



**Antioxidant, antimicrobial and anti-inflammatory activities valorisation of methanol extract of *Allium trichocnemis* J. Gay.**

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**Abstract**

*Allium trichocnemis* J. Gay, is North African endemic species, constitute an enigma toward their capacities. The antioxidant properties were evaluated through the ability of the extract to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals and the reducing power essay, the antimicrobial activity was tested with three bacterial strain and three fungi including yeast (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311, *Staphylococcus aureus* ATCC25923, *Aspergillus niger* 2CA936, *Aspergillus flavus* NRRL3357 and *Candida albicans* ATCC1024) and the anti-inflammatory activity, evaluated with proteins denaturation test. The results indicate a moderate to weak antioxidant and anti-inflammatory activities but a strong antifungal capacities.

**Key words:** Antioxidant, antimicrobial, anti-inflammatory, *Allium trichocnemis*.

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**INTRODUCTION**

*Allium* L. is probably the largest genus of the petaloid monocotyledons, comprising some 750 species [1]. The genus is characterized by having bulbs enclosed in membranous (sometimes finally fibrous) tunics, free or almost free tepals, and often a subgynobasic style. Most species produce remarkable amounts of cysteine sulphoxides causing the well-known characteristic odour and taste. The genus is naturally distributed only in the Northern Hemisphere, mainly in regions that are seasonally dry. It has a main centre of diversity in southwest and central Asia and a second smaller one in North America. [2].

According to Quezel and Santa. (1962), *Allium trichocnemis* J. Gay. (*Allium trichocnemis*), is an Algerian endemic species growth in littoral limestone rocks characterized by semi-cylindrical leaves (2-3 mm of diameter) clearly fistulous, Involucre with two spaths and Umbel multiflora. This plants can reach 25 – 60 cm [3].

The plants can be used as ornamentals, vegetables, spices, or as medicine. There are over 120 different documented uses of the *Allium* plants, and besides their remarkable medicinal powers, *Allium* plants are generally consumed for their flavours, while their nutritive values have been appreciated only recently [4][5].

These experiments aims to valorise the in vitro antioxidant, antimicrobial and anti-inflammatory activities to reveal the therapeutic efficacy of crude extract of *Allium tr.*

## EXPERIMENTAL SECTION

### Plant material

The areal parts of *Allium trichocnemis* were collected from the mountain of Megriss - Setif -Algeria in May 2014 and determined by Dr. Nouioua Wafa in Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Natural Life and Sciences, University Ferhat Abbas Setif, Algeria.

### Preparation of methanol extracts

The areal parts of *Allium trichocnemis* were powdered and macerated in 80 % methanol for 24, 48 and 72 hours, at room temperature (1:10 w/v, 10 g of dried herb). After maceration, the extracts were collected, filtered and evaporated to dryness under vacuum [6]. 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 [7]. The sample (0.2 mL) is mixed with 1 mL of the Folin-Ciocalteu reagent previously diluted with 10 mL of deionized water. The solution is allowed to stand for 4 minutes at 25 °C before 0.2 mL of a saturated sodium carbonate solution (75 mg/mL) is added. The mixed solution is allowed to stand for another 120 minutes before the absorbance at 765 nm is measured. Gallic acid is used as a standard for the calibration curve. The total phenolic compounds content is expressed as mg equivalent of Gallic acid per gram of extract (mg EAG/GE)

### Determination of total flavonoids contents

The flavonoids content in our extract was estimated by the Aluminium chloride solution according to the method described by Bahorun et al., (1996) [8]. 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 (0 – 40 µg/ml) were used as a standard. Results were expressed as mg equivalent Quercetin per gram of extract (mg EQ/GE).

### Determination of total tannins content

Tannins content was evaluated using the haemoglobin precipitation assay. An aliquot of 0.5 mL of the extract is mixed with 0,5 mL of haemolysis bovine blood to reach a final concentration of 1 mg/mL then the mixture was centrifuged at 480g for 20 minutes and the absorbance was measured at 578 nm [9]. In same time, tannic acid at various concentrations (100 – 600 µg / mL) was used in the same manner as the samples. Results were expressed as mg equivalent of tannic acid / gram of extract (E TA/GE).

