



## Cytotoxic effect of *Myrtus communis*, *Aristolochia longa*, and *Calycotome spinosa* on human erythrocyte cells

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### Abstract:

**Introduction.** *Myrtus communis*, *Aristolochia longa*, and *Calycotome spinosa* are medicinal plants frequently used in Algeria. Some plants can cause a fragility of the erythrocyte membrane and lead to hemolysis. Therefore, we aimed to study the cytotoxicity of aqueous extracts from the aerial part of these species against red blood cells.

**Study objects and methods.** The hemolytic effect was determined spectrophotometrically by incubating an erythrocyte solution with different concentrations of the aqueous extracts (25, 50, 100, and 200 mg/mL) at 37°C during one hour. In addition, we performed phytochemical screening and measured the contents of polyphenols and flavonoids.

**Results and discussion.** After one hour of incubation of human red blood cells with the aqueous extracts at different concentrations, the hemolysis percentage showed a significant leak of hemoglobin with *A. longa* (68.75 ± 6.11%; 200 mg/mL), the most toxic extract followed by *C. spinosa* (34.86 ± 5.06%; 200 mg/mL). In contrast, *M. communis* showed very low cytotoxicity (20.13 ± 3.11%; 200 mg/mL).

**Conclusion.** These plants are sources of a wide range of bioactive compounds but their use in traditional medicine must be adapted to avoid any toxic effect.

**Keywords:** *Myrtus communis*, *Aristolochia longa*, *Calycotome spinosa*, folk medicine, phenolic compounds, alkaloids, hemoglobin, cell toxicity, hemolytic activity

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### INTRODUCTION

Medicinal plants are an important pool of molecules with therapeutic potential for drug innovation [1]. According to Estella *et al.*, vulgarization of traditional herbal remedies is confronted with many predicaments due to the lack of information on their therapeutic and toxicological properties to guarantee their rational use [2]. According to Calixto [3], plants contain hundreds of phytotherapeutic agents with adverse effects and some of them are very toxic if inappropriately used.

In fact, Kharchoufa *et al.* have identified more than 89 toxic medicinal plants used as treatment in the North-Eastern region of Morocco [4]. These plants contain toxic compounds: alkaloids followed by glucosides, terpenoids, proteins, and phenolics. Their toxicity can lead to serious adverse reactions or interactions with

other plants. On the other hand, a misidentification of plants can lead to a toxicity that may also result from an uncontrolled or excessive use [5]. Therefore, before formulating and marketing a herbal medicine, appropriate scientific studies are essential, including those into pharmacological properties, toxicity, and side effects [6].

Algeria has more than 3000 species belonging to several botanical families distributed all along the Mediterranean, Saharan, and tropical regions. *Calycotome spinosa* (L.) belongs to the Fabaceae family, *Aristolochia longa* belongs to the Aristolochiaceae family, and *Myrtus communis* belongs to the Myrtaceae family [7]. Algeria is the only country that hosts both species, *M. communis* (L.) in the North and *Myrtus novelli* in the South [8]. For several centuries,



**Figure 1** 1 – *Myrtus communis*; 2 – *Calycotome spinose*; 3 – *Aristolochia longa*

*M. communis* has been used in folk medicine as treatment for many diseases due to its broad spectrum of pharmacological and therapeutic effects [9]. Species of *Aristolochia* are known for their toxicity and pose potential health risk associated with their content of aristolochic acids [10].

Since these species are widely used in the littoral zone of Algeria in traditional and folk medicine, there is a need for research into their toxicity. In this context, we aimed to evaluate the hemolytic effect on human erythrocyte cells induced by aqueous extracts of *M. communis* (Rayhan), *A. longa* (Bereztem), and *C. spinosa* (Guendoul). The last two species have been rarely studied.

## STUDY OBJECTS AND METHODS

Medicinal plants *Myrtus communis* (L.), *Calycotome spinosa* (L.), and *Aristolochia longa* (L.) were collected in many areas in the littoral of Algeria (March 2018), namely Damous (Tipaza), Benni Haoua (Chlef), and Bissa (Chlef) (Fig. 1). These species were identified by Dr. Belhacini, a teacher and researcher at Hassiba Benbouali University of Chlef (Algeria).

**Preparation of aqueous extracts.** For each species we used dried and powdered plant aerial parts according to traditional use in these areas, namely leaves for *M. communis* and *A. longa* and leaves and flowers for *C. spinosa*. Aqueous extracts were prepared by a decoction of the plant material. In particular, 10 g of the plant material was boiled with 100 mL of distilled water for 15 min and then the solution was filtered and dried at 39°C.

