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PHARMACOGNOSIE

Antioxidant Activity, Phenolic Content and Hematoprotective Effects of *Cleome arabica* Polyphenolic Leaf Extract

Activité antioxydante, contenu phénolique et effets hématoprotecteurs de l'extrait polyphénolique des feuilles de *Cleome arabica*

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Abstract In this study a polyphenolic extract from *Cleome arabica* leaves (CALE) was investigated for its antioxidant activity in vitro using DPPH•, metal chelating and reducing power methods and for its protective effects against AraC-induced hematological toxicity in vivo using Balb C mice. Results indicated that CALE exhibited a strong and dose-dependent scavenging activity against the DPPH• free radical (IC_{50} = 4.88 µg/ml) and a high reducing power activity (EC_{50} = 4.85 µg/ml). Furthermore, it showed a good chelating effects against ferrous ions (IC_{50} = 377.75 µg/ml). The analysis of blood showed that subcutaneous injection of AraC (50 mg/kg) to mice during three consecutive days caused a significant myelosupression ($P < 0.05$). The combination of CALE and AraC protected blood cells from a veritable toxicity. Where, the number of the red cells, the amount of hemoglobin and the percentage of the hematocrite were significantly high. On the other hand, AraC cause an elevation of body temperature (39 °C) in mice. However, the temperature of the group treated with CALE and AraC remained normal and did not exceed 37.5 °C. The observed biological effects of CALE, in vitro as well as in vivo, could be due to the high polyphenol and flavonoid contents. In addition, the antioxidant activity of CALE suggested to be responsible for its hematoprotective effect.

Keywords *Cleome arabica* · AraC · Hematological toxicity · Antioxidant activity · Polyphenolic extract

Résumé Dans cette étude, un extrait polyphénolique des feuilles de *Cleome arabica* (CALE) a été évalué pour son activité antioxydante in vitro en utilisant les tests du DPPH•, de chélation du fer et du pouvoir réducteur et pour ses effets protecteurs in vivo contre la toxicité hématologique induite par AraC chez la souris. Les résultats obtenus ont indiqué que CALE a exercé une forte et dose-dépendante activité antiradicalaire contre le radical libre DPPH• (IC_{50} 4,88 µg/ml), un puissant pouvoir réducteur (EC_{50} = 4.85 µg/ml) et une bonne activité chélatrice (IC_{50} = 377,75 µg/ml). L'analyse du sang a montré qu'une injection sous-cutanée d'AraC (50 mg/kg) à des souris pendant trois jours consécutifs a entraîné une myélosuppression significative ($p < 0,05$). La combinaison du CALE et d'AraC a protégé les cellules sanguines contre une véritable toxicité. En fait, le nombre des globules rouges, la quantité d'hémoglobine et le pourcentage de l'hématocrite sont significativement plus élevés. En plus, l'extrait a exercé une activité antipyrétique par le maintien de la température corporelle des souris au tour de 37 °C chez le groupe traité par la combinaison EFCA/AraC, contre 39 °C chez le groupe traité par l'AraC seule. En conclusion, les activités biologiques du CALE, observés in vitro ainsi qu'in vivo, sont probablement dues à sa richesse en polyphénols et en flavonoïdes. En outre, l'activité antioxydante du CALE semble être responsable de son effet hématoprotecteur.

Mots clés *Cleome arabica* · AraC · Toxicité hématologique · Activité antioxydante · Extrait polyphénolique

Abbreviations

BHT : butylated hydroxyl toluene
CALE : *Cleome Arabica* leaf extract
CMC : carboxymethyl cellulose
DPPH• : 1,1-diphenyl-2-picrylhydrazyl radical
Ferrozine : 3-(2-Pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine

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Introduction

Most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells, and consequently cause side effects such as immunosuppression, myelotoxicity, nausea, vomiting, diarrhea, anemia and alopecia. Myelotoxicity, including leukopenia and anemia, is the most serious among these side effects [1,2].

