Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Article
Volume 11, Issue 1, 2021, 8129 - 8140
https://doi.org/10.33263/BRIAC111.81298140

Phytochemical Characterization, *In Vitro* Antioxidant, Cytotoxic, and Antibacterial Effects of *Aristolochia longa* L.,

Amina El Yahyaoui El Idrissi [1], Aya Khouchlaa [1,*], Abdelhakim Bouyahya [1], Youssef Bakri, M'hamed Tijane [1]

[1] Laboratory of Human Pathologies Biology, Faculty of Science, Genomic Center of Human Pathologies, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Morocco
* Correspondence: aya.khouchlaa@gmail.com;

Scopus Author ID 57191631959
Received: 24.06.2020; Revised: 13.07.2020; Accepted: 14.07.2020; Published: 18.07.2020

Abstract: The aim of this study was devoted, in the one hand, to determine a preliminary phytochemical screening of methanolic and aqueous root extracts of *Aristolochia longa* L. In the other hand, polyphenols and flavonoids contents, *in vitro* antioxidant, antitumor, and antibacterial activities of the organic extract of *A. longa* were determined. Phytochemical screening was carried out using standard methods of precipitation and coloration reactions. The total phenol content and flavonoid contents were determined using the Folin-Ciocalteu method and Aluminum chloride colorimetric method, respectively. Antioxidant activity was studied using DPPH assay. The cytotoxic activity of extracts was evaluated against three cancerous cell lines using MTT assay, and antibacterial activity was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Rhodococcus equi*, and *Staphylococcus aureus* using the agar well diffusion assay. Preliminary phytochemical screening for aqueous and ethanolic extracts revealed the presence of starch, tannins, flavonoids, coumarins, and anthocyanins. The total phenol content of *A. longa* extracts showed that the methanol extract has the highest polyphenol and flavonoid concentrations with 101.4 mg GAE (Gallic acid equivalent)/g and 54.21 mg QE (Quercetin equivalent)/g extract, respectively. The methanolic extract also exhibited the highest antioxidant capacity of DPPH (IC_{50} = 1.32 mg/mL). While hexane extract exhibited both a potent inhibitory effect on VERO cell growth with IC_{50} = 15.125 µg/mL and a total inhibitory effect on *Staphylococcus aureus* with inhibition zone 8.5 mm. The high content of polyphenols in *A. longa* explains their antioxidant, antitumor, and antibacterial activity. Thus, this plant could be a significant source of natural compounds in preventing the development of cancer.

Keywords: *Aristolochia longa*; phenolic content; antioxidant; antibacterial; antitumor.

1. Introduction

Natural products design a new lead of drug discovery because of the presence of bioactive compounds that exhibited fewer side effects. Currently, naturally derived products used in pharmaceutical companies are originated from plants (25%), microorganisms (13%), and animals (25%) and present a promising opportunity to treat different diseases. Nowadays, herbal plants are primary health care, and the worldwide trade market of herbal medicine is annually growing at the rate of 10–15% [1,2]. Due to its geographical situation and heterogenic climate, Morocco presents a rich flora regroups more than 5200 species of plants, including 900 endemics [3].

Aristolochia is a genus that belongs to *Aristolochiaceae* family that contains about 500 species. This family has been reported in the forest of America, Asia, Africa, Europe, and rarely in other countries [4-6]. In Morocco, *Aristolochia longa*, known as Bereztem, is a species used in folk medicine to treat different diseases such as cancer, diabetes, asthma, and skin and intestinal affection using a different part of this plant with several forms *viz.* honey, milk and jus [7-12]. A recent ethnopharmacological study conducted by El Yahyaoui reported that root is the most used part of treating cancer in Morocco [13].

Chemical analysis of the extracts from different parts of *A. longa* revealed the presence of a wide range of bioactive compounds distributed in several phytochemical classes such as flavonoids, terpenoids, alkaloids, and fatty acid. Previous investigations carried out in several essential oils, organic and aqueous extracts of a different part of this plant, showed the presence of aristolochic acid (AA), maaliol, lycopene, limonene, palmitic acid, and *β*-caryophyllene [9,11-13]. In addition, several researches reported the pharmacological properties of *A. longa* and their compounds, which elicit pharmacological effects, including antitumoral, antibacterial, antifungal, anti-inflammatory, antidiabetic, antioxidant activities [9,14,15,17-19]. Furthermore, a recent study has evaluated the toxicity effect of this plant *in vivo* [20]. The aim of this study was devoted to determining phenolic and flavonoid content as well to evaluate the antioxidant, antitumor, and antibacterial activities of the root of *A. longa* from the Moroccan region.

2. Materials and Methods

2.1. Plant material.

The fresh root of *A. longa* was harvested in the cities Rabat (September 2014), Morocco. The plant, identified by Professor Kahouadji Azzeddine, Faculty of Sciences Rabat, Morocco, was ground and stored in watertight tubes at room temperature. Voucher specimens have been deposited in the Laboratory of Human Pathologies Biology, Mohammed 5 University, Faculty of Sciences, Rabat, Morocco.

2.2. Preparation of extract.

A. longa root extract was obtained by the maceration of the root powder (70 g of powder in 200 mL of solvent) for three days at room temperature and stirring at 70 revolutions/minute. This powder was successively extracted with hexane, dichloromethane, and methanol using a technique of continuous hot extraction, a Soxhlet extractor. The solvent obtained was then evaporated under reduced pressure to obtain the crude extract. The crude extracts were stored at -12°C until further uses.

2.3. Qualitative analysis.

Preliminary qualitative phytochemical analysis was carried out to identify phytochemical constituents of the plant in methanol and aqueous extracts. Following phytochemicals were tested starch, saponosides, tannins, flavonoids, anthocyans, coumarins, sterols, and triterpenes using standard methods, as reported in the literature [21,22].