**Phytochemical screening.** Phytochemical tests were performed on 5% infusion to detect certain secondary metabolites according to Takaidza *et al.* and Behbahani *et al.* [11, 12].

**Determination of total phenol contents.** A mixture of 250  $\mu$ L of Folin Ciocalteu phenol reagent, 50  $\mu$ L of the sample, and 500  $\mu$ L of 20%  $\text{Na}_2\text{CO}_3$  was prepared. The volume was adjusted to 5 mL with distilled water while shaking vigorously. After 30 min incubation, absorbance was read at 765 nm. A calibration curve of





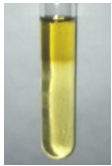




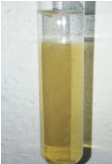




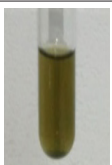
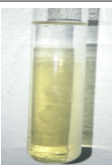

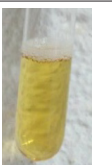
gallic acid (0–1 mg/mL) was done in parallel. The results were expressed in mg of gallic acid equivalent/g of dry matter (mg EAG/g DM) [13].

**Determination of total flavonoid contents.** The flavonoid assay was performed according to the method of Hmid *et al.* [14]. 1 mL of each extract was mixed with 1 mL of 2%  $\text{AlCl}_3$ . After 10 min incubation, absorbance was read at 430 nm. The flavonoid concentrations were calculated using a calibration curve established with quercetin (0–40  $\mu$ g/mL) and expressed in mg of quercetin equivalent/g of dry matter (mg EQ/g DM).

**Hemolytic activity determination.** A phosphate buffered saline (PBS) solution with pH = 7.4 was prepared by mixing the following compounds in appropriate concentrations:  $\text{Na}_2\text{HPO}_4$  (10 Mm),  $\text{KH}_2\text{PO}_4$  (1.8 Mm), KCl (2.7 Mm), and NaCl (137 Mm) [15]. A concentration range for each extract (*M. communis*, *C. spinosa*, and *A. longa*) was prepared by diluting in PBS: 25, 50, 100, and 200 mg/mL. An erythrocyte suspension was prepared from the blood of a healthy donor in a heparin tube. After centrifugation at 2400 rpm for 10 min, the plasma was removed and the pellet was washed twice with PBS and then filled up with the same volume of plasma removed. The erythrocyte suspension was diluted 20 times with PBS.

**Erythrocyte hemolysis assay.** The hemolytic effect test of the species studied was carried out according to the method described by Haddouchi *et al.* and Guo-Xiang and Zai-Qun [16, 17]. We mixed 2950  $\mu$ L of the erythrocyte suspension with 50  $\mu$ L of aqueous extract for each species in a hemolysis tube. The operation was repeated three times for each concentration. The tubes were incubated at 37°C for one hour. During this period, 500  $\mu$ L of each test was taken every 15 min (in 15, 30, 45, and 60 min) and added to 1.5 mL of PBS and then centrifuged again at 2400 rpm for 10 min. The absorbance of the hemoglobin leak in the supernatant was read at 548 nm against a blank containing PBS. A negative control tube was prepared under the same experimental conditions, 2950  $\mu$ L of the erythrocyte suspension and 50  $\mu$ L of the PBS buffer solution. On the other hand, a total hemolysis tube was prepared

**Table 1** Results of phytochemical screening tests for *Myrtus communis*, *Calycotome spinose*, and *Aristolochia longa*

Samples	Saponoside Test	Tanin Test	Flavonoid Test	Anthocyanin Test	Alkaloid test
 <i>Myrtus communis</i> (leaves)	 Foam > 1cm	 Catechin tannins	 Flavonones	 –	 +
 <i>Calycotome spinose</i> (leaves and flowers)	 Foam 0.2 cm	 –	 Flavones	 –	 +++
 <i>Aristolochia longa</i> (leaves)	 Foam > 1,8 cm	 Catechin tannins	 +/-	 +	 –

“+” Present, “++” Moderate presence, “+++” High presence, “+/-” Presence not evident, “–” Absent

containing 250  $\mu\text{L}$  of the erythrocyte suspension and 4750  $\mu\text{L}$  of distilled water. Each test was repeated three times. The hemolysis rate of various extracts was calculated as a percentage (%) of total hemolysis after 15, 30, 45, and 60 min of incubation, according to the following formula:

$$\% \text{ Hemolysis} = \frac{A(\text{extract at 60 min}) - A(\text{negative control at 60 min})}{A(\text{Total hemolysis at 60 min})} \quad (1)$$

**Statistical analysis.** Statistical analysis was done by One Way ANOVA. The data obtained were analyzed using the student's t-test. A P value less than 0.01 was considered statistically significant.