AraC (Cytarabine) is an anticancer drug of marine origin, isolated from the Caribbean sponge *Cryptothecacrypta*. Currently it is used in a routine treatment of patients with leukemia and lymphoma [3,4]. Some studies have suggested that AraC may be useful in inhibiting the growth of human colon carcinoma cell lines [5]. The main adverse event associated with AraC administration is the hematological toxicity. Thus, AraC is a potent myelosuppressive agent capable of producing severe leukopenia, thrombocytopenia and anemia [6]. Furthermore, high doses of AraC may result in skin, gastrointestinal and cerebellar toxicity [7,8].

Therefore, a search for compounds which can reduce the harmful side effects of anticancer drugs in normal tissues is necessary. Thus, herbal medicine and plant metabolites that can protect bone marrow cells may be useful to ameliorate the myelotoxicities caused by anticancer agents [2]. *Cleome arabica* is a desert plant abundantly distributed in the North Africa [9].The leaves of *Cleome arabica* are commonly used in the traditional medicine of North Africa populations as a sedative for abdominal and rheumatic pains [10]. It was reported by Ismail et al. [11] that *Cleome arabica* leaf and twigs extract is rich in glucosylated and rhamnosylated flavonoids. Several studies indicated that *Cleome arabica* leaf extract (CALE) possess anti-inflammatory, antioxidant and anticancer activities [12–14].

This study aimed to evaluate the antioxidant activity of CALE in vitro and to investigate its protective effects on AraC-induced hematotoxicity and fever in mice.

Materials and methods

Chemicals and reagents

Quercetin, carboxymethyl cellulose (CMC), trolox, butylated hydroxyl toluene (BHT), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) were obtained from Sigma (Germany). AraC was obtained from Pfizer (Mexico). All other reagents used were analytical grade.

Animals

Male and female Balb C mice weighting between 20 and 40 g were obtained from Pasteur Institute of Algiers, Algeria. They were housed in standard cages (48 cm × 35 cm × 22 cm) at room temperature (22 ± 2 °C) for 12-h light/dark cycle. Mice were acclimatized at least for 1 week prior use with free access to food and water. All procedures were performed in accordance with the European Union Guidelines for Animals Experimentation (2010/63/EU).

Plant material

Cleome arabica was collected from Boussaâda (Oued Maiter), Algeria, in March, 2007. Plant material was authenticated by an experienced botanist, Laboratory of Botany, University of Sétif, Algeria and where a voucher specimen (No. C.a. 2007-1) was deposited.

Preparation of polyphenolic extract

Polyphenols were extracted from *Cleome arabica* leaves according to Markham [15]. Briefly, the air dried leaves (100 g) of *Cleome arabica* were powdered using an electric mill and extracted three times with 200 ml of methanol–water (7:3 v/v) for 24 h. The collected filtrates were concentrated under reduced pressure (Rotavapor, Büchi) and 100 ml of boiled water was added to the residue and then incubated for 24 h at 4 °C. Addition of 150 ml of ethyl acetate to the aqueous layer yielded the formation of a yellow precipitate. This precipitate was washed with distilled water for several times and lyophilized to give a yellow powder (yield: 1% w/w).

Total polyphenol estimation

Total polyphenol contents were estimated by Folin–Ciocalteu method [16]. To 0.5 ml of the aqueous solution of the extract, 7 ml of distilled water and 0.5 ml of Folin–Ciocalteu reagent were added. After 3 min, 2 ml of 20 % Na_2CO_3 were added and the mixture was incubated at 100 °C for 1 min. After cooling in darkness, the absorption was measured at 685 nm. Results were expressed as gram per 100 g of dry matter with respect to tannic acid serving as standard.

Flavonoid content estimation

The flavonoid contents were estimated by the aluminum chloride method [16]. One milliliter of methanol extract was added to 1 ml of 2 % methanolic $AlCl_3$. After 10 min, the absorption was read at 430 nm. Results were expressed as gram per 100 g of dry matter with respect to quercetin serving as standard.

