ORIGINAL ARTICLE

In Vitro Antihemolytic Activity of *Echinops spinosus* Tannins Extracts against Human Erythrocytes

Gheffour Kamila[1], Boucherit Kebir[2], Boucherit Otmani Zahia[1], Azzi Rachid[1]

[1]Department of Biology, Laboratory Antibiotics, Antifungals, Physical Chemistry Synthesis and Biological Activity (LapSab), Abou Bekr Belkaid University, Tlemcen, Algeria, [2]Laboratory Antibiotics, Antifungals, Physical Chemistry Synthesis and Biological Activity, Center University of Ain Temouchent, Algeria

Abstract

Aim: In Algeria, especially in Tlemcen region, the research of medicinal plants with therapeutic uses presents a great interest. In this study, tannin extracts of the leaves, steams, and roots of *Echinops spinosus* which belong to the Asteraceae plant family of Tlemcen (north of Algeria) are screened for the antihemolytic activity toward human erythrocytes. The good results of the antioxidant activity of *E. spinosus* prompted us to lead a reflection on their antihemolytic activity. **Materials and Methods:** The hemolytic activity is performed by the modified spectroscopic method at five different inhibitory concentrations (IC_{50}, 2 IC_{50}, 10 IC_{50}, 20 IC_{50}, and 40 IC_{50}) of each extract. **Results:** The aqueous extract of *E. spinosus* contains very significantly the highest quantity of tannins being 1910*** mg EC/100 g dry matter and 704.34*** mg EC/100g dry matter in the aerial part and roots, respectively. This result is very significant ($P < 0.001$). All the extracts exhibit a very low hemolytic activity that does not exceed 5% of hemolysis, except ethyl acetate and n-butanol extracts of roots which reach 35 and 40%, respectively, compared to the total hemolysis. **Conclusion:** This extracts have a good antihemolytic activity and are considered as safe to human erythrocytes.

Key words: *Echinops spinosus,* extracts, hemolysis, human erythrocytes

INTRODUCTION

Toxicity of the active molecule is a key factor during drug designing, and hemolytic activity represents a useful starting point in this regard. It provides the primary information on the interaction between molecules and biological entities at the cellular level. Hemolytic activity of any compounds is an indicator of general cytotoxicity toward normal healthy cells.[1]

The use of tannins covers a wide range from bacteriology, virology, and hematology reflecting their importance in human medicine mainly. This is the reason for which we proposed to initiate the study on medicinal plants.

Earlier, in traditional medicine, *Echinops spinosus* is known for its many therapeutic properties, leaves are used as an anti-inflammatory to treat hemorrhoidal diseases,[2] warts,[3] while decoction roots are used against stomach pains and urinary ailments and are administered to women before delivery and to accelerate the delivery of the placenta.[4]

Published works show that *E. spinosus* has been reported for anti-inflammatory activity[5] and antioxidant activity.[6]

The interesting results of the antioxidant activity of tannin extracts of *E. spinosus* with very low inhibitory concentration (IC_{50}) ranging from 8.25 to 71.5 µg/mL prompted us to reflect on their toxicity toward erythrocytes.[7]

The aim of this study is to extract and determinate the amount of tannin extracts in the leaves, stems, and roots of *E. spinosus*

Address for correspondence:
Gheffour Kamila, Department of Biology, Laboratory Antibiotics, Antifungals, Physical Chemistry Synthesis and Biological Activity (LapSab), Abou Bekr Belkaid University, Tlemcen, Algeria.
E-mail: biochimie-gh@outlook.fr

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and to evaluate their antihemolytic activity against human erythrocytes.

MATERIALS AND METHODS

Plant material

Fresh roots and leaves stems of *E. spinosus* are collected (dry season) in Amieur town of Chetouane (West of Algeria). The plant material is withered, crushed, and stored in dark bottles.

Tannins extraction

The extraction of the tannins is carried out according to the method of Zhang *et al*., 2008. The shredded plant material (2.5 g) is extracted with 50 mL of acetone/water (35/15) for 72 h. The resulting solution is filtered and evaporated at 40°C using a rotary evaporator clogged type R-200 to remove the acetone, and the aqueous phase is washed with hexane to remove pigments and lipids. After removal of the organic phase, the aqueous phase is treated 3 times with ethyl acetate (V/V), the three organic phases obtained are combined and evaporated to dryness at 40°C using a rotavapor. The remaining aqueous phase is treated 3 times with n-butanol. N-butanol phases are evaporated to dryness to recover the extract in powder form.