## RESULTS AND DISCUSSION

**Phytochemical screening.** The phytochemical screening allowed us to highlight the presence of some secondary metabolites (saponosides, tannins, alkaloids, flavonoids, and anthocyanins). The phytochemical tests carried out on the infused flowers and leaves of the selected plants are shown in Table 1. The results obtained after shaking the infusion for 15 min showed that *Myrtus communis* and *Aristolochia longa* were rich in saponosides because the foam was greater than 1 cm. In the *Calycotome spinosa* leaf and flower infusion, the foam was unstable in the order of a few mm. The appearance of the orange and pink color after the addition of isoamyl alcohol indicated the presence of flavones in the *C. spinosa* leaf and flower infusion.

The purplish pink color indicated the presence of flavonones in the leaves of *M. communis*. In the *A. longa* infusion, the result was negative. The precipitate in the *C. spinosa* and *M. communis* infusions, which were previously acidified with sulfuric acid, after adding some drops of the Mayer reagent indicated the presence of alkaloids. However, the test was negative for *A. longa*. The appearance of a pink and red coloration after adding ammonia to the HCl-infused *A. longa* and *C. spinosa* indicated the presence of anthocyanins. However, this secondary metabolite was absent in the *M. communis* leaf infusion.

### Contents of total polyphenols and flavonoids.

The amount of polyphenols in the dry matter was expressed in mg gallic acid equivalent (mg EAG/g MS) and determined by the equation:  $y = 0.940x + b$ ;  $R^2 = 0.981$ . The amount of flavonoids in the dry matter was expressed in mg of quercetin equivalent (mg EQ/g MS) and determined by the equation:  $y = 0.055x + b$ ;  $R^2 = 0.996$  (Table 2). The total polyphenol content in the dry matter was  $234.89 \pm 0.80$ ,  $283.68 \pm 0.60$ , and  $346.27 \pm 2.00$  mg GEA/g for *C. spinosa*, *A. longa*, and *M. communis*, respectively. The content of total flavonoids in the dry matter was  $10.50 \pm 0.03$ ,  $34.86 \pm 0.06$ , and  $31.02 \pm 0.19$  mg EQ/g for *A. longa*, *C. spinosa*, and *M. communis*, respectively (Table 2).

**Hemolytic activity.** In the negative control tube (tube containing only PBS and erythrocyte suspension), the hemolysis rate was constant and did not exceed  $2.77 \pm 0.35\%$  after one hour of incubation. On the



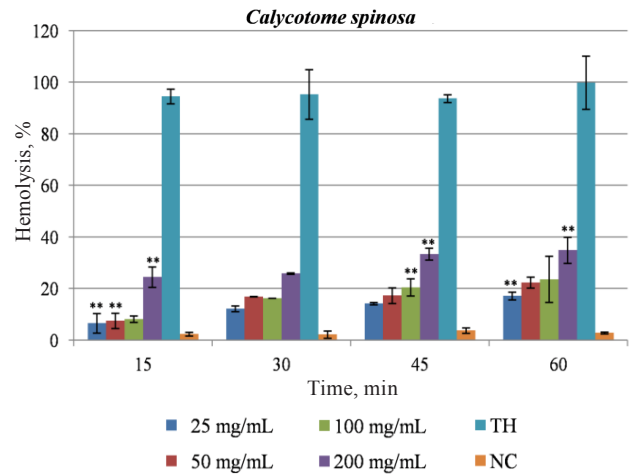
**Table 2** Polyphenol and flavonoid content in *Myrtus communis*, *Calycotome spinosa*, and *Aristolochia longa*

Species	Polyphenols mg GEA/g DM	Flavonoids mg EQ/g DM
<i>Myrtus communis</i>	346.27 ± 2.00	31.02 ± 0.19
<i>Calycotome spinosa</i>	234.89 ± 0.80	34.86 ± 0.06
<i>Aristolochia longa</i>	283.68 ± 0.60	10.50 ± 0.03

other hand, a total hemolysis of red blood cells was clearly observed in the total hemolysis tube. Indeed, we recorded a hemolysis rate that reached  $99.86 \pm 10.32\%$  at 60 min.