Antioxidant activity of CALE

DPPH• free radical scavenging activity

The method of Blois [17] was used with slight modifications in order to assess the DPPH$^{\bullet}$ free radical scavenging capacity

of CALE in comparison with trolox and quercetin as standard antioxidants. This method is based on scavenging of the stable free radical DPPH$^{\bullet}$ by antioxidants, which produces a decrease in absorbance at 517 nm. Briefly, 1,000 µl of DPPH$^{\bullet}$ solution in methanol (0.004 %) were mixed with 1,000 µl of plant polyphenolic extract solution (1–20 µg/ml, final concentrations). The mixture was then vortexed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and the result was expressed as a percentage DPPH$^{\bullet}$ scavenging activity relative to the control, using the following equation:

% Radical scavenging activity = [(Abs_{517} control − Abs_{517} sample)/Abs_{517} control] × 100

Ferrous ions (Fe^{++}) chelating activity

The chelating of ferrous ions by CALE and standard molecules was estimated by the method of Dinis et al. [18]. Briefly, CALE (200–1,000 µg/ml, final concentrations) prepared in methanol was added to a solution of 0.6 mM $FeCl_2$ (100 µl). The reaction was initiated by the addition of 5 mM of ferrozine (100 µl) and the total volume was adjusted to 1,600 µl with methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The chelating effect was calculated as a percentage using the following equation:

The chelating activity (%) = [($Abs_{562control}$/$Abs_{562sample}$)/$Abs_{562control}$] × 100

where $Abs_{562control}$ is the absorbance of control reaction mixture without the test compounds, and $Abs_{562sample}$ is the absorbance of the test compounds.

Total reducing capability using the potassium ferricyanide reduction method

The method of Oyaizu [19] was used to determine the reducing power of CALE. Different concentrations of CALE (2–12 µg/ml) in 1 ml of distilled water were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%) and incubated at 50 °C for 20 min. Thereafter, 2.5 ml of trichloro-acetic acid (10%) was added to the mixture, which was then centrifuged at 1,000 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. BHT was used as standard antioxidant. Increased absorbance of the reaction mixture indicates increase in reducing power.

Treatment of animals and collection of blood samples

Animals were divided into four groups consisting each of 6–8 mice. The two first groups (control groups) received intraperitoneal injection doses of the vehicle CMC (1%) during 6 days. From the 4th to the 6th day the first group was injected subcutaneously by AraC (50 mg/kg) and the second group was injected by the saline solution (NaCl, 0.9%). The 3rd and 4th groups (CALE and CALE combined with AraC, CALE/AraC) received intraperitoneal injection doses of CALE (100 mg/kg) during 6 days. From the 4th to the 6th day, they were injected subcutaneously by the saline solution (NaCl, 0.9%) or AraC (50 mg/kg), respectively. In the 7th day, mice were sacrificed and blood was obtained via cardiac puncture by means of a 5 ml hypodermic syringe and needle and placed in test tubes containing EDTA as an anticoagulant.

Hematological analysis

The red blood cell (RBC) and the white blood cell (WBC) counts, hemoglobin (Hb) and hematocrite were estimated using an automatic hemocytometer (Beck Man coulter, USA).

Body temperature measurement

Every day during the period of the experiments, the mice body temperature of the whole groups was taken by ear measurement using an electronic thermometer (THERMOVAL®).

Statistical analysis

The data are expressed as mean ± SEM (n = 3 independent experiments in vitro, n = 6–8 mice in vivo). A statistical analysis was performed using Student's t-test with the GraphPad INSTAT software system for Windows. In all cases, $P < 0.05$ was considered significant. Half inhibitory concentrations (IC_{50}) were calculated using Excel software.

Results and discussion

As previous study reported [12], the quantitative estimation of total polyphenols and flavonoids showed that CALE is relatively rich in polyphenols (322.1 ± 3.44 mg equivalent of gallic acid/g of extract) and, particularly, in flavonoids (245.6 ± 4.67 mg equivalent of quercetin/g of extract) using gallic acid and quercetin as standards, respectively.

DPPH$^{\bullet}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [20]. The reduction capability of DPPH$^{\bullet}$ radicals induced by antioxidants was determined by the decrease in its absorbance at 517 nm. Figure 1 illustrates a strong scavenging activity of CALE with an IC_{50} = 4.88 µg/ml. This value is comparable with those obtained with trolox and quercetin (IC_{50} = 4.77 µg/ml and IC_{50} = 4.78 µg/ml, respectively).