2.4. Determination of phenolic content.

2.4.1. Determination of total phenolic content.

The assessment of total polyphenols was performed according to the method of Singleton *et al*. [23] using the Folin-Ciocalteu as a reagent. On this base, the extracts are diluted to a concentration of 1 mg/mL. An amount of 100 µL of the diluted extract is placed in test tubes, and 500 µL of Folin-Ciocalteu reagent diluted 10 times in distilled water is added. After incubation for 1h at room temperature, 2 mL of sodium carbonate (Na_2CO_3) to 2% is added. The tubes are then shacked and placed in the dark for 30 minutes at room temperature. The same steps were followed to establish a reference range (0 to 100 µg/mL) prepared from an aqueous stock solution of gallic acid (0.5 g/L). The absorbance is measured using a UV-Visible spectrophotometer at a wavelength of 760 nm [24]. The absorbance values of each concentration enabled us to plot the calibration curve of gallic acid. The results are expressed in mg of gallic acid equivalents per gram of dry extract (GAE mg/g of dry extract). All manipulations are performed in triplicate.

2.4.2. Determination of total flavonoids content.

Flavonoids contents are measured using aluminum trichloride ($AlCl_3$) as a reagent. 1mL of the extract of *A. longa* is mixed with 1 mL of the solution of aluminum trichloride (2%) and 50 µL of acetic acid. The tubes are then gently mixed and incubated in the dark for 40 minutes at room temperature. Under the same conditions, a stock solution of quercetin mass concentration 0.2 g/L was prepared in methanol. From this stock solution, a standard range of concentrations from 0 to 25 mg/mL was prepared—the absorbance measured in the same spectrophotometer at a wavelength of 415 nm [23]. The obtained absorbance values enabled us to plot the calibration curve of quercetin. The results are expressed in mg of quercetin equivalent per g of dry extract (mg QE/g of dry extract). All manipulations are performed in three trials.

2.5. Antioxidant activity.

The antioxidant activity was determined according to the method Kubola and Siriamornpun with some modifications. Briefly, 1.8 mL of 0.1 mM DPPH solution is prepared in methanol and added into 0.2 mL tubes containing extract (methanol or ethanol) at increasing concentrations (0.31 mg/mL - 10 mg/mL for *A. longa* extracts) and are placed away from light at room temperature after agitation by a vortex. After 30 min, the absorbance is measured at 517 nm [25]. Ascorbic acid (vitamin C) was used as positive controls, while the methanol is used as a negative control. The scavenging activity of DPPH radical is calculated using the following formula: $A\ \% = (Abs_{control} - Abs_{sample}) \times 100/Abs_{control}$)

Abs control: Absorbance without antioxidant (containing all reagents except the test sample)

Abs sample: absorbance with the test sample.

IC_{50} is the concentration of the test sample required to reduce 50% of DPPH radicals. IC_{50} was graphically calculated by linear regression plots graphs, percentage inhibition as a function of different concentrations of the tested fractions and standards.

2.6. Cell viability assays.

The *in vitro* cytotoxic effect of the various extracts was evaluated on RD: Embryonal Rhabdomyosarcoma cancerous cell lines (ATCC N°CCL-136), and Vero: Monkey kidney cancerous cell lines (ATCC N°CCL-81) which obtained from the National Institute of Health, Rabat-Morocco. PBMC (Peripheral Blood Mononuclear Cell) isolated and purified from human blood was used as a positive control. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Streptomycin mixture. Cultures were maintained at 37°C in 5% CO_2 and 100% relative humidity atmosphere. The effect of the isolated extracts on cell viability was assessed using the 3-(4,5-dimethylthiazol -2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay, which measures the metabolic activity of mitochondria [26]. The tests were conducted on 96-well microplate. Before treatment with extracts, 100 µL medium DMEM (GIBCO) containing 3-4x106 cells/mL were placed in each well containing DMEM (GIBCO) and cultured at 37°C in 5% CO_2/humidified air for 24 h. After 24h incubation and attachment, cells were treated with crude extracts. Exactly from the stock solution (80 mg/mL), each extracted sample was applied in a series of 6 dilutions (final concentrations ranging from 12.5 µg/mL to 400 µg/mL) in Dimethyl sulfoxide (DMSO 1%). Test solution (100 µL) was added in decreasing concentrations in duplicate. The microplate was then incubated for 48 h at 37°C in the air condition of 5% CO_2. After, a 20 µL MTT solution (5 mg/mL) (SIGMA) was added to the wells containing cells. The cells were incubated for 4 - 5 h at 37°C in 5% CO_2. Tetrazolium salts are cleaved to formazan dye by a cellular enzyme (only in the viable cells). A solubilization solution (Isopropanol/hydrochloric acid) is added to dissolve the insoluble purple formazan product into a coloration solution. The absorbance was measured at 545 nm, using a microplate reader (Statfax 2100).

2.7. Antibacterial activity.

We used agar well diffusion methods to determine the antimicrobial activities of our extracts. Six species of bacteria were studied, 4 species of Gram-positive bacteria and two species of Gram-negative bacteria (Table 1). The test samples were first dissolved in dimethylsulfoxide (1%) who thus did not affect the microbial growth. Briefly, 8mL of medium agar was poured into sterile Petri plates. After solidification, 100 µL of fresh cultures of each bacterial (one microorganism per Petri dish) were swabbed on the respective plates. Then, 50 µL of extracts were placed in wells previously punched over the agar plates using sterile Pasteur pipette, at various concentrations (10 mg/mL; 20 mg/mL; 30 mg/mL; 40 mg/mL; 50 mg/mL). All Petri plates were then incubated at 37°C for 24 h. The diameters of inhibition zones were measured in millimeters. Moreover, commercially available antibiotics were used to compare with the antimicrobial activities of our plant extracts on all bacterial species studied. The antibiotic discs of Ampicillin were placed on the surface of the plates. DMSO 1% was used as a negative control. The plates were incubated at 37°C for 24h, and after incubation, the diameter of the inhibition zones was measured in mm and recorded [27].