Biological material

Blood samples are collected in dry sterile tubes, from sainfoin volunteer. Donors are screened to guarantee that none had ingested drugs such as aspirin or other anti-inflammatory agents for at least 10 days before donation since these compounds could be a source of non-systematic alteration in platelet function. The plasma is ejected, and the washing is repeated 2 times by PBS (pH = 7.4, 10 mM).

The blood samples are stored at 4°C and used within 24 h of collection.

Determination of tannin contents

Condensed tannins are determined by the method in the acidic medium vanillin.[8] This quantitative method is based on the ability of vanillin to react with units of condensed tannins in the presence of acid to produce a colorful complex measured at 500 nm. The reactivity of vanillin with tannin does not imply the first unit of the polymer. The amounts of tannins are estimated using the method described by vanillin.[9]

In brief, a volume of 50 µL of crude extract is added to 1500 µL of the vanillin/methanol solution (4% w/v) and then mixed using a vortex. Then, 750 µL of concentrated hydrochloric acid (HCl) is added. The mixture is allowed to react at room temperature for 20 min. The absorbance is measured at 550 nm against a blank using a UV-Visible spectrophotometer double beam (SPECTROD® 200 PLUS).

A standard curve is performed in parallel under the same operating conditions using catechin as a positive control.

The results of the plant studied are expressed in milligrams (mg) of catechin equivalent per 100 g of dry plant material (mg CE/100g).

Assessment of human erythrocyte suspension

For this experiment, 1% red-blood cell suspension in pH 7.4 phosphate buffer is used throughout in the preparation of experimental (test) and control tubes (WHO, 1998).

In a series of five tubes are mixed successively: 8.5mL of PBS (10 mM, pH = 7.4), 0.5 mL of each type of blood suspension, and 1 ml of extracts according to the dose.

In parallel, the positive and negative control is prepared as follow: The first one only 1 mL of extract is replaced by distilled water and the second is replaced by PBS. The different tube contents are made under continuous agitation using a magnetic stirrer orbital shaker thermo format 37°C for 2 h (120 min) with different concentrations. The washing solution used is $MgCl_2$, NaCl (2 mM, 150 mM).

Different tubes are mixed and centrifuged for 5 min at 4000 r.p.m at room temperature by a centrifuge SIGMA 2 - 16PK. The absorbance (or optical density [OD]) of the supernatant is read with a UV-visible spectrophotometer (SPECTROD® 200 PLUS) at 548 nm using the mixture of $MgCl_2$, NaCl, and PBS as blank. This procedure is repeated 3 times to achieve a statistical analysis (average student test and standard deviation), and the average is determined for each dose of each extract from measurements of OD.

The rate of hemolysis inhibition of different extracts is determined with the following formula:

$$\% \text{ Hemolysis inhibition} = DO_0\text{-}DO_i/DO_0 \times 100$$

DOi: Absorbance of sample extract.

DO_0: Optical density of the control solution.

Statistical analysis

The results obtained are expressed as mean ± SD, all measurements are carried out in triplicate. The data are performed by analysis of variance (ANOVA, $P < 0.05$) (Schwartz, 1993).

In this test, the average absorbance of the tubes at different concentrations, incubated for 120 min, is compared at time 0 min.

The value of *t* gives us the degree of *P* meaning read on table student. The difference between the two averages is:

- Not significant: $P < 0.05$ (*).
- Significant: $P < 0.01$ (**).
- Very significant: $P < 0.001$ (***).
- Highly significant: $P < 0.0001$ (****).

RESULTS

Determination of tannins

Condensed tannins content in two parts of *E. spinosus* is shown in Table 1 and Histogram 1.

Standard curve prepared is used for the determination of condensed tannins content using different concentrations of catechin ($Y = 2.6283x$, $R^2 = 0.977$) [Figure 1].

Observation shows that the aqueous extract of aerial part contains the highest content of tannins (1910 ± 0.003 mg of EC/100 g of DM) ($P < 0001$) followed by the root part of the same extract (704.34 ± 0.0004 mg of EC/100 g of DM) ($P < 0.001$), while methanol extract (70%) contest significantly the lowest quantity of tannins in the two-part of the plant being 296 ± 0.01 and 57.5 ± 0.02 mg EC/100 g in the aerial part and roots, respectively, as compared of aqueous extract ($P < 0.05$).

