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# Anticancer activity, phytochemical screening and acute toxicity evaluation of an aqueous extract of *Aristolochia longa* L.

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**ABSTRACT**

*Aristolochia longa (Aristolochiaceae) is widely used in traditional medicine. The present study was carried out to investigate the cytotoxic activity and the acute toxicity of an aqueous extract of A. longa roots. Also, the phytochemical composition of the extract was evaluated. The cytotoxic effects of the aqueous extract in triple negative breast cancer MDA-MB-231 and HBL-100 cell lines was evaluated by MTT assay. A. longa roots were screened for the presence of phytochemical constituents using the standard qualitative phytochemical procedures. The acute oral toxicity (5000 mg/kg limited dose test) was evaluated. Our results showed that both cells were inhibited in a dose-dependent manner by A. longa aqueous extract. The $IC_{50}$ of A. longa aqueous extract was estimated after 72h treatment at 40µg/ml and 97µg/ml in HBL100 and MDA-MB-231 cell lines, respectively. A. longa aqueous extract at a concentration of 500µg/ml suppressed effectively the cell growth of HBL100 and MDA-MB-231 cells. TLC analysis revealed the presence of flavonols, flavones and/or flavonoid glycosides as major compounds in the extract. Results of the acute toxicity study suggest the non-toxicity of the A. longa aqueous extract to the liver. Interestingly, the renal function was not affected by the extract administration at 5000mg/kg. A. longa aqueous extract could be toxicologically safe when administered orally in rats in a single dose. A. longa could be considered as a promising and safe source for developing novel therapeutics against breast cancer.*

**Keywords:** *Aristolochia longa*; breast cancer; phytochemical; acute toxicity; TLC.
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## INTRODUCTION

Medicinal plants and other natural products are considered as important sources of new promising bioactive anticancer compounds [1-3]. Numeros potential anticancer molecules such as resveratrol, anthocyanin, damnacanthal, morindone, garcinol, didemnins, plitidepsin bryostatins and dolastatins derive from medicinal plants [4]. *Aristolochia longa* belongs to the genus *Aristolochia* (Aristolochiaceae), the largest genus of the Aristolochiaceae family which is widespread throughout the North Africa, Europe and Asia [5]. Over the last 20 years, there has been considerable interest in members of this genus, which have been the subject of many chemical and pharmacological studies. Anticancer activities have been reported for some species of *Aristolochia* [6]. *Aristolochia longa* is used as an antidote for snakebites, in weight-loss regimens and to prevent arthritis [7]. *Aristolochia longa*, commonly known as "Berrostom" to the local population in Algeria, is widely used in traditional medicine. It has been reported that the most widely uses of *Aristolochia longa* in Algeria are in cancer treatment [8]. The use of this plant as anticancer has been also reported in Morocco [9]. Recently, we have demonstrated that *A.*

*longa* aqueous extract triggered the mitochondrial (intrinsic) pathway of apoptosis in Burkitt's lymphoma BL41 cell line [10].

The present study aimed to investigate the cytotoxic effect of an aqueous extract of *A. longa* roots against breast cancer cell lines and evaluate its acute toxicity in Wistar albino rats. Also, the phytochemical composition of the extract was evaluated.

## MATERIALS AND METHODS

### 2.1 Preparation of *A. longa* aqueous extract

Roots of *A. longa* were collected in March 2009; in "Tissemssilet", an administrative region located in western Algeria. Botanic identification and authentication were made by Dr. Kada Righi (Department of Agriculture, Faculty of Nature and Life Sciences, Mascara University, Algeria). The roots were dried, pulverized and finely sieved. The aqueous extract of *A. longa* was prepared as follows: the dried roots were boiled for 20 min at 100°C, cooled to room temperature, and then filtered. The solution passing through the filter was collected, concentrated, lyophilized and stored in a desiccator at +4°C until use.

### 2.2 MTT assay

The human triple- negative breast cancer MDA-MB-231 and HBL-100 cell lines were cultured in medium with Glutamax supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere with 5% $CO_2$ in air at 37 ◦C. The experiments were performed three times using cells in the exponential growth phase. The effects of the *A. longa* aqueous extract on viability were determined by the colorimetric MTT assay as described previously. Briefly, MDA-MB-231 and HBL-100 cells were seeded at a density of $8\times10^3$ cells/well in 96-well plates and incubated for 24 h at 37 °C. Thereafter, cells were treated with increasing concentrations (from 0.00 to 500µg/ml) of *A. longa* aqueous extract for 24 h, 48h and 72h. At the end of the treatment, 50µl of MTT (0.5mg/ml) were added and the cells were incubated at 37°C, 5% $C0_2$ for 1 hour. After medium removal, 500µl of DMSO were added to each well to dissolve the formazan formed during the reaction and the plate was then shaken for 10min under obscurity. The absorbance was recorded at 570nm using a 96-well plate reader (ASYS-UVM-340). All the experiments were performed in triplicate.