Table 1. Bacterial species used and their origins.

Bacterial species	**Origins**
Escherichia coli	Food Microbiology Laboratory, UCL, Belgium (MBLA)
Pseudomonas aeruginosa IH	Rabat Institute of Hygiene
Staphylococcus aureus	German Collection of DSM Microorganisms
Rhodococcus equi (GK1, GK2, GK3)	Laboratory of Human Pathologies Biology, UM5, Rabat

3. Results and Discussion

3.1. Qualitative analysis.

The phytochemical screening of *A. longa* roots revealed the presence of starch, tannins, flavonoids, coumarins, anthocyans, and the absence of saponins, sterols, and triterpenes (Table 2). These findings showed similarity with the earlier study, which reports that the phytochemical screening of *A. longa* revealed the presence of flavonoids, and saponins, and the absence of sterols and triterpenes [14]. In 2019, Bouteldja *et al*. [28] evaluated the phytochemical screening of *A. longa* methanolic extracts from the aerial part found in Algeria. This extract revealed the presence of tannins, and flavonoids while anthocyanins, steroids, and terpenoids were absent.

In addition, several studies reported the phytochemical composition of species in the same genus. Aerial parts of *A. indica* from India showed the presence of terpenes, saponins, tannins, and flavonoids [29]. In recent studies, Bourhiya *et al*. [30,31] assessed the phytochemical screening roots from *A. baetica,* and *A. paucinervis* and revealed the presence of flavonoids, polyphenols, alkaloids, tannins, and saponins and the absence of anthraquinone, sterols, and terpenes. Despite the part use, the plant localization, the type of extract, the groups, namely sterols, steroids, terpenes, triterpenes, anthocyanins, anthraquinone, were absent in the genus of *Aristolochia.*

Table 2. Preliminary qualitative phytochemical analysis of *A. longa* methanol and aqueous root extracts.

Plants constituents	Methanol extract	Aqueous extract
Starch		++
Saponosides		-
Tannins	+	+
Flavonoids	++	
Coumarins	+	
Anthocyans	+	
Sterols	-	
Triterpenes	-	

+++: highly present, ++: moderately present, +: Low, -: absent.

3.2. Phenolic content.

The contents of total polyphenols have been determined by colorimetric assay using the Folin-Ciocalteu reagent. The results are expressed in mg GAE/g extract by referring to the previously established calibration curve with gallic acid (polyphenol correlation: $R^2 = 0.998$). Total flavonoids are determined by the trichloride reagent aluminum, and the results are expressed in mg QE/g extract based on the established calibration curve with quercetin (flavonoid correlation: $R^2 = 0.985$). The results of the polyphenol and flavonoid contents of extracts of *A. longa* are illustrated in Table 3.

The total phenolic content of various extracts of *A. longa* from root parts was varying widely between 29.54 to 101.41 mg GAE/g extract (Table 3). The methanolic extract was demonstrating higher total polyphenols content (101.41 ± 0.85 mg GAE/g) followed by ethanol and hexane extracts, which were 89.41 ± 4.96 mg GAE/g, and 76.41 ± 8.74 mg GAE/g, respectively. Results of flavonoids' contents show that methanolic extract resulted a high value of another fraction (54.2 ± 10.12 mg QE/g).

The concentration of polyphenols and flavonoids of our extract is widely higher compare to other studies. In a recent study, El Omari *et al*. [9] reported that the methanolic fraction of *A. longa* from root contains 24.48 ± 1.63 mg GAE/g of polyphenols and 7.00 ± 0.61

mg RE (Rutin equivalent)/g of flavonoids. While Djeridane *et al*. [32] studied *A. longa* roots and reported a total phenolics and flavonoids content as 1.47 ± 0.02 mg GAE/g and 0.81 ± 0.02 mg RE/g, respectively. Previous studies reported that the methanolic extract from *Aristolochia* species (*A. indica*, *Aristolochia baetica*, and *Aristolochia paucinervis*, and *Aristolochia bodamae*) contains a high amount of phenols and flavonoids. However, phenols and flavonoids content vary from different studies [30,33,34]. Several factors may affect their content. Studies have shown that extrinsic factors (such as geographic and climatic factors), genetic factors, but also the degree of maturation of the plant and the storage time, have a strong influence on the content in polyphenols [35-38].

Table 3. Total phenolic and flavonoids content of root extract of *A. longa*.

	A. longa	
	Polyphenols (mg of GAE/g of extract)	**Flavonoids (mg of QE/g of extract)**
Dichloromethane extract	89.41 ± 4.96	34.02 ± 1.87
Methanolic extract	101.41 ± 0.85	54.21 ± 0.17
Hexanic extract	76.41 ± 8.74	29.54 ± 0.95

GAE: Gallic acid equivalents; QE: Quercetin equivalent

3.3. Antioxidant activity.

Antioxidant activity was performed using *in vitro* DPPH methods. The results are expressed in ascorbic acid equivalents. The DPPH radical scavenging activity of different extract of *A. longa* at various concentrations are illustrated in Figure 1, and all extracts showed a scavenging activity which increases with samples concentration. A lower value of IC_{50} indicates greater antioxidant activity. Among the solvent used, methanolic extract was registered a high antioxidant activity, IC_{50} = 1.32 mg/mL. Hexane and dichloromethane extract of *A. longa* have an IC_{50} > 5 mg/mL (Table 4). IC_{50} of ascorbic acid was 27.20 ± 0.17 µg/mL. High antioxidant activity of ascorbic acid can be explained by his use as a pure molecule compares to our extract, which contained several molecules.

