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Research Article

## Antioxidant, antimicrobial and anti-inflammatory activities development of methanol extract of *Cyclamen africanum* B. et R., growth in Jijel - Algeria

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### ABSTRACT

The genus *Cyclamen* L. (Primulaceae) is represented by 20 species, where inter alia *Cyclamen africanum* B. et R. is the only endemic species presented in Algeria. The study of their biological potentialities was evaluated through antioxidant, antimicrobial and anti-inflammatory activities. The antioxidant activity was evaluated by DPPH and the reducing power essay, the antimicrobial activity was tested with three bacterial strains and one yeast (*Staphylococcus aureus* ATCC25923, *Klebsiella pneumonia* ATCC700603, *Bacillus subtilis* ATCC6633 and *Candida albicans* ATCC1024) and anti-inflammatory activity with the Human Red Blood Cell (HRBC) membrane stabilization method. The results demonstrate a perfect antioxidant activity and an excellent anti-inflammatory power at very small concentrations.

**Keywords:** *Cyclamen*; crude extract; reducing power; antimicrobial; HRBC.

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### INTRODUCTION

*Cyclamen* L. is a small genus of the Primulaceae [1]. Consists of more than 20 species with highest diversity [2] distributed the Mediterranean region, Central Europe, the Caucasus and northern Somalia [1]. Some species have been cultivated in Western European countries since the 18<sup>th</sup> century [3]. Although, *Cyclamen* species are in the list of CITES (Convention on International Trade in Endangered Species of wild fauna and flora) [4], several species are widely used in traditional folk medicine for their laxative and abortive [5], sedative, purgative, emmenagogue and anti-helminthic properties [6].

Botanically, *Cyclamen africanum* B. et R., is a perennial plant with tuberculous strain. Their leaves are cordifolites petiolate and their flowers are scapiform with petals reflected above the tube, nutantes at anthesis. It flowering in autumn, with flower preceding the leaves (6-15 cm), spotted with red more or less dented, crenelated on the margins. Tubers are very bulky. Large flowers (2-3,5 cm), with toothed groove. Peduncle retracting into a cork after flowering. This species is a North African endemic prefer the forests and the scrub, can be found in the Tell, Algerian littoral and Constantine [7].



Figure 1: *Cyclamen africanum* Boiss. & Reuter original illustration from Curtis's Botanical Magazine (1869)

In this study, we adapted these protocols for *C. africanum*. In detail, the aim was to valorise their biological activities to enrich the Algerian pharmacopeia and to preserve this endemic species from extinction.

## MATERIALS AND METHODS

### Plant material

The random sampling were used during the harvesting, the areal parts of *Cyclamen africanum* B. et R., were harvested from Taza National Park Jijel – Algeria (36° 35' 16"- 36° 48' 12" north Latitude and between 5° 29' 07" and 5° 40' 11" Longitude East); Determined by Dr. Nouioua Wafa.

### Preparation of methanol extract

The areal parts were powdered and macerated in 80 % methanol for 24, 48 and 72 hours, at the laboratory temperature (1:10 w/v, 10 g of dried herb). After maceration, the extracts were collected, filtered and evaporated to dryness under vacuum [8]. The dry extract was stored at a temperature of -18 °C for later use.

### Determination of Total Phenolic Content

For total polyphenol determination, the Foline Ciocalteu method was used [9]. The samples (0.2 mL) were mixed with 1 mL of the Folin-Ciocalteu reagent previously diluted with 10 mL of deionized water. The solutions were allowed to stand for 4 minutes at 25 °C before 0.2 mL of a saturated sodium carbonate solution (75 mg/mL) was added. The mixed solutions were allowed to stand for another 120 minutes before the absorbance were measured at 765 nm. Gallic acid was used as a standard for the calibration curve. The total phenolic compounds content was expressed as mg equivalent of Gallic acid per gram of extract (mg EAG/GE).

### Determination of total flavonoids content

The flavonoids content in crude extract were estimated by the Aluminium chloride solution according to the method described by Bahorun *et al.*, (1996) [10]. Briefly, 1 mL of the methanol solution of the extract was added to 1 mL of 2 % AlCl<sub>3</sub> in methanol. After 10 minutes, the absorbance was determined at 430 nm. Quercetin was used as a standard. Results were expressed as mg equivalent Quercetin per gram of extract (mg EQ/GE).