For the aqueous extract of the *M. communis* leaves, we observed a significantly low hemolysis rate during the first 15 min ( $P < 0.01$ ). The hemolysis rates were  $5.07 \pm 0.21$ ,  $7.85 \pm 1.20$ , and  $12.57 \pm 2.89\%$  for the concentration of 25 mg/mL;  $6.04 \pm 1.90$  ( $P < 0.01$ ),  $6.46 \pm 0.77$ , and  $20.42\%$  for 50 mg/mL;  $8.05 \pm 1.41$ ,  $11.32 \pm 5.72$ , and  $19.51 \pm 6.71$  for 100 mg/mL; and  $12.01 \pm 0.21$ ,  $12.22 \pm 0.26$ , and  $20.14 \pm 3.11\%$  ( $P < 0.01$ ) for 200 mg/mL, respectively, compared to total hemolysis (Fig. 2).

For *C. spinosa*, we found a significant increase in hemolysis rates over time (15, 30, 45, 60 min). Also, the rates were considerably higher with higher concentrations of the extract. For the concentrations of 25 and 50 mg/mL, hemolysis rates ranged between  $6.52 \pm 3.78$  and  $17.12 \pm 1.50\%$ , as well as  $7.50 \pm 2.95$  and  $22.36 \pm 2.12\%$ , respectively. However, a significant hemolytic effect was recorded in 100 (45 min) and 200 mg/mL (15, 45, and 60 min) of the *C. spinosa* extract. This rate increased from  $8.14 \pm 1.23\%$  at 15 min to  $23.61 \pm 8.94\%$  at 60 min in the presence of a 100 mg/mL concentration and from  $24.44 \pm 3.95\%$  at



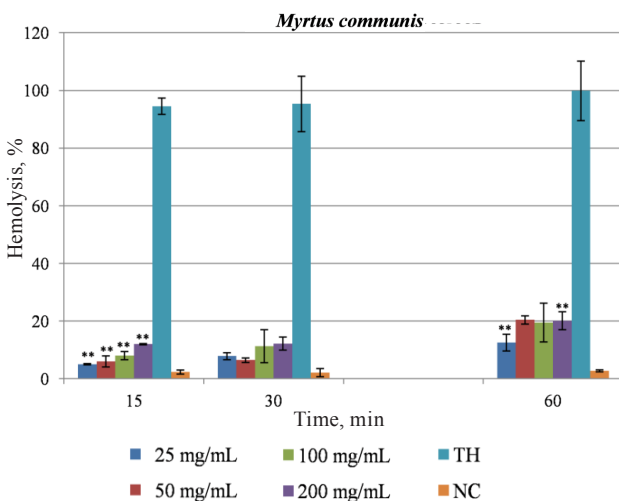
TH: Total Hemolysis. NC: Negative Control. The means of 3 replicates.  $P < 0.01$ . \*\* significant

**Figure 3** Hemolytic effect of four concentrations of *Calycotome spinosa* extract at 15, 30, 45, and 60 min

15 min to  $34.86 \pm 5.05\%$  at 60 min in the presence of a 200 mg/mL concentration (Fig. 3).

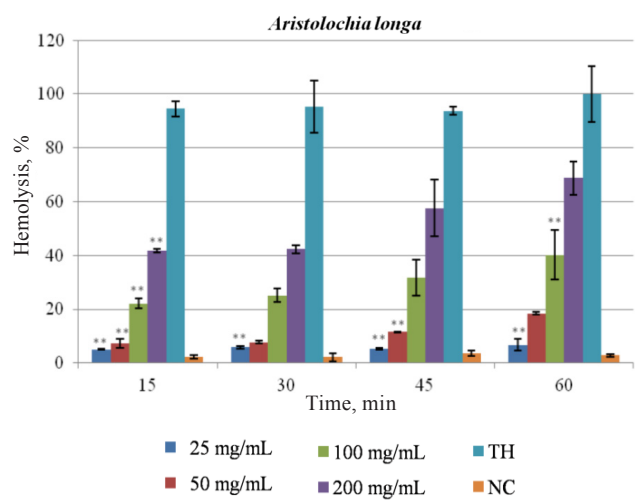
For the extract of the *A. longa* leaves (Fig. 4), we found an increase in hemolysis rates over time (15, 30, 45, and 60 min). As the concentration increased, the percentage of hemolysis increased as well. At concentrations of 25 and 50 mg/mL, a hemolysis percentage ranged from 5 (15 min) to 6.71% (60 min) and from 7.22 (15 min) to 18.47% (60 min), respectively. The hemolysis rate was significant at 15 and 45 min ( $P < 0.01$ ).