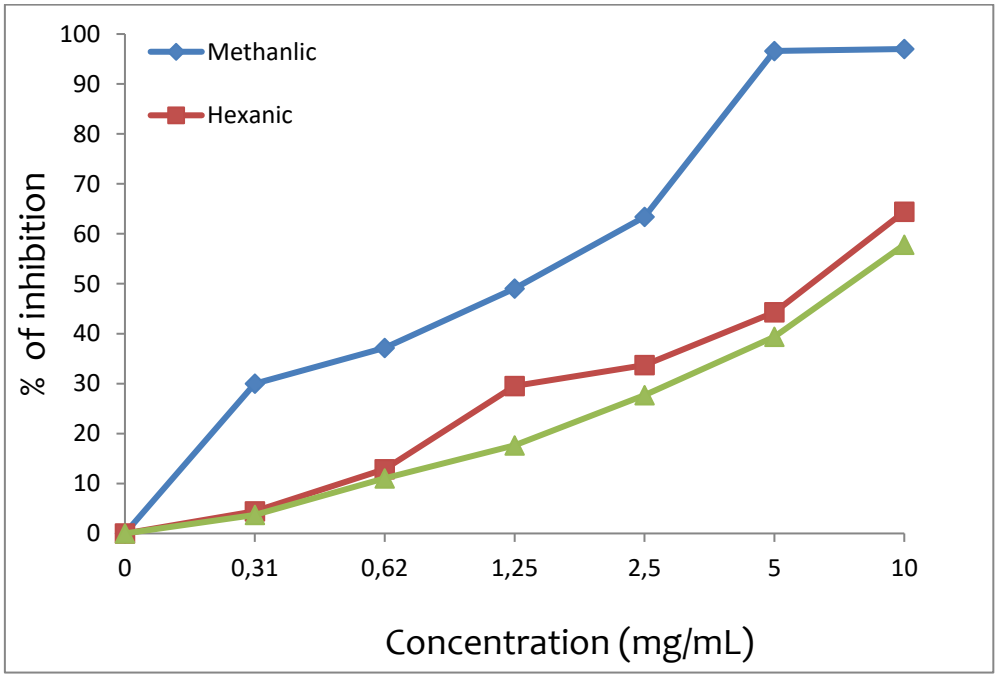

Figure 1. DPPH radical scavenging activity of *A. longa* organic extract.

Table 4. Free radical (DPPH) scavenging activity of *A. longa* organic extract.

	Extracts	IC_{50} (mg/mL)
A. longa	Methanol	1.32
	Hexane	6.58
	Dichloromethane	8.26

Numerous studies investigated the ability of organic extract of a different part of *A. longa* to scavenge free radical using DPPH scavenging activity. A recent Moroccan study reported the highest inhibition of *A. longa* root aqueous fraction with IC_{50} = 125.40 ± 2.40 μg/mL [9]. An *in vitro* research carried out by Merouani *et al*. showed that aerial parts methanol extract exhibited the highest dose-dependent reducing DPPH with IC_{50} = 55.04 μg/mL with no significant ($p>0.05$) [15]. In Algeria, Djeridane *et al*. [32] reported an IC_{50} = 55.04 ± 0.4 μM. The antioxidant activity of these extracts is related to the highly important charges of phenolic compounds polyphenols and flavonoids. Indeed, several *in vitro* and *in vivo* researches reported the correlation between phenolic compounds and antioxidant effects and also with the synergy effect with other compounds [39-41].

3.4. Cytotoxicity effects.

The cytotoxic potential of *A. longa* organic extracts was conducted on two tumor cell lines: RD, and VERO. These lasts were treated with different concentrations of extracts from 15.12 µg/mL to 1000 µg/mL. Assay by the MTT assay indicates that extracts revealed different cytotoxic activities towards two cancer cell lines investigated (RD and VERO). The results have been compared to control (PBMC) to compare the antiproliferative effects of extracts. The obtained results are listed in Table 5. In general, a dose-dependent decrease in the survival of the two cancerous cell lines is illustrated in figure 2 and figure 3. Amongst these tested plant extracts, hexane extracts exhibited potent and selective antiproliferative activities against VERO and RD cell lines with the same IC_{50} (IC_{50} = 15.12 µg/mL) (Figure 3, and Figure 2).

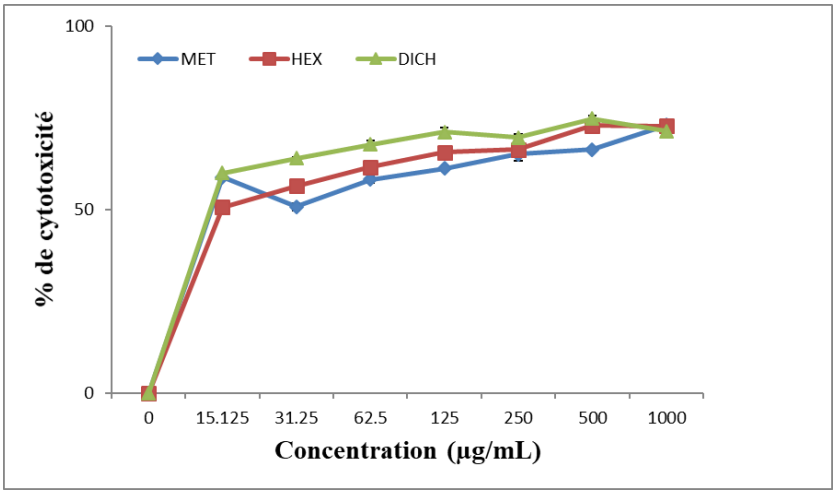

Figure 2. Cytotoxic activity of root extract of *A. longa* against RD cell lines.

