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## Antimicrobial, antiplasmodial, haemolytic and antioxidant activities of crude extracts from three selected Togolese medicinal plants

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### ABSTRACT

**Objective:** To investigate the antioxidant, antimicrobial, antiplasmodial, acute toxicity and haemolytic activities of methanolic extracts of three plants. Phytochemical analysis to determine the phenolic contents was also carried out. **Methods:** The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging, NCCLS broth microdilution and *Plasmodium* Lactate Dehydrogenase (pLDH) assays were used to determine antioxidant, antimicrobial and antiplasmodial activities, respectively. Haemolysis assay was conducted on A<sup>+</sup> human red blood cells and acute toxicity on male Swiss albino mice. Phenolics were quantitatively determined using spectrophotometric methods. **Results:** The DPPH assay yielded interesting antioxidant activities of methanolic extract of *Parinari curatellifolia* (*P. curatellifolia*) and *Entada africana* (*E. africana*) (IC<sub>50</sub> were 0.20±0.01 μg/mL and 0.47±0.01 μg/mL, respectively). This activity was highly correlated with phenolic contents of extracts. The antimicrobial tests displayed minimal inhibitory concentrations (MICs) values ranging from 0.90 to 1.80 mg/mL for *Serratia marcescens* (*S. marcescens*) the most susceptible bacterial strain. MIC value was 1.20 mg/mL for susceptible fungal strains including *Mucor rouxi* (*M. rouxi*), *Fusarium oxysporum* (*F. oxysporum*) and *Rhizopus nigricans* (*R. nigricans*). pLDH assay showed moderate antiplasmodial activity of *Balanites aegyptiaca* (*B. aegyptiaca*) (IC<sub>50</sub> = 24.56±3.45 μg/mL), however this extract was highly haemolytic and toxic in mice (LD<sub>50</sub> = 625±128 mg/kg). **Conclusions:** Our results support in part the use of the selected plants in the treatment of microbial infections. In addition the plant showed interesting antioxidant activity that could be useful in the management of oxidative stress.

## 1. Introduction

Using plants for medical purposes dated back to prehistory and people of all continents have this old tradition. Until today, plant-based medicine continues to play a key role in healthcare systems in many regions worldwide, principally in Africa where modern drugs are not affordable<sup>[1,2]</sup>. Indeed, it has been estimated to 80%, the percentage of people who rely on Traditional Medicine (TM) for their primarily health care in Africa<sup>[3]</sup>. Subsequently, a scientific interest for phytotherapy increased in several medical fields such as immunology, oncology, haematology and the bioguided

fractionation of plants extracts has let to the identification of several compounds with interesting pharmacological properties<sup>[4–6]</sup>. Consequently, despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in industrialized countries derived directly or indirectly from plants<sup>[7]</sup>.

Togo is a country located in Western Africa with a border on the Atlantic Ocean. The north of the country belongs to tropical zone with one rainy season and one dry season. The vegetation is essentially constituted of tree and bush savannah. The south of the country belongs to sub-equatorial zone with a long rainy season, and short rainy season. The region consists of disparate forests, relics of gallery forests, savannahs, coastal thickets, meadows or halophilic marshy<sup>[8]</sup>. Globally the country benefits of an excellent biodiversity of medicinal plants<sup>[9]</sup>.

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As in the other sub Saharan countries, the population is mostly rural and depend on TM for their health care. Various ethnobotanical surveys have already been conducted in Togo. However, few pharmacological investigations have been carried out to endorse the flora of Togo. Therefore, the screening of these plants is urgently needed<sup>[9–11]</sup>.

*Balanites aegyptiaca* (*B. aegyptiaca*) (L.) Delile (Balantiaceae), *Entada africana* (*E. africana*) Gill. & Perr. (Fabaceae) and *Parinari curatellifolia* (*P. curatellifolia*) Planch. ex Benth. (Chrysobalanaceae) are three plants widely distributed in the country. These plants are used the treatment of several microbial infections and malaria<sup>[9]</sup>. The present study aimed to evaluate medicinal and therapeutic potentials of these three plants. The antimicrobial, antiplasmodial, acute toxicity, haemolytic and antioxidant properties of these medicinal plant crude extracts were investigated. Phytochemical analysis for the total phenolic and proanthocyanidins contents was also carried out.