At 10 µg/ml, CALE, quercetin and trolox exerted scavenging activities against DPPH$^{\bullet}$ radical more than 94%.

The chelating activity of ferrous ions (Fe^{2+}) by CALE was determined. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents such as antioxidant compounds, the complex formation is disrupted, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows an estimation of the metal chelating activity of the coexisting chelator. In this test, just high concentrations of CALE (200–1,000 µg/ml) showed a good chelating activity with an IC_{50} of 377.75 µg/ml. Therefore, at 1,000 µg/ml the chelating activity of CALE was 93.3%. However, small concentrations of CALE (10–100 µg/ml) showed a very week chelating activity (Fig. 2A). The EDTA is a very strong chelating agent (IC_{50} = 4.79 µg/ml) and it showed approximately 100% chelating activity at 20 µg/ml (Fig. 2B).

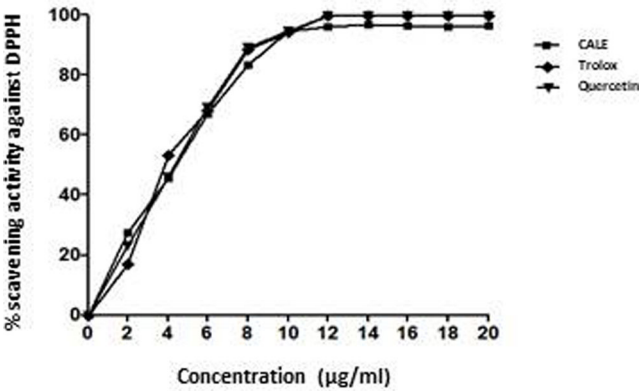

Fig. 1 DPPH$^{\bullet}$ radical scavenging activity of CALE (2–20 µg/ml) compared with quercetin and trolox. Values are expressed as means ± SEM (N = 3)

Measurement of reducing potential can reflect some aspects of antioxidant activity in the extract. In this method ferric ions (Fe^{+3}) are reduced to ferrous ions (Fe^{+2}) with color change, from yellow to bluish green. The intensity of the color depends on the reducing potential of the compounds present in the medium. Greater the intensity of the color, greater will be the absorption, consequently, greater will be the antioxidant activity [21]. Results indicate that CALE exerted a stronger reducing power (EC_{50} = 4.85 µg/ml) than BHT (EC_{50} = 8.62 µg/ml) (Fig. 3). It is known that polyphenolic compounds appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products [22].

The chemoprotection effect of CALE against hematological toxicity induced by AraC was studied. The analysis of mice blood indicated that the subcutaneous injection of 50 mg/kg AraC during three consecutive days caused a remarkable myelodepression. Indeed, the number of red and white blood cells decreased significantly ($P \leq 0.05$) (Fig. 4). Moreover, the amount of hemoglobin and the percentage of hematocrite decreased remarkably ($P \leq 0.05$) (Fig. 5). However, CALE did not exert any hematological toxicity. Furthermore, the combination of AraC with CALE protected red cells from a real cytotoxicity. On the other hand, the amount of hemoglobin and the percentage of hematocrite remained the same as in the control group. However, the combination of AraC with CALE showed that CALE slightly protected the white blood cells against the toxicity of AraC but still insignificant. Platelets number