At the same time, there is no significant effect of dichloromethane and methanol extracts. The cytotoxic activity of hexane extracts showed low cytotoxicity against normal cells (PBMC) with IC_{50} values of 62.57 ± 16.295 µg/mL (Table 5, and Figure 4). In our study, the presence of polyphenols and flavonoids in hexane extracts (76.41 ± 8.74 mg of GAE/g, and 29.54 ± 0.95 mg of QE/g, respectively) may explain the cytotoxic effect on RD and VERO tumor cell lines. Several researches reported the activity of *A. longa* extracts against various

cancerous cell lines [40,42]. Benarba *et al*. reported an IC_{50} =15.63 µg/mL for *A. longa* aqueous extract against Burkitt's lymphoma BL41 cells [42]. A previous study reported the effect of tuber extracts of this plant on the same cell line used in this study. In our laboratory, the cytotoxic effect of *A. longa* tuber extracts has been studied by Aneb *et al*. [40]. They screened the cytotoxic effect of hexane and dichloromethane extract, which presented a cytotoxic capacity IC_{50} = 30 µg/mL and IC_{50} = 15 µg/mL, respectively, on RD cell lines [40]. The mechanism of action of extracts through which compounds act in their cytotoxic effect is not fully understood. However, the authors suggested that these compounds act thought the membrane disruption, apoptosis pathways, and inactivation of telomerase [40,43].

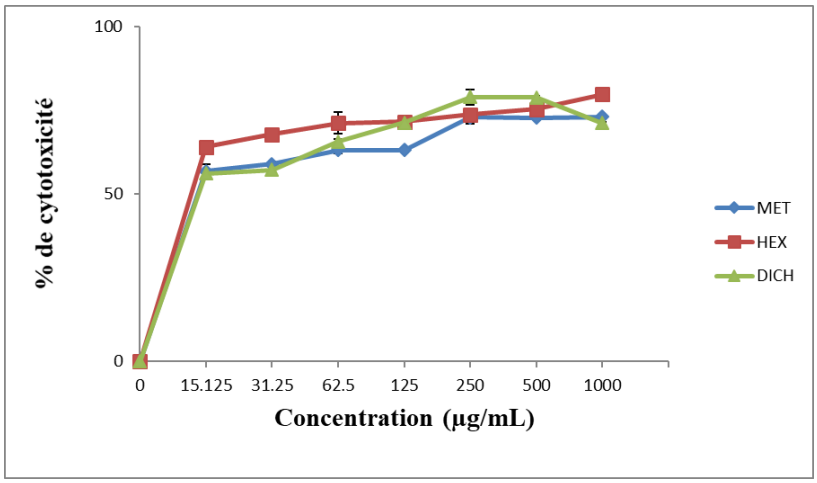

Figure 3. Cytotoxic activity of root extract of *A. longa* against VERO cell lines.

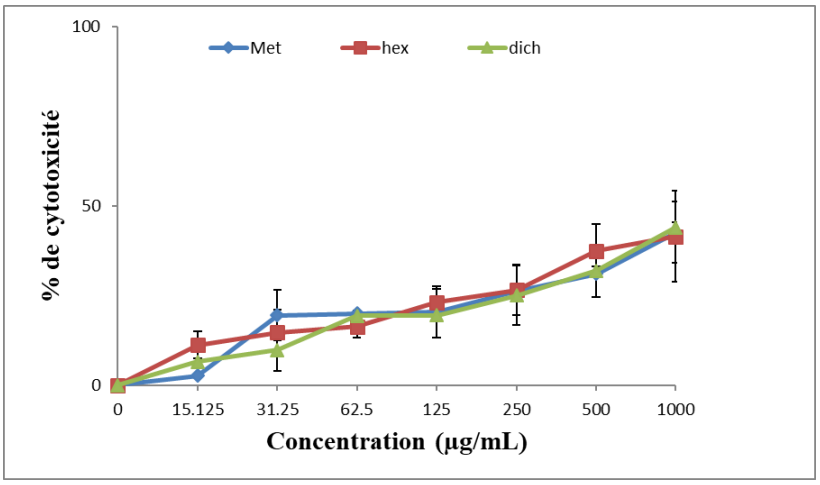

Figure 4. Cytotoxic activity of *A. longa* root extracts against PBMC cell lines.

Table 5. Inhibition concentration (IC_{50} in µg/mL) values from *A. longa* towards RD, VERO cancerous cell lines, and PBMC (control) as determined by the MTT assay.

		A. longa		
		Hexane	**Dichloromethane**	**Methanol**
IC_{50} (µg/ml)	RD	15.12 ± 66.32	125.43 ± 71.17	62.5 ± 61.24
	PBMC	62.57 ± 16.295	14.16 ± 6.48	31.25 ± 09.765
	VERO	15.125 ± 64.01	31.25 ± 57.17	125.3 ± 63.13

3.5. Antibacterial activity.

This study evaluated the antibacterial activity of *A. longa* hexane, dichloromethane, and methanolic extracts on Gram-positive and Gram-negative strains. The results of agar well diffusion methods showed that each extract has a different degree of growth inhibition (Table

6). At 10 mg/mL, *S. aureus* showed a high sensitivity against hexane extract with diameters inhibition 8.5mm. The effect of three bacterial strains of genus *Rhodococcus* were tested. At 50 mg/ml, hexane and dichloromethane extracts were found to possess a higher antibacterial activity against *Rhodococcus* sp GK1 with a diameter of inhibition 1.55 mm and 1.9 mm, respectively. While for Gram-, a lower activity inhibition effect was observed for *E. coli*, and *P. aeruginosa*.

Previous studies reported that *A. longa* has a significant *in vitro* antibacterial activity against *Rhodococcus* sp, *S. aureus*, *E. coli*, and *P. aeruginosa* [15,40]. Aneb *et al*. reported the highest inhibition activity of hexane and methanolic extract against *R. equi* (25 mm, 12 mm, respectively). While in Algeria, the fruit methanolic extract and aerial part aqueous extract showed the highest inhibitory effects against *S. aureus* with inhibition zone diameter of 22 mm, and 20 mm, respectively [15]. The variability of these results was influenced by, the solvent used, the extract used, the extraction methods as well as bioactive compounds [15,40]. These last act in targeting different mechanisms pathways such as cell membrane and the cytoplasm and also by modifying cell morphology and gene expression [44].

