines. Consequently, their IC_{50} values on the corresponding sensitive cells were determined, and the results are summarized in **Table 1**. In the US-NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value following incubation between 48 and 72 h is less than 20 µg/mL. Boik [25] defined

this cutoff point as 4 µg/mL for compounds. Herein, IC_{50} values below or around 20 µg/mL were recorded for the crude extract from *P. limbatum* as well as for fraction PLA3 on two of the five studied cancer cell lines and for fraction PLA1 on 3/5 cell lines. For compounds, IC_{50} values below 4 µg/mL were obtained with **1** and **4** on leukemia THP-1 cells. Such samples can therefore be considered as potential anticancer drugs, especially against leukemia. In fact, the leukemia cell line was found to be more sensitive than other cell lines to the compounds isolated from *P. limbatum*, the lowest IC_{50} values of 1.8 and 3.5 µg/mL recorded with **1** and **4** being lower than that of paclitaxel (4 µg/mL), highlighting their interesting anti-leukemic potentials. These data are consistent with the previously reported study [26] and reflect the clinical situation with established anticancer drugs, as it is well known that leukemia is more sensitive to chemotherapy than other malignancies.

The overall results of the present work indicate that *P. limbatum* and the isolated compounds could mostly be considered as potential antiproliferative rather than antimicrobial drugs. However, the role of flavonoids as anticancer compounds has been demonstrated. A number of molecules of the class of flavonoids have been shown to exhibit significant inhibition of cancer development *in vitro* and in various animal models and have also produced the most compelling data for the antitumor activities of plant secondary metabolites in various types of cancers [27]. In addition, they were found to induce apoptosis and cell cycle arrest in cancer cells and were documented as good inhibitors of the angiogenesis process [27].

To the best of our knowledge, the cytotoxicity of *P. limbatum* is being reported herein for the first time. Nevertheless, the anticancer activity of plants of the genus *Polygonum* was reported. The chloroform and hexane fractions from another plant of the genus *Polygonun, P. bistorta*, showed moderate to very good activity against P388, HL60, and LL2 cancer cell lines [28]. The anticancer activity of *P. limbatum* is therefore in accordance with that of other plants from the genus *Polygonum.* Besides, the cytotoxicity of compounds such as cardamonin (**1**) was demonstrated [29], and this can also explain the activity of this plant extract as observed in this study. In addition, another compound isolated in *P. limbatum*, (−)- pinostrobin (**2**), was reported for its antitumor activity against human mammary carcinoma [30]. Compound **2** was found to inhibit DNA topoisomerase I activity, suggesting that this could be its possible anticancer mechanism of action [30]. Nevertheless, the topoisomerase I-mediated DNA cleavage induced by pinostrobin was still lower than that of a known inhibitor such as campthotecin [30]. In addition, the presence of chrysin (compound **6**) in the extract from *P. limbatum* can also explain the cytotoxic activity of this plant as it is a well-known antineoplastic agent [31].

Regarding the structure-activity relationship, it appears that compounds **1** (chalconoid) and **3** (flavanone) on one hand, **4** (chalconoid) and **5** (flavanone) on another hand are isomers. In the antimicrobial study, compound **4** showed both antibacterial and antifungal activity whilst compound **5** was not active. Compound **4** was more active on leukemia THP-1 cells than compound **5**; meanwhile the difference in the two isomers seems to play a role in their selectivity to adherent HeLa and PC-3 cell lines. Besides, it can be observed that the chalconoids **1** and **4** appeared to be more active than their corresponding flavanone isomers **3** and **5**.

Finally, the results of the present investigation provided baseline information for the possible use of *Polygonum limbatum* as well as some of the isolated compounds for the control of cancer diseases and mostly leukemia.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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