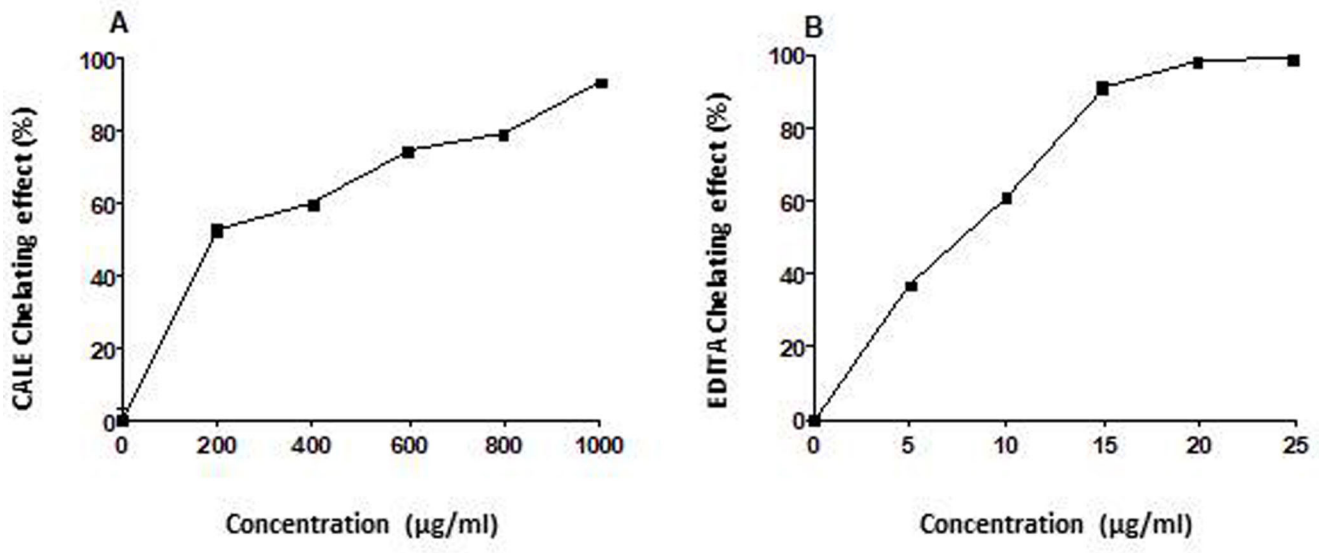

Fig. 2 Metal chelating activity of CALE (A) and the standard chelating agent EDTA (B). Values are expressed as means ± SEM (N = 3)

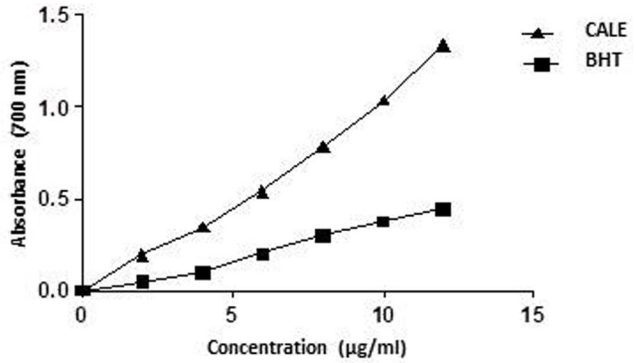

Fig. 3 Ferric reducing power of CALE compared to the standard antioxidant BHT. Values are expressed as means ± SEM ($N = 3$)

was not influenced by subcutaneous injection of 50 mg/kg AraC (results not shown).

Some studies showed that the toxicity of AraC toward normal cells is due to its interference with the metabolism of nucleotides in cells with high turnover like bone marrow and intestinal mucosa cells [23]. In addition, Kanno et al. [24] demonstrated that AraC use another mechanism to induce apoptosis in cells via reactive oxygen species (ROS). In this study we suggest that the decrease in red blood cells number is due principally to ROS effect. Thus, red blood cells are vulnerable to oxidation because of their role in carrying oxygen and close proximity to chemicals or drugs in circulation. Oxidation occurs in many areas of the cell including cell membrane lipids, proteins in the globin chains or cell skeleton, and iron in the hemoglobin. Oxidation of cell membrane lipids causes peroxidation of unsaturated fatty acids and polymerization of phospholipids and proteins. This affects cell deformity and increases membrane permeability to cations such as K^+ causing the red blood cells to swell and dying by hemolysis. Less deformable erythrocytes are prone to damage when passing through the microcirculation and are subsequently removed by splenic macrophages. Therefore, antioxidants such as flavonoids blocked the ROS production caused by AraC inhibited the apoptosis and cytotoxicity effect [24]. Furthermore, it was indicated that red blood cells have the ability to store flavonoids [25]. This study showed that CALE is very rich in polyphenols mainly flavonoids and exhibited a strong scavenging activity against the free radical $DPPH^{\bullet}$ and a strong reducing power. As a result, we suggest that the protection effect of CALE toward red blood cells is due principally to its antioxidant activity.