## 2. Material and method

### 2.1. Chemical and biochemicals

Culture medium RPMI 1640, bovine foetal serum, HEPES, chloroquine phosphate, catechin, gallic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), phosphate buffer saline (PBS) and Folin reagent were obtained from Sigma Chemical Company (St. Louis). Ascorbic acid and sodium carbonate were from Merck (Germany). L-Glutamine and streptomycin/penicillin were obtained from Gibco BRL. Media for bacteria and fungi were from Biorad (France). All the other chemicals were of analytical grade.

### 2.2. Preparation of extracts

The plant materials were harvested in June 2010 at Sokodé, in Central Region of Togo. The collected samples were aerial parts of *B. aegyptiaca* L. Delile. (Balantiaceae), *E. africana* Guill. & Perr. (Mimosaceae) and *P. curatellifolia* Planch. ex Benth. (Chrysobalanaceae). These samples were botanically authenticated at the Department of Botany of University of Lomé where voucher specimens were referenced and deposited (voucher numbers 10DSK-008, 10DSK-009 and 10DSK-006, respectively). The fresh plant materials were then air-dried at ambient temperature in laboratory and pulverized using a mechanical grinder. The powdered materials were extracted with 70% methanol by cold maceration with constant agitation for 24 h, then filtered through Whatman No.1 filter paper and freeze-dried.

### 2.3. Phytochemical analysis

Total phenolic compounds from lyophilized samples were quantified using Folin-Ciocalteu's method<sup>[12]</sup>. The assay was performed in 96 well-plates. Gallic acid was used as standard and results were expressed as milligram of gallic acid equivalent (GAE) per gram of lyophilized sample.

Proanthocyanidins content was determined using Butanol/HCl method in 96 well plates with catechin as standards<sup>[13]</sup>. Results were expressed as milligram of catechin equivalent (CE) per gram of lyophilized sample.

### 2.4. DPPH radical scavenging activity

The antioxidant activity of the plant extracts was assessed using the stable 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging with ascorbic acid as standard<sup>[14]</sup>. For each test 50  $\mu$  L of the extract or standard (0.01 to 625  $\mu$  g/mL) dissolved in methanol were added to 150  $\mu$  L of 1 mmol/L methanolic DPPH in 96 well plates. After incubation at 37 °C for 30 min, the absorbance was determined at 517 nm. The assay was conducted in duplicate. The free radical scavenging activity (RSA) was calculated as follows:

$$\text{RSA (\%)} = 100 \times (1 - A_s) / A_c$$

Where  $A_s$  was the absorbance of the sample at 517 nm and  $A_c$  the absorbance of the control at 517 nm.  $EC_{50}$  value was the concentration of the sample required to scavenge 50% DPPH free radical.

### 2.5. Antimicrobial test

The antimicrobial activity of the extracts was assayed by determining the minimal inhibitory concentrations (MICs) using the broth microdilution in 96 well-plates according to National Committee for Clinical Laboratory recommendation<sup>[15]</sup>. The following strains were tested: *Escherichia coli* (*E. coli*) CIP 105182, *Proteus mirabilis* (*P. mirabilis*) CIP 588104, *Serratia marcescens* (*S. marcescens*) CIP 6755, *Shigella flexneri* (*S. flexneri*) CIP 8248, *Salmonella paratyphi* (*S. paratyphi*) A. CIP 5539, *Salmonella enteritidis* (*S. enteritidis*) CIP 8297, *Klebsiella pneumoniae* (*K. pneumoniae*) CIP 52144, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Mucor rouxi* (*M. rouxi*) ATCC 24905, *Fusarium oxysporum* (*F. oxysporum*) CIP 525 72 and *Rhizopus nigricans* (*R. nigricans*) ATCC 622713.

### 2.6. Haemolytic assay

Haemolytic assay was carried out on human A+ red blood cells by adopting the method recommended by the World Health Organisation<sup>[16]</sup> with slight modifications. The red blood cells were suspended in the PBS at 2% haematocrit. Fifty microlitres of serial dilution of extracts were added to 150  $\mu$  L of this cell suspension in 96 well plates. The extracts were dissolved in DMSO and diluted with PBS to have a final concentration below 1% in the first wells. Control experiment was conducted separately by replacing the extract by DMSO to ensure that DMSO had no significant influence on cell haemolysis. Another control experiment was performed with extract diluted in PBS without red blood cells to correct for interfering compounds absorbance at 450 nm. The plates were incubated at 37 °C under permanent agitation for 2 h afterwards absorbance was measured at 450 nm. Saponin R was used as standard and results were expressed as saponin equivalent (SE). The experiment was carried out in duplicate.