Assessment of hemolytic activity

To evaluate the biological activities of natural extracts, requires a study of the hemolytic activity, even if the plant has an antioxidant activity, their use in food and pharmaceutical preparations will be impossible due to the presence of these hemolytic effects which confirm the presence of saponins;[10] thus, hemolytic assays are performed because compounds possessing potent biological activity may not be useful in pharmacological preparations if they possess hemolytic effect.[11]

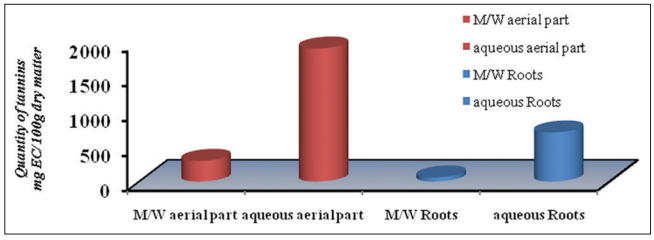
In addition, it is important to note that IC50 values, found during the evaluation of the antioxidant activity of different extracts of *E. spinosus*, do not exceed 71 µg/mL in all the extracts.

The observation of Figures 2-5 of each extract shows that ethyl acetate and n-butanol extracts of aerial part have not exhibited any hemolytic activity at 40 times higher of IC_{50}, and hemolytic power does not exceed 5% hemolysis except two extracts, n-butanol and ethyl acetate of roots where the percent of hemolysis is 35 and 40%, respectively.

Therefore, it can be expected that extracts of this plant have very low cytotoxicity toward erythrocytes comparing to total hemolysis.

DISCUSSION

The phytochemical analysis of many studies indicates that Asteraceae family contains a higher content from polyphenolic tannins and non-tannins and flavonoids and their biological activities are direct reflections of the effect and nature of the phytochemicals it contains.[12]



Histogram 1: Quantity of tannins of two parts of *Echinops spinosus*

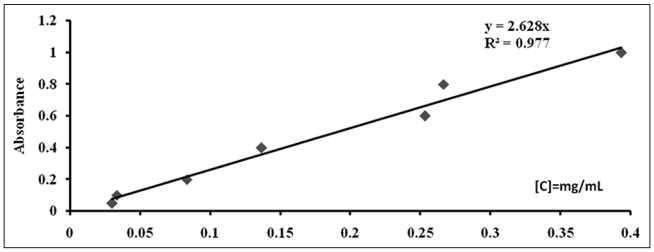


Figure 1: Calibration curve for the determination of tannin contents

Table 1: Quantity of tannins of aerial part and roots of *Echinops spinosus*

Plant material	Extracts	*Quantity* of tannins (mg EC/100g dry matter) (mean±SD)
Echinops spinosus	Aerial part	
	Methanol/water	296±0.01
	Aqueous	1910±0.003
	Roots	
	Methanol/water	57.5±0.02
	Aqueous	704.34±0.0004

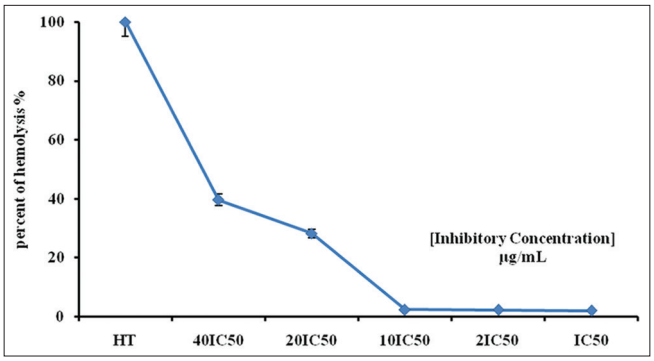


Figure 2: Evolution of hemolysis rate (%) of different inhibitory concentrations of ethyl acetate extract of tannins of the root part of *Echinops spinosus*, after 120 min of incubation with respect to the total hemolysis

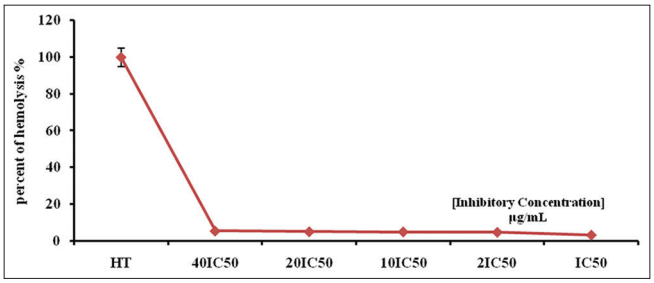


Figure 3: Evolution of hemolysis rate (%) of different inhibitory concentrations of ethyl acetate extract of tannins of the aerial part of *Echinops spinosus*, after 120 min of incubation with respect to the total hemolysis

The results show that aqueous extract of aerial part contains the highest content of tannins.