On the other hand, we observed an important hemolytic effect of the *A. longa* aqueous extract at concentrations of 100 and 200 mg/mL. This rate went



TH: Total Hemolysis. NC: Negative Control. The means of 3 replicates.  $P < 0.01$ . \*\* significant

**Figure 2** Hemolytic effect of four concentrations of *Myrtus communis* extract at 15, 30, 45, and 60 min



TH: Total Hemolysis. NC: Negative Control. The means of 3 replicates.  $P < 0.01$ . \*\* significant

**Figure 4** Hemolytic effect of four concentrations of *Aristolochia longa* extract at 15, 30, 45, and 60 min

from  $22.12 \pm 1.95$  (15 min) to  $40.23 \pm 9.13\%$  (60 min) and from  $41.71 \pm 0.75$  (15 min) to  $68.75 \pm 6.11\%$  (60 min), respectively. This increase in hemolytic effect remained inferior to total hemolysis.

#### Hemolytic effect of the plants studied at 60 min.

Figure 5 shows the evolution of the hemolytic effect or the leakage of Hb after 60 min for the *A. longa*, *C. spinosa*, and *M. communis* extracts at four concentrations (25, 50, 100, and 200 mg/mL) in a PBS buffer medium (pH 7.4) containing an erythrocyte suspension incubated at 37°C, compared to a negative control tube (PBS + suspension) and a total hemolysis tube (distilled water + suspension).

The *M. communis* species showed a significantly low hemoglobin leakage rate compared to the other species, as well as a  $20.14 \pm 3.11\%$  total hemolysis. This species had a lesser effect on the cell membrane of erythrocytes ( $P < 0.01$ ). However, *C. spinosa* caused a significant intermediate leakage of hemoglobin, compared to *M. communis* and *A. longa*, at 200 mg/mL (60 min), namely in the range of  $34.86 \pm 5.06\%$  ( $P < 0.01$ ). Nevertheless, the most important cytotoxic effect on red cells was produced by the aqueous extract of the *A. longa* leaves, where the leakage rate was  $68.75 \pm 6.11\%$  at 200 mg/mL (60 min) and close to total hemolysis ( $P > 0.01$ ), indicative of the species' high toxicity. These results are phenotypically observable in the supernatant.

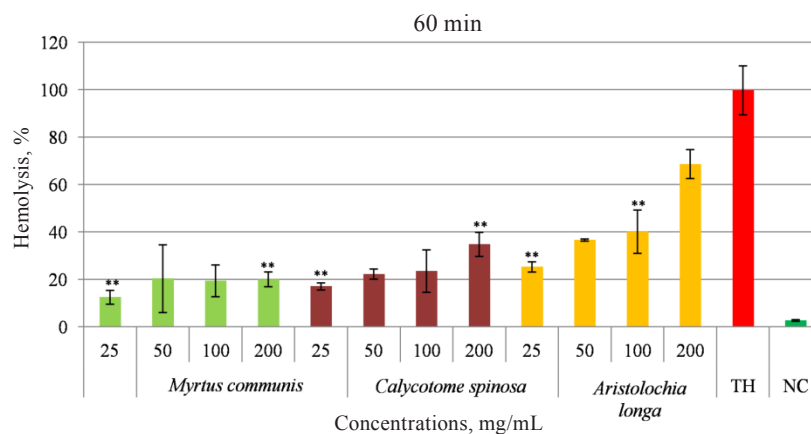
For millennia, humans have been searching for drugs in barks, seeds, fruit organs and other parts of plants to heal themselves and alleviate pain [18]. Nowadays, several studies have been conducted on plants to create new drugs and, to some extent, to evaluate their toxicity and identify their components. Polyphenolic compounds of *M. communis* L. extracts are grouped in three major chemical classes: phenolic acids, tannins, and flavonoids [19]. Our results of the phytochemical screening of *C. spinosa* are consistent with those reported by Cherfia *et al.*, who identified polyphenols, flavonoids, alkaloids, tannins, and saponosides [20]. The *Aristolochia* species

are a source of various active compounds such as aristolochic acid, alkaloids (aporphines, protoberberines, protopines), quinolines, amides, chlorophylls, terpenoids, lignans, flavonoids, tetralones, and steroids [21].