### 2.7. Antiplasmodial assay

*P. falciparum* was a strain from Burkina Faso grown in 96-well plates as described by Trager and Jensen<sup>[17]</sup>. The culture medium was RPMI 1640 supplemented with L-glutamine (4.2 mmol/L), HEPES (25 mmol/L), bovine foetal serum (10% (v/v)), streptomycin (100  $\mu$  g/mL) and penicillin (100 IU/mL). The parasitemia was 2%. Lyophilised extracts were dissolved in DMSO and then diluted with culture

medium to a final concentration of 0.5% (v/v) DMSO in the first wells. Chloroquine phosphate was dissolved in distilled water. The antiplasmodial activity was quantified by the lactate dehydrogenase assay used by Mesia *et al*[18]. After 48h incubation in a CO<sub>2</sub> incubator at 37 °C under humid atmosphere and 5% CO<sub>2</sub>, the plates were frozen and stored at –20 °C. The plates were thawed, and centrifuged at 2 500 rpm/min for 15 min. In new 96 well plates, 20 μ L of the haemolysed cell suspensions were treated with 180 μ L Cypress® reagent containing 65 mmol/L of imidazol, 0.6 mmol/L of pyruvate and 0.18 mmol/L of NADH. The plates were developed in dark for 2 h, and the absorbance was measured at 340 nm. Results were expressed as the percent reduction in *P. falciparum* present in the extract treated wells compared with the untreated controls.

### 2.8. Acute toxicity

Male albinos Swiss mice (provided by Pasteur Institute, Tunis), 6–8 weeks old and (29.00±4.35) g mean body weight, were synchronized under light/dark cycle: 12/12 in autumn[19,20]. Animals were randomly assigned in different groups to the experimental set up. They were housed in groups of 10 to live in plastic cages with wire-grid bottoms with food and water ad libitum, in a light-controlled room at a temperature of (23±2) °C and 55%–75% selective humidity.

In order to assess for the lethal toxicity (LD<sub>50</sub>) of the three methanolic extracts, mice were intraperitoneally injected with a single dose in a fixed fluid volume (10 mL/kg of body weight). Extracts were dissolved in DMSO and diluted in saline water and four doses were prepared for each extract. Each dose was tested on 10 mice. Animals receiving the vehicle (DMSO–saline water) served as control. Animal's behaviors (signs and symptoms) were monitored constantly during the first three hours following injection. The number of deaths was computed 72 h later and the LD<sub>50</sub> was determined by Miller and Tainter method[21].

### 2.9. Statistical analysis

Results were expressed as mean followed by standard deviation. Statistical significance was determined by one-way analysis of variance Fisher's test, with the level of significance at  $P < 0.05$ . Linear regression analysis was used to determine IC<sub>50</sub> values and to observe the correlation between the phenolic contents and antioxidant activities.