### 2.3 Phytochemical screening

*A longa* aqueous extract was screened for the presence of phytochemical constituents, such as alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins, steroids and glycosides, with the standard qualitative phytochemical procedures described by [11].

### 2.4 Thin layer chromatography (TLC)

TLC of the extract was performed on *Merck Silica gel* 60 F254, 20 x 20 cm. TLC spots were viewed under ultraviolet light at 254 and 366 nm and the Rf values of individual bands were calculated.

### 2.5 Acute toxicity study

Healthy adult albino rats (Wistar strain), of either sex, weighing 165-200g, were used in this study. The rats were used after 14-day period of acclimation to the laboratory environment. Standard diet (ONAB, O/Tlilet, Oran, Algeria) and water were supplied *ad libitum*. The acute oral toxicity (5000 mg/kg limited dose test) was evaluated according to Organization of Economic Cooperation and Development (OECD) guideline 423 [12]. A single high dose of 5,000 mg/kg of *A. longa* extract suspended in vehicle (distilled water) was administered orally to six male rats and six female rats in the treatment groups. The rats were observed for signs of acute toxicity, such as changes in behavior and death, over a 75-hour period. Six male rats and six female rats received 10 ml distilled water/kg body weight as a control. The animals were monitored for apparent signs of toxicity for 14 days. At the end of the experiment, all surviving animals were fasted overnight and killed following institutional guidelines. Blood was collected intracardially in centrifuge tubes, without heparin, for biochemical analyses. The organs, such as the liver and kidneys, were excised and weighed. Macroscopic pathological observations of these tissues were carried out. Standardized diagnostic kits (*SPINREACT*®) were used for spectrophotometric determination of the following biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and urea concentrations.

### 2.6 Statistics

Mean data values are presented, with their standard deviations (mean ± SD). All statistical comparisons were made by Student's *t* test, and statistical significance was defined as $p< 0.05$.

## RESULTS

### 3.1 Anticancer activity of the aqueous extract of *Aristolochia longa*

In this study, we investigated the effects of an aqueous extract of *A. longa* roots on cell viability in vitro, by incubating HBL100 and MDA-MB-231 breast cancer cells with various concentrations of the extract. After 48h and 72h, cell viability was determined using MTT assay. We determined survival as a percentage of that for untreated cells. Our results (Fig.1) show that both cells were inhibited in a dose-dependent manner by *A. longa* aqueous extract after 72h of incubation.