### Quantitative Estimation of Alkaloids:

To 1 mL of methanolic extract, 5 mL phosphate buffer (pH 4.7) and 5 mL BCG solution were added, and shake a mixture with 4 mL of chloroform. The extract was collected in a 10 mL volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above. Atropine is used as a standard [10]. Result are expressed as mg equivalent of atropine / gram of extract (E ATR/GE).

### Determination of carotenoids

Total carotenoids content in the extract was determined by a spectrophotometric assay described by Youngmin Choi et al., (2006) [11]. Approximately 5 mL of extract were mixed with equal volume of distilled water and 15 mL of hexane/acetone/methanol (50/25/25, v/v) solution. The mixture was then homogenized with a Polytron and centrifuged at 3000 rpm (940g) for 10 minutes. The absorbance of the top layer of hexane was measured at 450 nm. Total carotenoids content of the sample was calculated as 1 g β-carotene per 100 g of sample using an extinction coefficient of  $E_{1cm}^{1\%} = 2505$  [12]. Results are expressed as mg equivalent of β-carotene / gram of extract E (β-carotene/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) [13]. One millilitre of the extract at different concentrations was added to 0.5 mL of a DPPH-methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 minutes in the dark. The absorbance of the resulting solution was then 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:

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

Where  $A_0$  is the absorbance of the control at 30 minutes and  $A_1$  is the absorbance of the sample at 30 minutes. BHT was used as standard [14].

### Reducing power

The reducing power was determined according to the method of Oyaizu (1986) [15]. 2.5 mL of the extract was mixed with 2.5 mL of sodium phosphate buffer (pH 6.6 ; 200 mmol/L) and 2.5 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50 °C for 20 minutes. After cooling, 2.5 ml of trichloroacetic acid (100 mg/ml) were added; the mixture was centrifuged at 200g for 10 minutes. The upper layer (5 ml) was mixed with 5ml of deionized water and 1ml 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.  $EC_{50}$  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. Ascorbic acid was used for comparison [16].

### Antimicrobial activity:

Strains of bacteria were obtained from the American Type Culture Collection: Gram-positive bacteria (*Staphylococcus aureus* ATCC25923) and Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 13311). Two fungi: *Aspergillus niger* 2CA936 and *Aspergillus flavus* NRRL 3357; and one yeast: *Candida albicans* ATCC1024.

Muller Hinton agar was used for bacteria culture, the potato dextrose agar for fungi culture and Sabouraud for yeast.

### Anti-bacterial Activity

Agar disc diffusion method was employed for the determination of antibacterial activities of *Allium trichocnemis* methanol extract [17] [18].

Briefly, a suspension of the tested microorganism ( $10^8$  CFU / mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10  $\mu$ L of different concentration of the extract and placed on the inoculated plates.

These plates were incubated at 37 °C for 24 hours. Gentamicin (10  $\mu$ g/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) [19]:

- <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 [17]. The potato dextrose agar plates were inoculated with each fungal culture (*Aspergillus niger* 2CA936, *Aspergillus flavus* NRRL 3357), 8 days old by point inoculation.

The spore suspension was prepares in an emulsion of 0.5 % tween 80 adjusted to a concentration of  $2-3 \times 10^6$  spores/mL, corresponding to 0.15 to 0.17 absorption at 530 nm [20]. However, *Candida albicans* ATCC1024 suspension is obtained in physiological saline 0.9 % of a culture in Sabouraud 24 hours at 37 °C, adjusted to  $10^5$  CFU / ml.

One hundred microliter of suspension was placed over agar in Petri dishes and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10  $\mu$ L of each sample at different concentrations.

Nystatin 100 $\mu$ g, clotrimazon 50  $\mu$ g and amphotericin 100  $\mu$ g were used as standard and dimethylsulfoxide DMSO as control. Inhibition zones were determined after incubation at 27 °C for 48 hours.