Moreover, the results showed that AraC caused an elevation of body temperature which reached 39 °C in the group treated with AraC alone. In contrast, the body temperature of the group treated with AraC combined with CALE remained normal and did not exceed 37.5 °C (Fig. 6). This latter result could be explained by the anti-inflammatory effects of CALE. Indeed, it has been reported that CALE inhibited inflammation induced by carrageenan in rat paw and reduced neutrophil chemotaxis and degranulation [12].

During the execution of the experiments, some adverse effects of AraC were observed in mice such as diarrhea,

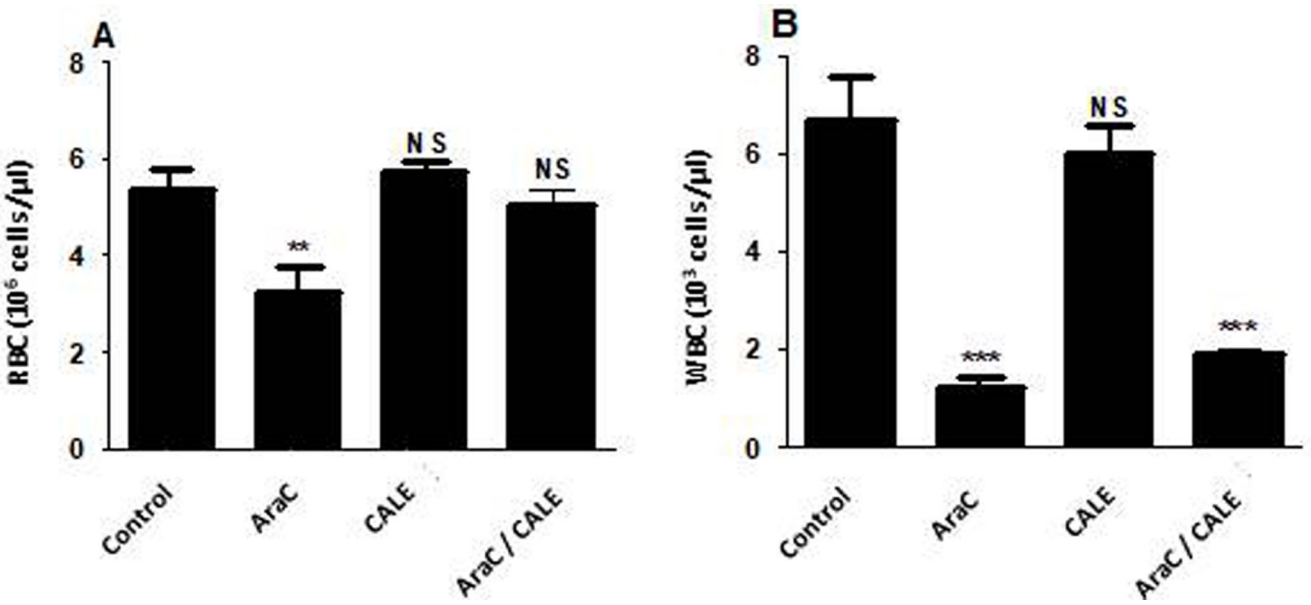

Fig. 4 Effect of CALE, AraC alone or combined with CALE (AraC/CALE) on the numbers of RBC (A) and WBC (B) in mice blood. AraC (50 mg/kg) and CALE (100 mg/kg) were administered to mice by subcutaneous and intraperitoneal injection. Hematological RBC and WBC were counted using an automatic hemocytometer. Values represent mean ± SEM of the data from 6–8 mice. $^{**}P < 0.05$ and $^{***}P < 0.001$ significantly different from control group