### DPPH Assay

The donation capacity of extract was measured by bleaching of the purple-coloured solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato *et al.*, (1998) [11]. One millilitre of the extract at different concentrations was added to 0.5 mL of a DPPH-methanol solution. The mixtures were shaken vigorously and left standing at the laboratory temperature for 30 minutes in the dark. The absorbance of the resulting solutions were measured at 517 nm. The antiradical activity was expressed as IC<sub>50</sub> (micrograms per millilitre). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where: A<sub>0</sub>: the absorbance of the control at 30 minutes

A<sub>1</sub>: is the absorbance of the sample at 30 minutes. BHT was used as standard [12].

### Reducing power

The reducing power was determined according to the method of Oyaizu (1986) [13]. The extract (2.5 mL) was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer

(pH 6.6) and 2.5 mL of 10 mg/mL potassium ferricyanide. The mixtures were incubated at 50 °C for 20 minutes; after cooling, 2.5 mL of 100 mg/mL trichloroacetic acid were added and the mixtures were centrifuged at 200g for 10 minutes. The upper layer (5 mL) was mixed with 5mL of deionized water and 1 mL of 1 mg/mL ferric chloride, and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. EC<sub>50</sub> value (mg extract/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. BHA was used as standard [14].

### Antimicrobial activity

Bacteria Strains were obtained from the American Type Culture Collection: Gram-positive bacteria (*Staphylococcus aureus* ATCC25923), Gram-negative bacteria (*Klebsiella pneumoniae* ATCC700603 and *Bacillus subtilis* ATCC6633) and yeast: *Candida albicans* ATCC1024. Muller Hinton agar was used for bacteria culture and Sabouraud for yeast.

### Anti-bacterial Activity

Agar disc diffusion method was employed for the determination of antibacterial activities of *C. africanum* methanol extract [15] [16]. Briefly, a suspension of the tested microorganism (10<sup>8</sup> CFU / mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µL (100 mg/mL) of the extract and placed on the inoculated plates. These plates were incubated at 37 °C for 24 hours. Gentamicin (10 µg/disc) was used as a standard and dimethylsulfoxide DMSO as a control.

The antibacterial activity was determined by measuring of inhibition zone diameters (mm) and was evaluated according the parameters suggested by Alves *et al.* (2000) [17]:

- <9 mm, inactive ;
- 9–12 mm, less active ;
- 13–18 mm, active;
- >18 mm, very active.

### Antifungal activity

The antifungal activity was tested by disc diffusion method with modifications [15]. *Candida albicans* ATCC1024 suspension was obtained in physiological saline 0.9 % from a culture in Sabouraud (incubated before 24 hours at 37 °C), adjusted to 10<sup>5</sup> CFU / mL.

One hundred microliter of the suspension was placed over agar in Petri dishes and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10 µL (100mg/mL) of each sample. Amphotericin 100 µg was used as standard and dimethylsulfoxide DMSO as control. Inhibition zones were determined after incubation at 27 °C for 48 hours.

### The Human Red Blood Cell (HRBC) membrane stabilization method

To prepare the HRBC suspension, fresh completely human blood (10 mL) was collected and transferred into the centrifuge tubes. These lasts were centrifuged at 3000 rpm for 10 minutes thrice and washed with equal volume of normal saline each time. The volume of blood was measured and reconstituted as 10 % v/v suspension with normal saline.

The principle involved here was stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The mixture contain 1mL phosphate buffer (pH 7.4, 0.15 M), 2 mL hypo saline (0.36 %), 0.5 mL HRBC suspension (10 % v/v) and 0.5 mL of plant extract or standard drug (diclofenac sodium) at various concentrations (10, 50, 100, 250, 500  $\mu\text{g}/\text{mL}$ ). The control was distilled water instead of hypo saline to produce 100 % haemolysis.

The mixtures were incubated at 37 °C for 30 minutes and centrifuged at 2500 rpm for 5 minutes. The absorbance of haemoglobin content in the suspensions were estimated at 560 nm. The percentage of haemolysis of HRBC membrane can be calculated as follows:

$$\text{Haemolysis (\%)} = \left( \frac{\text{Optical density of Test sample}}{\text{Optical density of Control}} \right) \times 100$$

However, the percentage of HRBC membrane stabilization can be calculated as follows:

### Statistical analysis

Results were expressed as mean  $\pm$  standard deviation in triplicates. Data was statistically analysed using t test of Student as primary test followed by Fisher test with the criterion of  $P < 0.05$  to determine whether there were any significant differences between methanol extract of *C. africanum* and standards, using Graphpad prism 5 Demo Software.