Table 6. Antibacterial activity of *A. longa* extract against *Rhodococcus* sp., *S. aureus*, *E. coli*, and *P. aeruginosa* was determined by agar well diffusion (Ø mm).

	Extract	**Concentrations/ species**	**Zone of inhibition (Ø mm)**					
			GK1 +	**GK2**	**GK3**	***S. aureus*** +	***E. coli*** -	***P. aeruginosa*** -
A. longa	DICH	10 mg/mL	0	0	0	1.05	1	0
		20 mg/mL	0	0.55	0	0.95	0.8	0.23
		30 mg/mL	1	0.6	0	1.2	0	0.26
		40 mg/mL	0.35	0.45	0	0.85	1.1	0
		50 mg/mL	1.55	0.85	0	0.55	0	0
	HEX	10 mg/mL	0	1.3	1.45	8.5	0.6	0
		20 mg/mL	0.8	1.45	1.15	5	1.15	0.36
		30 mg/mL	0	1.1	0	6	1.1	0.5
		40 mg/mL	1.65	1.35	1.8	5	0.75	0
		50 mg/mL	1.9	0	1.35	7	0	0
		60 mg/mL	0	1.25	0	-	-	0.98
		70 mg/mL	0	0	1.45	-	-	0.55
		80 mg/mL	0	0	1.15	-	-	0
	METH	10 mg/mL	1.1	1.25	1	0.15	1	0.3
		20 mg/mL	1.25	1.05	0.75	1	0.8	0.23
		30mg/mL	0.3	1.25	1.2	0.8	0	0
		40 mg/mL	0.6	0	0.85	0.8	1.1	0
		50 mg/mL	0.75	0	0	0.65	0	0
Amoxycillin		50 mg/mL	6	6	7	8	6.5	7
DMSO		1 000 mg/mL	7	6	7	7.5	6	8

Tests were carried out in 3. Bacterial cells were incubated with plant extracts at 37°C for 24 h. Data are expressed as means of inhibition zone in mm ± SD of 3 independent experiments.
(DICH: dichloromethane, HEX: hexane, METH: methanol)

4. Conclusions

The present study showed that methanolic extract, rich in polyphenols and flavonoids, showed remarkable antioxidant activity. However, hexane extract exhibited the highest antibacterial activity against *R. equi* and *S. aureus*, and exhibited the highest anticancer activity against VERO cancerous cell lines. These results justify the use of this plant by the Moroccan population as a beneficial folk plant in the treatment of cancer and as a source of natural compounds in the pharmaceutical industry. Therefore, further research should be conducted to separate these phenols compounds, elucidate their structure, and identify and isolate the

bioactive compounds. These researches are necessary to fully understand the antibacterial and antitumor mechanism of *A. longa*.

Funding

This research received no external funding.

Acknowledgments

This research has no acknowledgment.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Calixto, J.B. The role of natural products in modern drug discovery. *An Acad Bras Cienc* **2019**, *91*, https://doi.org/10.1590/0001-3765201920190105.
2. Patra, J.; Shukla, A.; Das, G. *Advances in Pharmaceutical Biotechnology*. Publisher: Springer, Singapore, **2020**; pp. 3-13, https://doi.org/10.1007/978-981-15-2195-9.
3. El Hachlafi, N.; Chebat, A.; Bencheikh, R.S.; Fikri-Benbrahim, K. Ethnopharmacological study of medicinal plants used for chronic diseases treatment in Rabat-Sale-Kenitra region (Morocco). *Ethnobot Res Appl* **2020**, *20*, http://dx.doi.org/10.32859/era.20.2.1-23.
4. Heinricha, M.; Chan, J.; Wanke, S.; Neinhuis, C.; Simmonds, M.S. Local uses of *Aristolochia* species and content of nephrotoxic aristolochic acid 1 and 2-A global assessment based on bibliographic sources. *J Ethnopharmacol* **2009**, *125*, 108-144, https://doi.org/10.1016/j.jep.2009.05.028.
5. Dey, A.; De, J.N. *Aristolochia indica* L.: A Review. *Asian J Plant Sci* **2011**, *10*, 108-116, https://doi.org/10.3923/ajps.2011.108.116.
6. González, F.; Pabón-Mora, N. Sinopsis actualizada de *Aristolochia* (*Aristolochiaceae*, Piperales) en Panamá. *Acta Bot Mex* **2018**, *122*, 109-140, http://dx.doi.org/10.21829/abm122.2018.1249.
7. Benarba, B. Medicinal plants used by traditional healers from South-West Algeria: An ethnobotanical study. *J Intercul Ethnopharmac*ol **2016**, *5*, 320-330, https://doi.org/10.5455/jice.20160814115725.
8. Dhouioui, M.; Boulilaa, A.; Chaabanea, H.; Zina, M.S.; Casabianca, H. Seasonal changes in essential oil composition of *Aristolochia longa* L ssp *paucinervis* Batt (*Aristolochiaceae*) roots and its antimicrobial activity. *Ind Crops Prod* **2016**, *83*, 301-306, https://doi.org/10.1016/j.indcrop.2016.01.025.
9. El Omari, N.; Sayah, K.; Fettach, S.; El Blidi, O.; Bouyahya, A.; Fouzia, M.E.A.; Kamal, R.; Barkiyou M. Evaluation of *in vitro* antioxidant and antidiabetic activities of *Aristolochia longa* extracts. *eCAM* **2019**, *2019*, https://doi.org/10.1155/2019/7384735.
10. Najem, M.; Belaidi, R.; Slimani, I.; Bouiamrine, E.H.; Jamal Ibijbijen, J.; Nassiri, L. Pharmacopée traditionnelle de la région de Zerhoun - Maroc - : connaissances ancestrales et risques de toxicité. *Int J Biol Chem Sci* **2018**, *12*, 2797-2807, https://dx.doi.org/10.4314/ijbcs.v12i6.25.
11. Touiti, N.; Houssaini, T.S.; Iken, I.; Benslimane, A.; Achour, S. Prevalence of herbal medicine use among patients with kidney disease: A cross-sectional study from Morocco. *Nephrol ther* **2020**, *16*, 43-49, https://doi.org/10.1016/j.nephro.2019.01.007.
12. Zakariya, I.; Ifezouane, J.; Addaoui, A.; Skalli, S.; Bouslimane, Y. Evaluation of the therapeutic and toxicological knowledge of herbalists on the most notified plants in the poison control and pharmacovigilance center of Morocco. *J Pharmacognosy Phytother* **2018**, *10*, 126-132, https://doi.org/10.5897/JPP2018.0507.
13. El Yahyaoui El idrissi, A.; Talbaoui, A.; Bouyahya, A.; Khouchlaa, A.; Bakri, Y.; Tijane, M. Ethnobotanical study on the Bereztem Plant (*Aristolochia longa*) used in the treatment of some diseases in the cities of Rabat, Sale and Temara (Morocco). *JMES* **2018**, *9*, 1914-1921.
14. Merouani, N.; Belhattab, R.; Sahli, F. Evaluation of the biological activity of *Aristolochia longa* L. Extracts. *IJPSR* **2017**, *8*, 1978-1992, https://doi.org/10.13040/IJPSR.0975-8232.8(5).1978-92.
15. Dhouioui, M.; Boulila, A.; Jemli, M.; Schiets, F.; Casabianca, H.; Zina, M.S. Fatty acids composition and antibacterial activity of *Aristolochia longa* L. and *Bryonia dioïca* Jacq. Growing wild in Tunisia. *J Oleo Sci* **2016**, *65*, 655-661, https://doi.org/10.5650/jos.ess16001.
16. Benarba, B.; Pandiella, A.; Elmallah, A. Anticancer activity, phytochemical screening and acute toxicity evaluation of an aqueous extract of *Aristolochia longa* L. *Int J Pharm Phytopharm Res* **2016**, *6*, 20-26.