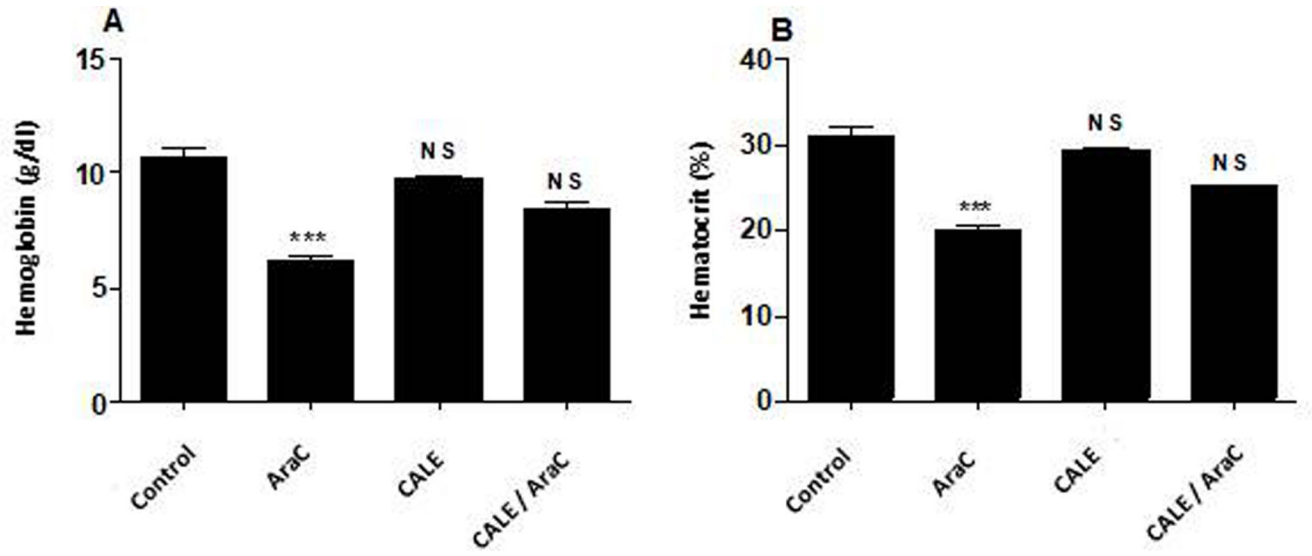

Fig. 5 The toxicity effect of AraC (50 mg/kg) on the amount of hemoglobin (A) and the percentage of hematocrite (B) and the protection effects of CALE (100 mg/kg). Values represent mean ± SEM of the data from 6–8 mice. $^{**}P < 0.05$ and $^{***}P < 0.001$ significantly different from control group

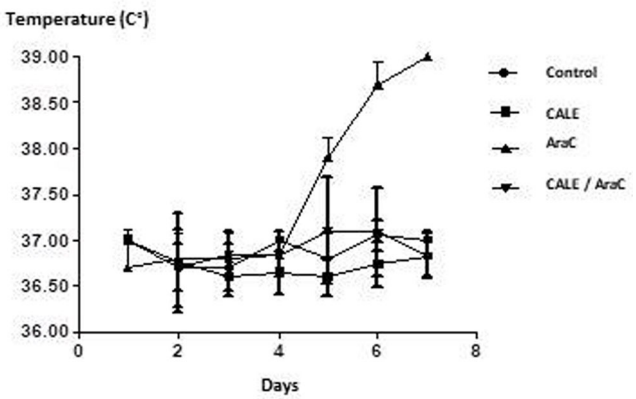

Fig. 6 The antipyretic effect of CALE (100 mg/kg) in Balb C mice with subcutaneous injection of 50 mg/kg of AraC

myalgia and alopecia. These symptoms were disappeared when the drug was discontinued.

Conclusion

CALE exerted a protective effect toward red blood cells. We suggest that the protection effect of CALE is due principally to its antioxidant activity. However, it is known that medicinal plants extracts are believed to contain different chemoprotective or chemotherapeutic compounds, which possess more than one mechanism of action.

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Conflicts of interests: the authors have no conflicts of interests to declare.

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