### RESULTS AND DISCUSSION

The results obtained from the extraction method reveal an important yield (30%) containing  $43,49 \pm 1,04$  mg EAG/GE of polyphenols and  $2,48 \pm 0,08$  mg QG/GE of flavonoids.

The DPPH radical was widely used in the model system to investigate the scavenging activities of several natural compounds [19]. The method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen-donating antioxidant [20]. Figure 1 shows the dose-response curve for the radical-scavenging activity of *C. africanum* compared with BHT at the different concentrations by using the DPPH colouring method:

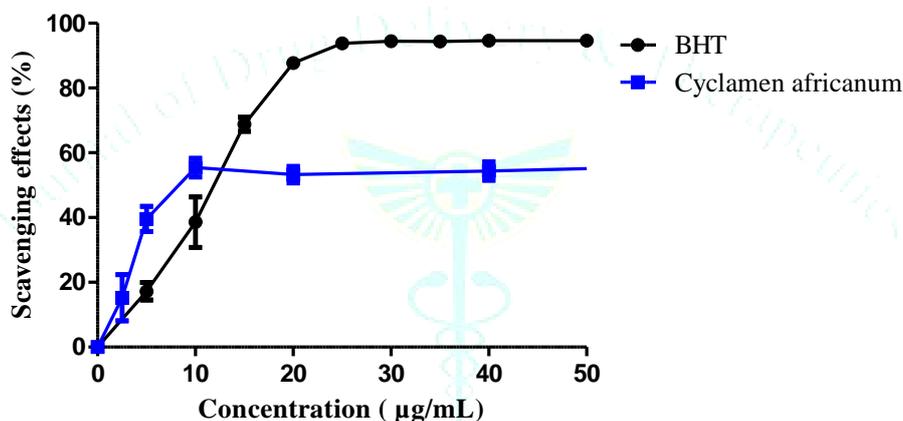


Figure 1: DPPH test of methanol extract of *Cyclamen africanum* B. et R.

A relatively higher antioxidant activity was observed in crude extract of *C. africanum* ( $\text{IC}_{50} = 36,85 \pm 14,99$   $\mu\text{g}/\text{mL}^{***}$ ) against the BHT ( $\text{IC}_{50} = 6,29 \pm 1,12$   $\mu\text{g}/\text{mL}$ ). The methanol extract of *C. africanum* showed  $55,90 \pm 5,86$  % of antiradical effect at 80  $\mu\text{g}/\text{mL}$ , lower than standard drug.

It is well accepted that the DPPH radical scavenging by antioxidants is attributable to their hydrogen-donating ability [21].

The measure of the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  ions is another method to observe the antioxidant activity, which is illustrated in Figure 2:

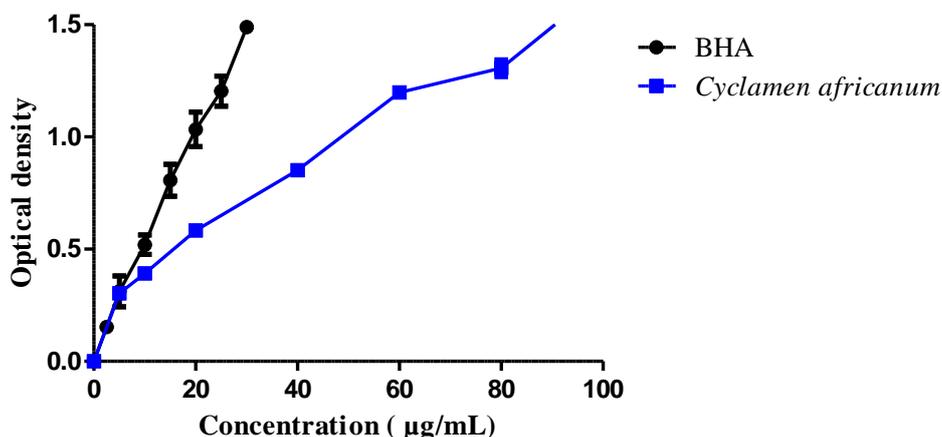


Figure 2: Reducing power activity of methanol extract of *Cyclamen africanum* B. et R.