17. Benarba, B.; Meddah, B. Ethnobotanical study, antifungal activity, phytochemical screening and total phenolic content of Algerian *Aristolochia longa*. *J Inter Ethnopharmacol* **2014**, *3*, 150-154, https://doi.org/10.5455/jice.20140826030222.
18. Derouiche, S.; Zeghib, K.; Gharbi S.; Khelef, Y. Protective effects of *Aristolochia longa* and *Aquilaria malaccensis* against lead-induced oxidative stress in rat cerebrum. *Asian J Pharm Sci* **2019**, *9*, 57-63, https://doi.org/10.5958/2231-5659.2019.00010.9.
19. Benzakour, G.; Amrani, M.; Oudghiri, M. A Histopathological analyses of *in vivo* antitumor effect of an aqueous extract of *Aristolochia longa* used in cancer treatment in traditional medicine in Morocco. *Int J Plant Res* **2012**, 2, 31-35, https://doi.org/10.5923/j.plant.20120202.06.
20. Merouani, N.; Belhattab, R. Acute toxicity of *Aristolochia longa* L. of aqueous extract in mice. *JDDT* **2020**, 10, 4-0, https://doi.org/10.22270/jddt.v10i3.4055.
21. Andersen, O.M.; Markhan, K.R. *Flavnoids: chemistry, biochemistry and applications.* Publisher: CRS press, Taylor and Francis group, Boca Raton, **2006**; https://doi.org/10.1021/np068246q.
22. Bruneton, J. *Pharmacognosy, phytochemistry, medicinal plants*. 4th eds. Publisher: Laviosier Publishing, Paris, **2009**.
23. Borgi, W.; Chouchane, N. Anti-inflammatory activity of saponosides and flavonoids of the root bark of *Zizyphus lotus* L] *Rev Régions Arides* **2007**, *1*, 283-286, https://doi.org/10.1016/j.sajb.2008.01.009.
24. Singleton, V.; Orthofer, R.; Lamuela-Raventos, R.A. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* **1999**, *299*, 152-175, https://doi.org/10.1016/S0076-6879(99)99017-1.
25. Hannache, B.; Bazin, D.; Boutefnouchet, A.; Daudon, M. Effect of plant extracts on the *in vitro* dissolution of cystine stones: A study at the mesoscopic scale. *Prog Urol* **2012**, *22*, 577-582, https://doi.org/10.1016/j.purol.2012.06.001.
26. Mosmann, T. Rapid colorometric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immuno Methods* **1983**, *65*, 55-63, https://doi.org/10.1016/0022-1759(83)90303-4.
27. Daniyan, S.Y.; Mahammad, H.B. Evaluation of the antifungal activities and phytochemical properties of extracts of *Tamaridus indica* against some diseases causing bacteria. *Afri J Biotec* **2008**, *7*, 2451-2453.
28. Bouteldja, R.; Doucene, R.; Bouzid, R.; Moulay, M.; Aggad, H. Biological activity of *Aristolochia longa* L. against some pathogenic bacteria and phytochemical screening. *Facultatea de Medicină Veterinară, Timisoara* **2019**, *52*, 23-30.
29. Subramaniyan, V.; Saravanan, R.; Baskaran, D.; Ramalalingam, S. *In vitro* free radical scavenging and anticancer potential of *Aristolochia indica* L. against MCF-7 cell line. *Int J Pharm Pharm Sci* **2015**, *7*, 392-396.
30. Bourhia, M.; Laasri, F.E.; Moussa, S.I.; Ullah, R.; Bari, A.; Saeed Ali, S.; Kaoutar, A.; Haj Said, A.A.; El Mzibri, M.; Said, G.; Khlil, N.;Benbacer, L. Phytochemistry, Antioxidant Activity, Antiproliferative Effect, and Acute Toxicity Testing of Two Moroccan *Aristolochia* Species. *eCAM* **2019,** *2019,* https://doi.org/10.1155/2019/9710876.
31. Bourhia, M.; Ait Haj Said, A.; Chaanoun, A.; El Gueddari, F.; Naamane, A.; Benbacer, L.; Khlil, N. Phytochemical screening and toxicological study of *Aristolochia baetica* Linn roots: Histopathological and biochemical evidence. *J Toxicol* **2019,** *2019*, https://doi.org/10.1155/2019/8203832.
32. Djeridane, A.; Yousfi, M.; Nadjemi, B.; Maamri, S.; Djireb, F.; Stocker, P. Phenolic extracts from various Algerian plants as strong inhibitors of porcine liver carboxylesterase. *J Enzym Inhib Med Ch* **2006**, *21*, 719-726, https://doi.org/10.1080/14756360600810399.
33. Sivaraj, D.; Shanmugam, S.; Rajan, M.; Sasidharan, S.P.; Sathyanarayanan, S.; Muniyandi, K.; Thangaraj, P.; de Souza Araújo, A.A. Evaluation of *Aristolochia indica* L. and *Piper nigrum* L. methanol extract against centipede Scolopendra moristans L. using Wistar albino rats and screening of bioactive compounds by high pressure liquid chromatography: a polyherbal formulation. *Biomed Pharmacother* **2018**, *97*, 1603-1612, https://doi.org/10.1016/j.biopha.2017.11.114.
34. Ozen, T.; Bora, N.; Yenigun, S.; Korkmaz, H. An investigation of chemical content, enzyme inhibitory propert, antioxidant and antibacterial activity of *Aristolochia bodamae* Dingler (develiotu) (*Aristolochiaceae*) root extracts from Samsun, Turkey. *Flavour Fragr J* **2019,** *00*, 1-14, https://doi.org/10.1002/ffj.3559.
35. Generalić, I.; Ljubenkov, I.; Možina, S.S, Abramović, H.; Šimat, V.; Katalinić, A.; Novak, T.; Skroza, D. Abiotic factors during a one-year vegetation period affect sage phenolic metabolites, antioxidants and antimicrobials. *Ind Crops Prod* **2019,** *141*, https://doi.org/10.1016/j.indcrop.2019.111741.
36. Zhang, C.; Yang, D.; Liang, Z.; Liu, J.; Yan, K.; Zhu, Y, Yang, S. Climatic factors control the geospatial distribution of active ingredients in *Salvia miltiorrhiza* Bunge in China. *Sci Rep* **2019**, *9*, https://doi.org/10.1038/s41598-018-36729-x.
37. Boudieb, K.; Ait Slimane-Ait Kaki, S.; Amellal-Chibane, H. Effect of maturation degree on the fixed oil chemical composition, phenolic compounds, mineral nutrients and antioxidant properties of *Pistacia lentiscus* L. fruits. *Not Bot Horti Agrobo* **2019**, *47*, 836-847, https://doi.org/10.15835/nbha47311534.