## 3. Results

### 3.1. Phenolic content and free radical scavenging

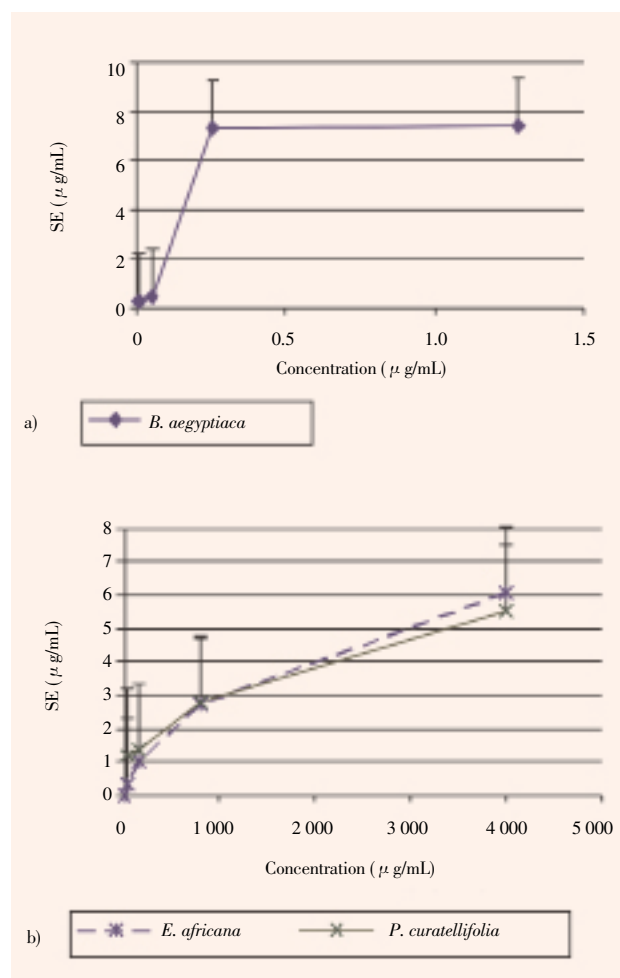
The results of chemical analysis are summarised in Table 1. According to the table, the highest concentration of total phenolics was detected in leaves of *P. curatellifolia* and *E. africana* (34.67±1.10) mg GAE/g and (20.34±1.26) mg GAE/g, respectively). Similarly these extracts had a higher amount of proanthocyanidins, (49.08±3.10) mg CE/g for *E. africana* and (25.82±2.58) mg CE/g for *P. curatellifolia*. However, there was low correlation between total phenolics content and proanthocyanidins content ( $r^2 = 0.574$ ). Indeed extract with highest total phenolics contents; *P. curatellifolia* did not contain largest amounts of proanthocyanidins.

The radical scavenging activity was expressed as a

percentage decrease in absorbance at 517 nm. All extracts showed a significant percentage radical scavenging activity in dose dependent manner. The assay yielded interesting IC<sub>50</sub> values with *P. curatellifolia* (0.20±0.01) μ g/mL and *E. africana* (0.47±0.01) μ g/mL. These two extracts displayed very high antioxidant activity compared with the standard antioxidant, ascorbic acid. This activity was highly correlated with the total phenolic content ( $r^2 = -0.902$ ) and the proanthocyanidins content ( $r^2 = -0.870$ ).

### 3.2. Haemolytic activity

The haemolytic assay revealed that total haemolysis occurred with concentrations high than 6 μ g/mL saponin R and the measured optical density was above 210 at 450 nm. The results of the assay are presented in Figure 1. According to the figure the haemolytic effect was dose dependent and the extract of *B. aegyptiaca* had the highest haemolytic activity. The haemolytic capacity of this extract was more than 15 times greater than saponin R.



**Figure 1.** Haemolytic effects of plant extracts on human A+ red blood cells.

### 3.3. Antibacterial activity

Methanolic extracts of plants screened in the present study yielded MIC values in the range of 0.90 to 1.80 mg/mL for *S. marcescens* CIP 6755 which was susceptible to all extracts (Table 2). Some bacteria were selectively inhibited by one

**Table 1**

DPPH radical scavenging and phenolic content of extract.

Extract	Total phenolic content GAE (mg/g)	Proanthocyanidins content CE (mg/g)	DPPH inhibition IC <sub>50</sub> (μg/mL)
<i>Balanites aegyptiaca</i>	1.76±0.19	1.38±0.13	52.53±12.89
<i>Parinari curatellifolia</i>	34.67±1.10	25.82±2.58	0.20±0.01
<i>Entada africana</i>	20.34±1.26	49.08±3.10	0.47±0.01
Ascorbic acid	–	–	4.79±0.02

**Table 2**

Antibacterial activity of plant extracts expressed as minimal inhibitory concentrations (mg/mL).