These results are consistent with results of many researchers whom indicate that the aqueous extract records the highest levels of condensed tannins followed by the methanol extract.[13]

A study conducted by Singh, in 2012, shows that *Artemisia absinthium* which belongs to the Asteraceae family has a value of tannins of aqueous extract estimated to 30.44 ± 1.08 mg/EAG/g of extract.

This value is very low compared to our study which confirms that *E. spinosus* is rich in tannins.[14]

To maximize the extractive capability of tannins content from plant material is considerably depended on their chemical nature, the solvent used and the operating conditions. However, condensed tannins content may vary also due to several factors such as tannins sensitivity to several degradative pathways (oxidative, light.), cultural, climatic, soil, or predation stress.[15]

The erythrocyte model has been widely used as it presents a direct indication of toxicity of injectable formulations as well as a general indication of membrane toxicity that cells are easy to isolate from the blood.[16]

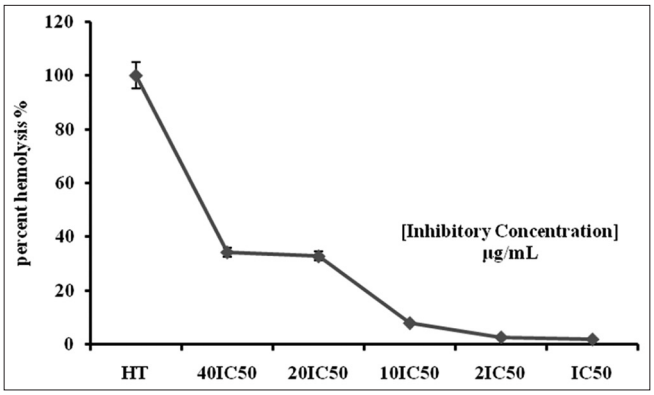


Figure 4: Evolution of hemolysis rate (%) of different inhibitory concentrations of an n-butanol extract of tannins of the root part of *Echinops spinosus*, after 120 min of incubation with respect to the total hemolysis

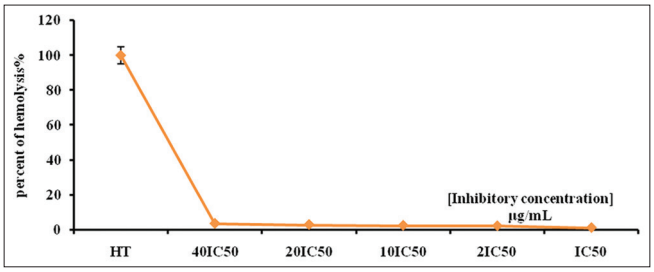


Figure 5: Evolution of hemolysis rate (%) of different inhibitory concentrations of an n-buthanol extract of tannins of the aerial part of *Echinops spinosus*, after 120 min of incubation with respect to the total hemolysis

Many research indicates that plants with none lysis in the human blood red blood cells did not contain cardiac glycosides, alkaloids, saponins, and phlobatannins as which are responsible for the lysis of the erythrocytes,[17] and the inhibition of hemolysis increase with an increase in the concentration of extract.[18]

Our results agree with the findings of Rengasamy and Zubair where the extracts of various plants tested showed low hemolytic effects against human erythrocytes.[19]

Under the same conditions, El Alaoui records an order hemolysis rates of 88% after incubation of isolated erythrocytes in PBS (pH = 7.4) in the presence of 1mg/mL crude hydroalcoholic extract of *Nigilla sativa*.[20]

Thus, Senguttuvan *et al*. indicated that the root water extracts of *H. radicata* possessed effective antihemolytic activity (68.45%).[21] Moreover, a recent study conducted by Shobana *et al*. indicates that leaves of *Abutilon indicum* show a percentage of hemolysis equal to 42.85 ± 1.85% at the extract concentration equal to 1 mg/mL.[22]

Some reports improve that high total phenol and tannins contents in the extract led to its potent antihemolytic activity.[23]

The toxicity of a substance in the body depends on the nature of the substance, dose, and exposure time. The direct hemolytic effect of different toxic agents is due to a variety of non-specific mechanisms, for example, surfactants cause hemolysis through the dissolution of the erythrocyte plasma membrane which ruptures due to increased fragility or due to osmotic lysis caused by increased permeability of the plasma membrane.[24]

CONCLUSION

In the light of the results obtained, we concluded that the aerial part of *E. spinosus,* in the Tlemcen, is very rich of tannins, and has very low toxicity against human erythrocytes especially the aerial part that possesses a very significant antihemolytic activity. It can be a very important source in the therapeutic and pharmacological to relieve various diseases.[25-31]

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