38. Mashabela, M.; Mahajan, P.V.; Sivakumar, D. Influence of different types of modified atmosphere packaging films and storage time on quality and bioactive compounds in fresh-cut cauliflower. *Food Packag Shelf Life* **2019**, *22*, https://doi.org/10.1016/j.fpsl.2019.100374.
39. Saad, F.; Mrabti, H.N.; Sayah, K.; Bouyahya, A.; Salhi, N.; Cherrah, Y.; El Abbes, F.M. Phenolic content, acute toxicity of *Ajuga iva* extracts and assessment of their antioxidant and carbohydrate digestive enzyme inhibitory effects. *S Afr J Bot* **2019**, *125*, 381-385, https://doi.org/10.1016/j.sajb.2019.08.010.
40. Aneb, M.; Talbaoui, A.; Bouyahya, A.; EL Boury, H.; Amzazi, S.; Benjouad, A.; Dakka N.; Bakri, Y. *In vitro* cytotoxic effects and antibacterial activity of Moroccan medicinal plants *Aristolochia longa* and *Lavandula multifida*. *EJMP* **2016**, *16*, 1-13, https://doi.org/10.9734/EJMP/2016/28534.
41. Bouyahya, A.; Dakka, N.; Talbaoui, A.; Et-Touys, A.; El-Boury, H.; Abrini, J.; Bakri, Y. Correlation between phenological changes, chemical composition and biological activities of the essential oil from Moroccan endemic Oregano (*Origanum compactum* Benth). *Ind Crop Prod* **2017**, *108*, 729-37, https://doi.org/10.1016/j.indcrop.2017.07.033.
42. Benarba, B.; Ambroise, G.; Aoues, A.; Meddah, B.; Vazquez, A. *Aristolochia longa* aqueous extract triggers the mitochondrial pathway of apoptosis in BL41 burkitt's lymphoma cells. *IJGP* **2012**, *6*, 45-49.
43. Guaouguaou, F.Z.; Alien Ahl Bebaha, M.; Taghzouti, K.; Bouyahya, A.; Bakri, Y.; Dakka, N.; Es-Safi, N.E. Cytotoxicological investigation of the essential oil and the extracts of *Cotula cinerea* and *Salvia verbenaca* from Morocco. *BioMed Reas Inter* **2018**, *2018*, https://doi.org/10.1155/2018/7163961.
44. Bouyahya, A.; Bakri, Y.; Et-Touys, A.; Talbaoui A.; Khouchlaa, A.; Charfi, S.; Abrini, J.; Dakka, N. Resistance to antibiotics and mechanisms of action of essential oils against bacteria. *Phytothérapi*e **2017**, *16*, 173-183, https://doi.org/10.1007/s10298-017-1118-z.