Microorganisms	<i>B. aegyptiaca</i>	<i>E. africana</i>	<i>P. curatellifolia</i>
<i>Escherichia coli</i> CIP 105182	>2.50	>2.50	>2.50
<i>Proteus mirabilis</i> CIP 588104	>2.50	1.80	1.80
<i>Serratia marcescens</i> CIP 6755	0.90	1.80	1.80
<i>Shigella flexneri</i> CIP 8248	>2.50	>2.50	1.80
<i>Salmonella paratyphi A</i> . CIP 5539	>2.50	>2.50	1.80
<i>Salmonella enteritidis</i> CIP 8297	>2.50	>2.50	>2.50
<i>Klebsiella pneumoniae</i> CIP 52144	>2.50	1.80	>2.50
<i>Staphylococcus aureus</i> ATCC 25923	>2.50	>2.50	>2.50
<i>Mucor rouxi</i> ATCC 24905	1.20	1.20	>2.50
<i>Fusarium oxysporum</i> CIP 52572	>2.50	>2.50	>2.50
<i>Rhizopus nigricans</i> ATCC 622713	>2.50	>2.50	1.20

**Table 3**

Antiplasmodial activity and acute toxicity of the plant extracts.

Extract	IC <sub>50</sub> (μg/mL)	LD <sub>50</sub> (mg/kg)
<i>Balanites aegyptiaca</i>	24.56±3.45	625±128
<i>Entada africana</i>	>100	950±197
<i>Parinari curatellifolia</i>	>100	1 225±195
Chloroquine phosphate	0.15±0.03	–

Values are the means of two measurement followed by standard deviations.

or two extracts. Indeed, *Proteus mirabilis* CIP 588104 was inhibited by *E. africana* and *P. curatellifolia* (MIC = 1.80 mg/mL for both two extracts), while. *S. flexneri* CIP 8248 and *S. paratyphi A* CIP 5539 were inhibited by *P. curatellifolia* (MIC = 1.80 mg/mL for both two strains). *K. pneumoniae* CIP 52144 was inhibited by *E. africana*. The other bacteria such as *E. coli* CIP 105182, *S. aureus* ATCC 25923 and *S. enteritidis* CIP 8297 resisted to all tested extracts (MIC > 2.50 mg/mL). Fungal strains tested also showed selective susceptibility to extracts. Thus *F. oxysporum* CIP 525 72 resisted to the three tested extracts while *M. rouxi* ATCC 24905 was susceptible to *B. aegyptiaca* and *E. africana* (MIC = 1.20 mg/mL for both two extracts). *R. nigricans* ATCC 622713 resisted to *B. aegyptiaca* and *E. africana*, although it was inhibited by *P. curatellifolia* (MIC = 1.20 mg/mL).

### 3.4. Antiplasmodial activity

The results of the antiplasmodial assay are displayed in Table 3. According to the table, the lowest IC<sub>50</sub> was recorded with the methanolic extract of *B. aegyptiaca* (24.56±3.45) μg/mL. The other extracts yielded IC<sub>50</sub> values greater than 100 μg/mL.

### 3.5. Acute toxicity

The lethal doses killing 50% of treated mice (LD<sub>50</sub>) of plant methanolic extracts are displayed in Table 3. The lowest LD<sub>50</sub> was recorded with the extract of *B. aegyptiaca* followed by *E. africana* and *P. curatellifolia*. Most of deaths were observed within five hours for mice treated with *E. africana*

and *P. curatellifolia*; or seven hours for mice treated with *B. aegyptiaca* following injection. The survival rate remained subsequently unchanged until day 3. Tail reaction, and decrease respiration was observed with mice treated with *E. africana* and *P. curatellifolia*. For the mice treated with the extracted of *B. aegyptiaca* the main symptoms were jumping, running, and respiration decrease before death.

## 4. Discussion

The present study aimed to ascertain few pharmacological properties of methanolic extracts of three medicinal plants. The phenolic compounds quantified in the extracts seemed to be responsible for the free radical scavenging activity of the extracts according to correlation coefficients. Phenolics are a group of highly hydroxylated compounds present in the extractive fraction of several plant materials. Polyphenols in plants include hydroxycoumarins, hydroxycinnamate derivatives, flavanols, flavonols, flavanones, flavones, anthocyanins and proanthocyanidins often called condensed tannins. The antioxidant activity of polyphenols is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching oxygen, or decomposing peroxides. Indeed, the antioxidant activity of some plant extracts is highly correlated with their phenolics content<sup>[22–24]</sup>. Crude methanolic extracts of *P. curatellifolia* and *E. africana* leaves exhibited significant antioxidant activity which may be relevant in the treatment of oxidative stress, although it is difficult to extrapolate this



directly to preparations used by the traditional healers. The moderate antioxidant activity of *B. aegyptiaca* could be due then be in relation with its low content in phenolics, however Speroni *et al*[25] found good antioxidant activity of both methanol and butanol extracts of the plant and single saponin compounds isolated from the plant, using Briggs–Rauscher reaction. Investigation of fixed oil composition of fruits of the plant and evaluation of its biological activity also resulted in interesting antioxidant activity probably because of synergistic effect of oleic and linoleic acid in addition to sterols present in the oil[26].