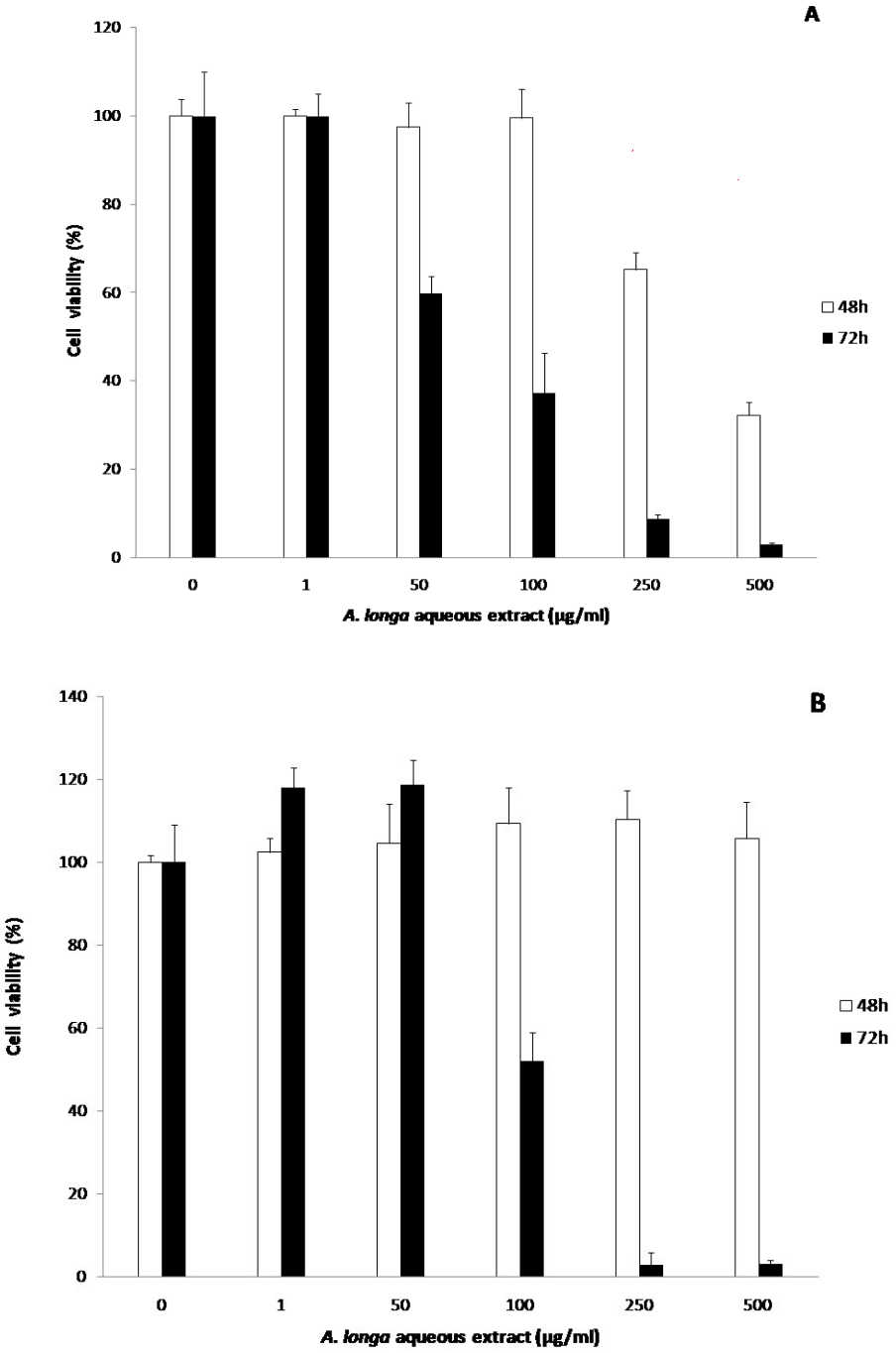


**Fig.1. Effect of *A. longa* aqueous extract on viability of HBL100 (A) and MDA-MB-231 (B) cells**

*Cells were treated with increasing concentrations (from 0.00 to 500 µg/ml) of A longa aqueous extract and cell viability was measured by MTT assay as described in M&M. Per cent survival was determined as compared to untreated cells. The difference in cell viability between untreated and A longa aqueous extract - treated cells were found to be highly significant ($p < 0.001$).*

As shown in Fig.1, at the concentration of 500µg/ml, *A. longa* aqueous extract induced 91.99% and 96.97% cell death of HBL100 and MDA-MB-231 cells, respectively. The $IC_{50}$ of *A. longa* aqueous extract was estimated after 72h treatment at 40µg/ml and 97µg/ml in HBL100 and MDA-MB-231 cell lines, respectively.

### 3.2 Phytochemical screening of *Aristolochia longa*

Phytochemical screening of *A. longa* aqueous extract showed the presence of polyphenols, flavonoids, tannins, c-heterosides, carbohydrates, and saponins. However, alkaloids, coumarins and o-heterosides were not detected. As shown in table 1, results of TLC analysis revealed the presence of flavonoid compounds in the *A. longa* aqueous extract. Regarding Rf values and spots colors, flavonols, flavones and/or flavonoid glycosides are the major compounds in the extract [13-14].

**Table 1: TLC analysis of *A. longa* aqueous extract (Rf values and spots colors)**

| Rf | Color | | Suspected compound |
|---|---|---|---|
| | UV (366nm) | $AlCl_3$ - UV (366nm) | |
| 0.46 | Yellow | Yellow | Flavonol/ flavonoid glycoside |
| 0.80 | Violet/fluorescent | Yellow | Flavone |
| 0.88 | Yellow | Yellow | Flavonol / flavonoid glycoside |

### 3.3 Acute toxicity

We next evaluated the *in vivo* toxicity during oral administration of *A. longa* to rats. No death was observed in the first 24 h and throughout the period of experiment (14 days). The *A. longa* aqueous extract did not produce any signs of sedation like quiescence and reduced locomotion.

In addition, although treated rats exhibited a slight increase in body weight (table 2) no significant modifications were observed between these two groups concerning relative weight of liver and kidneys (table 3).