The metal chelating activities of methanol extract of *C. africanum* and standards were concentration-dependent, indicate an excellent EC<sub>50</sub> (12,73±0,57 µg/mL<sup>\*\*\*</sup>) against BHA (9,51±0,12 µg/mL).

Reducing power serves as a significant and sensitive parameter to assess potential antioxidant activity of plant extracts [22]. Hence, the ability of a compound to transfer electron is a significant indicator of its potential as an antioxidant [23]. Therefore, the methanol extract of *C. africanum* possess an excellent ability to transfer electrons.

A very weak antimicrobial power was registered (7,17±0,24 mm<sup>\*\*\*</sup> for *Bacillus subtilis* and 9,33±0,47 mm<sup>\*\*\*</sup> for *Klebsiella pneumonia*) against gentamicin (23,00±0,82 mm and 25,67±1,70 mm respectively).

The anti-inflammatory activity of *C. africanum* extract was confirmed by erythrocyte membrane stabilization test. The results (figure 3) show that human erythrocyte membranes were protected against hypotonic solution-induced lysis at different concentrations of extract, especially the smallest ones.

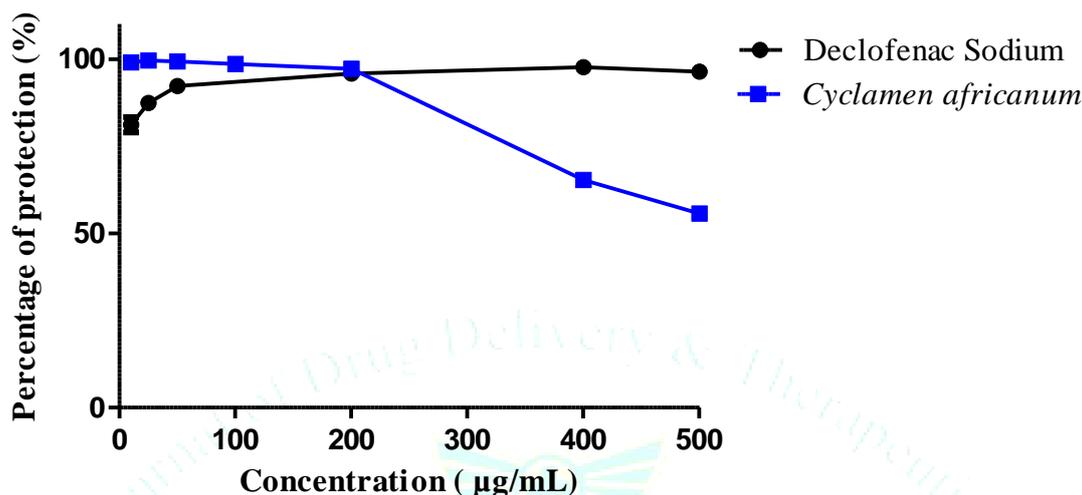


Figure 3: The percentage inhibition of hypotonicity induced haemolysis of HRBCs (%) of standard and methanol extract of *Cyclamen africanum B. et R.*

Until a concentration of 200 µg/mL the extract shows an excellent capacities of erythrocytes membrane stabilisation, better than standard. This capacity decreases in high concentration (400 - 500 µg/mL) i.e the extract at 500 µg/mL shows 55,72±0,63 %\* of erythrocytes protection against 96,40±0,10 % of protection in case of Declofenac Sodium.

Membrane-stabilizing attributes were acknowledged for their power to interpose with release of phospholipases that activate the establishment of inflammatory intercessors [24]. During inflammation, lysosomal enzymes and hydrolytic components are released from the phagocytes to the extracellular space, which causes damages of the surrounding organelles and tissues and also assists a variety of disorders [25]. Hence, methanol extract of *Cyclamen africanum* act as an anti-inflammatory agent at a very small concentrations to give maximum effect.

## CONCLUSION

*Cyclamen africanum B. et R* is a north African endemic species where few studies have been done. This investigation reveals the biological potentialities of the crude extract of this species demonstrate a perfect capacity of donating electrons and hydrogen, also an excellent anti-inflammatory activity in very small concentrations. However, a very weak antimicrobial power was observed. Hence, these results indicate that *Cyclamen africanum* crude extract can be a potential candidate for the development of new therapies for the treatment of inflammatory infections and a perfect antioxidant molecules sources.

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