The polyphenol and flavonoid contents that we found in the *M. communis* leaves were higher than those obtained by Bouaziz *et al.*, who reported  $157.70 \pm 2.65$  mg EAG/g MS and  $2.64 \pm 0.22$  mg EQ/g of dry matter [22]. In another study, the hydromethanolic extract of *C. spinosa* leaves had a polyphenol content of  $228.42 \pm 8.86$  and a flavonoid content of  $4.87 \pm 0.12$  [23]. According to Djeridane *et al.*, the methanolic extract of *A. longa* contained  $1.47 \pm 0.20$  mg/g EAG polyphenols and  $0.81 \pm 0.02$  mg/g EQ flavonoids [24]. Our results were in agreement with Merouani *et al.*, who found  $396.88 \pm 8.86$  mg/g EAG polyphenols and  $9.92 \pm 0.23$  mg/g EQ flavonoids [25].

Plants contain toxic compounds in high doses, which makes the evaluation of their hemolytic power indispensable for their correct use in traditional therapy, as well as for choosing the right mode of administration and preserving the integrity of membranes. According to Haddouchi *et al.*, the hemolysis test should be performed even if a plant has a powerful antioxidant power, since its use in traditional medicine and in pharmacological preparations will be impossible in the presence of their hemolytic effect, which is an indicator of cytotoxicity [16]. "Free radicals induce several effects on erythrocytes, such as hemolysis, fluidity of the membrane, changes in morphometry and lipid peroxidation, among others. Erythrocytes potentially promoting the oxidative process are extremely sensitive to oxidative damage because of the polyunsaturated fatty acid content in their cell membranes and their high content of oxygen and hemoglobin" [26].

Many secondary metabolites were revealed in our extracts that may be a cause of cytotoxicity. We found a major lysis of red blood cells treated with *C. spinosa*, which was more prominent when treated with *Aristolochia*, testifying to severe toxicity. We found



TH: Total Hemolysis. NC: Negative Control. The means of 3 replicates.  $P < 0.01$ . \*\* significant

**Figure 5** Hemolytic effect of four concentrations of *Myrtus communis*, *Calycotome spinosa*, and *Aristolochia longa* extracts at 60 min

very few studies on *A. longa* and *C. spinosa*, trying to examine a relationship between the extracts' chemical composition and toxicity.

According to Bissinger *et al.*, saponins – a secondary metabolite identified in aqueous extracts of the plants under our study – may lead to the stimulation of hemolysis as well as to suicidal erythrocyte death [27]. Alkaloids are present in many plants which may be toxic and affect human health [28]. Mahdeb *et al.* reported that alkaloids are capable of disrupting the permeability of the membranes of erythrocytes [29].

As stated by Galati and O'Brien, many adverse effects were associated with dietary polyphenol consumption or exposures such as hemolytic anemia [30]. The authors added that before using these polyphenols for therapy, they need to be assessed for safety.

According to Grollman *et al.*, the toxicity of *Aristolochia longa* is due to a toxin that is a major component of all *Aristolochia* species, namely the aristolochic acid responsible for nephropathic syndromes, although the therapeutic use of *Aristolochia* has rarely taken into account its intrinsic toxicity before [31]. These findings corroborate the study of Touiti *et al.*, which showed that *Aristolochia longa* was incriminated in nephrotoxicity [32].

## CONCLUSION

Some herbs used in traditional therapy in high doses can reveal toxic properties and harm human health. It appears essential to determine their hemolytic capacity as a marker of toxicity for rational adaptation to traditional therapy. We found that *Aristolochia longa* and *Calycotome spinosa* caused significant lyses of red blood cells and a potent leakage of hemoglobin. Therefore, these species cannot be used without control as a therapeutic or pharmacological tool to treat diseases. Furthermore, it is important to perform antitumoral tests on cancer cells with these plant extracts or their chemical compounds to develop anti-cancer drugs.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## CONTRIBUTION

L. Gadouche conceived and designed the analysis, performed the biological experiments, and wrote the paper. A. Zidane and K. Zerrouki contributed to data analysis and revised the paper. K. Azouni and S. Bouinoun performed the biological experiments. All the authors revised the manuscript for publication.


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