**Table 2: Effect of aqueous extract of *A. longa* on body weight (g) in control and treated rats**

| Groups | Initial weight | Final weight |
|---|---|---|
| Control | 180.86±20.32 | 210.21±09.88* |
| *A. longa* aqueous extract 5000 mg/kg | 165.23±19.98 | 193.87±46.6** |

*Values are expressed as mean ±S D*

**: Significantly different statistically from the control at p< 0.05, t-test*

***: Significantly different statistically from the control at p< 0.01, t-test*

*Rats (6 male and 6 females) were treated with oral administration of A longa aqueous extract (5000 mg/kg) or control extracts for 14 days. Both groups showed a similar increase in body weight.*

**Table 3: Effect of aqueous extract of *A. longa* on relative weight (g) of liver and kidneys in control and treated rats**

| | | Liver | Kidneys |
|---|---|---|---|
| Control | Absolute weight | 7.07±0.99 | 0.72±0.05 |
| | Relative weight | 0.04±0,005 | 0.004±0,0004 |
| *A. longa* aqueous extract 5000 mg/kg | Absolute weight | 5.89±1.23 | 0.70±0.24 |
| | Relative weight | 0.031±0.01 | 0.005±0.0005 |

*Rats (6 male and 6 females) were treated with oral administration of A longa aqueous extract (5000 mg/kg) or control extracts for 14 days. Treated and control groups did not show any significant effect on the relative weight of various vital organs (liver and kidneys), and all organs were macroscopically (size, color, consistency) comparable to the control (data not shown). Values are expressed as mean ±S D.*

The liver function was explored by measuring levels of hepatic enzymes such as serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). As shown in table 4, expression of hepatic biomarkers was not affected by the *A. longa* aqueous extract administration. There were no significant differences of SGOT levels between the control group (260.93±31.77U/L) and the treated group (251.86±22.39 U/L). Similarly, the levels of SGPT did not vary statistically (control: 55.60±08.24 U/L *vs* 68.61±02.93 U/L). On the other hand, *A. longa* aqueous extract did not affect the renal function as assessed by serum levels of urea and creatinine. Indeed, urea levels were almost the same in control and treated group (7.85±1.75*vs* 8.77±1.36mmol/L). Similar findings are noticed for creatinine (table 4).The results indicated that the medium lethal dose ($LD_{50}$) is higher than 5000 mg/kg for both male and female rats.

**Table 4: Effect of aqueous extract of *A. longa* on some serum biochemical markers in control and treated rats**

| Groups | Hepatic biochemical parameter | | Renal biochemical parameter | |
|---|---|---|---|---|
| | SGOT (UI/l) | SGPT (UI/l) | Urea (mmol/l) | Creatinine (µmol/l) |
| Control | 260.93±31.77 | 55.60±08.24 | 7.85±1.75 | 48.79±5.89 |
| *A. longa* aqueous extract (5000 mg/kg) | 251.86±22.39 | 68.61±02.93 | 8.77±1.36 | 56.71±6.77 |

## DISCUSSION

Cancer has become a major public health concern in Algeria [15]. We have reported that breast cancer was the most reported among Algerian women [16]. Most of the anticancer drugs in current use or being tested in clinical trials is derived from natural sources [17]. *A. longa* is widely used in traditional medicine in Algeria. The genus *Aristolochia* contains many species reported to have anticancer activities [6].

In the present study, cytotoxic effects of *A. longa* aqueous extract in triple negative breast cancer HBL100 and MDA-MB-231 cells were investigated. Our study revealed that *A. longa* aqueous extract had a growth inhibitory effect on HBL100 and MDA-MB-231 cells in a dose – dependent manner. *A. longa* aqueous extract at 500.00µg/ml suppressed effectively the proliferation of HBL100 and MDA-MB-231 cells. The $IC_{50}$ of *A. longa* aqueous extract was estimated to be approximately 40µg/ml and 97µg/ml in HBL100 and MDA-MB-231 cell lines, respectively. These results are in consistence with those we previously obtained. In our previous study we demonstrated that the aqueous extract of *A. longa* induced cell death of Burkitt's lymphoma BL41 cells in a dose-dependent manner. The $IC_{50}$ of *A. longa* aqueous extract was estimated at about15.63µg/ml. The extract induced apoptosis, a loss of mitochondrial membrane potential and the activation of caspases-9 and -3 followed by PARP cleavage [10]. It has been reported that polar extracts of plants belonging to the genus *Aristolochia* induced growth inhibition of human cell lines in a dose dependent fashion. Methanolic extract of *A. macroura* (from Argentina) inhibited growth of Hep G2 cells line in a concentration-dependent manner; the $IC_{50}$ was estimated to be 513±91 µg/ml [18]. Recently, Chaouki *et al.* [19] reported similar activity of four polar extracts of *A. baetica* against MCF-7 cells.