Many plants used in traditional medicine worldwide contain saponins, which can often account for their therapeutic action including antibacterial, antiviral, anti-inflammatory, antiprotozoal and antitumor activities[29].

However, the most characteristic property of saponins is their ability to cause haemolysis. When added to a suspension of blood, they produce changes in erythrocyte membranes causing haemoglobin diffusion into surrounding medium. Previous studies showed that saponin-lysed erythrocytes do not reseal, suggesting that saponin damage to the lipid bilayer is irreversible[27]. In the present study, *B. aegyptiaca* showed highest haemolytic activity.

Several reports indicated the presence of high amount of steroidal saponins in the plant[25,28]. Although saponins are extremely toxic to cold-blooded animals, their oral toxicity to mammals is low[30]. It is evidence that the main route of administration of traditional concoctions is oral, but in the case of *B. aegyptiaca* this plant is used to treat wound by directed application in some cases[31–33]. Concoctions based on this plant should be therefore managed with precaution to avoid notable adverse effects, according to our results.

A key factor in curing chronic wound, gastroenteritis and infectious diseases is the failure to combat multi-factorial infections including *E. coli*, *Staphylococcus*, *Pseudomonas* and *Proteus* species[34–36]. According to Fabry *et al*[37] for crude extracts of plants to be considered as potentially useful therapeutically, they must have MIC values < 8 mg/mL, whilst Gibbons[38] suggests that isolated phytochemicals should have MIC < 1 mg/mL.

According to our results *B. aegyptiaca* was active on one fungal strain and two bacteria. Similarly Maregesi *et al*[39–43] reported selective antimicrobial activity on both fungal and bacterial strains. Indeed the methanol extract of the aerial part of the plant was active on bacterial strains principally gram positive ones while the bark extract was active on *C. albicans*. *E. africana* inhibited three bacteria and one fungal strain and *P. curatellifolia* four bacteria and one fungal strain. Although data are insufficient to state on the antimicrobial activity of these extracts, phenols could be partially or totally incriminated according to their high amount. The antimicrobial properties of phenols are well documented[44]. Indeed, phenols have been reported to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins. The activity may be due to iron deprivation or hydrogen bonding with vital proteins such as microbial enzymes[45,46].

According to our previous results, antiplasmodial activity was classified as follows: highly active at  $IC_{50} < 5 \mu\text{g/mL}$ , promising at  $5\text{--}15 \mu\text{g/mL}$ , moderate at  $15\text{--}50 \mu\text{g/mL}$  and inactive at  $> 50 \mu\text{g/mL}$ [47,48]. In vitro screening against *P. falciparum* strain in the present study, supplied one extracts (*B. aegyptiaca*) showing moderate activity, the two other extracts (*E. africana* and *P. curatellifolia*) displaying

no significant activity (Table 3). However Clarkson *et al*[49] found a good antiplasmodial activity of Dichloromethane extract of the roots of *P. curatellifolia* with  $IC_{50}$  values in the range of 7 and  $5 \mu\text{g/mL}$ . In our knowledge the other plants were not yet screened for antiplasmodial activity. As mentioned above, the methanolic extract of *B. aegyptiaca* is a saponin-rich extract that causes erythrocytes lyses. This property could partially contribute to the moderate antiplasmodial activity.

According to the  $LD_{50}$  value of the methanolic extract of *B. aegyptiaca*, this extract could be considered as toxic. Once again the saponin could be incriminated since the administration of the extract was by intra peritoneal injection.

The results of the present study support partially the use of the selected plants in traditional medicine notably in the treatment of microbial infections. However the extract of *B. aegyptiaca* should be used with care since this extract showed high haemolytic activity and acute toxicity.

### Conflict of interest statement

We declare that we have no conflict of interest.

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