Phytochemical screening of *A. longa* aqueous extract revealed the presence of polyphenols, flavonoids, tannins, c-heterosides, carbohydrates, and saponins. Moreover, TLC analysis revealed the presence of three phytochemicals identified as flavonoids (folavonol, flavones and/or flavonoid glycoside). It is well documented that biological activities of medicinal plants are closely related to their chemical compounds, thus the cytotoxic activity of the *A. longa* aqueous extract shown in this study may be attributed to the flavonoids. Indeed, the potent anticancer activity of extracts from medicinal plants has been associated with their components of phenolic compounds [20]. Natural phenolic compounds play an important role in cancer prevention and treatment. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and also contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signalling pathways [21]. Flavonoids possess strong cytotoxic and apoptogenic activities against several cancer cell lines, including those of the breast [22-23]. Luteolin has been shown to enhance paclitaxel-induced apoptosis in human breast cancer MDA-MB-231 cells by blocking STAT3, and resulted in a decrease in orthotropic tumour growth in nude mice. Wang *et al*. [24] demonstrated that Baicalein (a flavonoid derived from the root of *Scutellaria baicalensis*) suppressed adhesion, migration and invasion of MDA-MB-231 human breast cancer cells. Recently, it has been reported that Quercetin-3-O-glucuronide inhibited invasion of MDA-MB-231 human breast cancer cells by blocking β2-adrenergic signaling [25]. *In vivo*, flavonoids interact with various enzymatic systems. Their inhibition of the enzymes cyclooxygenase and lipooxygenase results in cancer chemoprevention [26]. In our previous study, the total phenolic content of the *A. longa* aqueous extract was found to be 6.07 mg (GAE)/g [27].

Results of the acute toxicity study revealed that, up to 5000 mg/kg (body weight), no death of rats was neither recorded in the control nor in the treated groups. All of the rats gained weight and displayed no significant changes in behavior. No significant variations in the levels of hepatic biomarkers (SGOT and SGPT) were observed, which suggests the non-toxicity of the *A. longa* aqueous extract to the liver. Interestingly, the renal function was not affected by the extract administration at 5000mg/kg. Creatinine is known as a good indicator of renal function. Indeed, creatinine and urea levels did not vary between the control and treated groups. We demonstrate that the aqueous extract could be free of aristolochic acids, the primary constituent of *Aristolochia* [28]. Aristolochic acids are nephrotoxic agents that cause acute renal failure and tubular lesions in experimental animals and humans [29]. In experimental animals, studies showed that the kidneys are the primary site of toxicity by aristolochic acids [30]. Recently, in a retrospective study we demonstrated that the intake of *A. longa* roots by breast cancer post menopausal women is detrimental for kidney function and resulted in high bone resorption, maybe due to the reduction in renal function caused by the aristolochic acids contained in the roots [31]. Furthermore relative weight and gross examination of liver and kidney were also found to be normal, demonstrating that the aqueous extracts did not interfere with the organs. The $LD_{50}$ of our extract is thus higher than 5,000 mg/kg. Plants or plant products with $LD_{50}$ values higher than 5000 mg/kg are considered free of any toxicity [32, 12]. The non toxicity of *A. longa* aqueous extract may be attributed to the low toxicity of its main bioactive compounds [33] or the absence of alkaloids [34]. Regarding these findings, we can state that the *A. longa* aqueous extract could be toxicologically safe when administered orally in rats in a single dose. Similar conclusions have been reported recently by Cherif *et al.*

[35] and Benzakour *et al.* [9]. However, the sub-acute and chronic toxicity studies should be carried out to validate the safety of *A. longa* aqueous extract on long term use.

## CONCLUSION

Results of the present study confirm the ability of *Aristolochia longa* aqueous extract to induce a cytotoxic effect in tumour cells. *A. longa* aqueous extract at a concentration of 500µg/ml suppressed effectively the cell growth of triple negative breast cancer HBL100 and MDA-MB-231 cells. The $IC_{50}$ of *A. longa* aqueous extract was estimated at 40µg/ml and 97µg/ml in HBL100 and MDA-MB-231 cell lines, respectively. This anticancer effect may be due to a synergistic effect among the secondary metabolites revealed by phytochemical screening and identified as flavonoids (flavonol, flavones and/or flavonoid glycoside). Moreover, results of the acute toxicity study revealed that the *A. longa* aqueous extract could be toxicologically safe when administered orally in rats in a single dose. Thus, *A. longa* could be considered as a promising and safe source for developing novel therapeutics against breast cancer.

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