### Inhibition of proteins denaturation

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate-buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of extracts so that final concentrations became 100, 200, 400, 500 and 800  $\mu$ g/mL. A similar volume of double distilled water served as the control. Next, the mixtures were incubated at  $37 \pm 2^\circ\text{C}$  for 15 minutes and then heated at  $70^\circ\text{C}$  for five minutes. After cooling, the absorbance was measured at 660 nm by using the vehicle as a blank. Diclofenac sodium in the final concentrations of 100, 200, 400,

500 and 800 µg/mL was used as the reference drug and treated similarly for the determination of absorbance [21]. The denaturation of protein inhibition by the extracts and standard was expressed as percentage by using the formula:

$$\text{Percentage of inhibition} = (\text{Control} - \text{Test}) / \text{Control} \times 100 \text{ [22]}.$$

### Statistical analysis

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

## RESULTS AND DISCUSSION

*Allium trichocnemis* methanol extract give yield of 13,87 %, the quantification of different secondary metabolites groups are shown in table 1:

Table 1: *Allium trichocnemis* secondary metabolites quantification

	Polyphenols mg EAG/GE	Flavonoids mg EQ/GE	Tannins mg E TA/GE	Alkaloids mg E ATR/GE	Carotenoids E ( $\beta$ - carotene/GE)
Methanolic extract	111,15 $\pm$ 4,51	103,24 $\pm$ 2,48	66,89 $\pm$ 0,45	12,53 $\pm$ 1,40	17,195 $\pm$ 1,84

Owing to the complex reactive facets of phytochemicals, the antioxidant activities of plant extracts cannot be evaluated by only a single method, but at least two test systems have been recommended for the determination of antioxidant activity to establish authenticity [23]. For this reason the antioxidant activity of *Allium trichocnemis* crude extract was determined by DPPH and reducing power tests.

The DPPH testing is a rapid and easy method to measure antioxidant activity. The DPPH free radical-scavenging activity of *Allium trichocnemis* reach 64,13 $\pm$ 0,11% (figure 1), and the corresponding IC<sub>50</sub> values required to scavenge 50% of free radicals was 150,95 $\pm$ 8,24 µg/ mL<sup>\*\*\*</sup> against 8,76 $\pm$ 0,69 µg /mL for BHT.

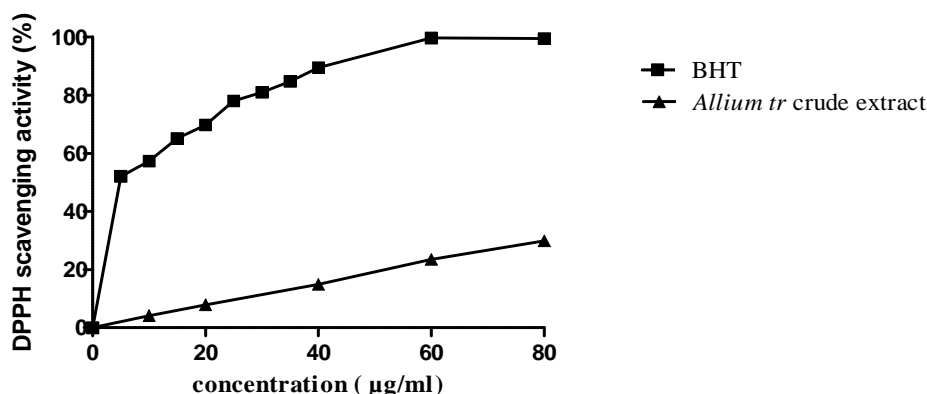


Figure 2: *Allium trichocnemis* methanol extract DPPH scavenging activity.

The scavenging activity of the extract was moderate to weak in comparison with BHT and the power of the extract increased with increasing concentration.

The discrepancy among the DPPH radical-scavenging activities in different studies may be partially due to different antioxidant potentials of different compounds, the antioxidant activity of the plant extracts strongly depends on the extraction solvent [24].

In this study, the organic solvent selected for the experiment was methanol due to the solubility of allicin. Nevertheless, the half-life of allicin can vary depending on the concentration, temperature, extraction solvent, and storage [25]. Hence, the use of 45 °C in Rotavapor significantly decrease the amount of allicin which act in synergy with phenolic compounds, consequently decrease the scavenging ability of the extract.

Compounds with reducing power are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [26]. The reducing power ability of *Allium tr* methanol extracts was determined using ascorbic acid as standard (figure 2). Presence of reducers causes the conversion of the  $Fe^{3+}$ /ferricyanide complex used in this method to the ferrous form.

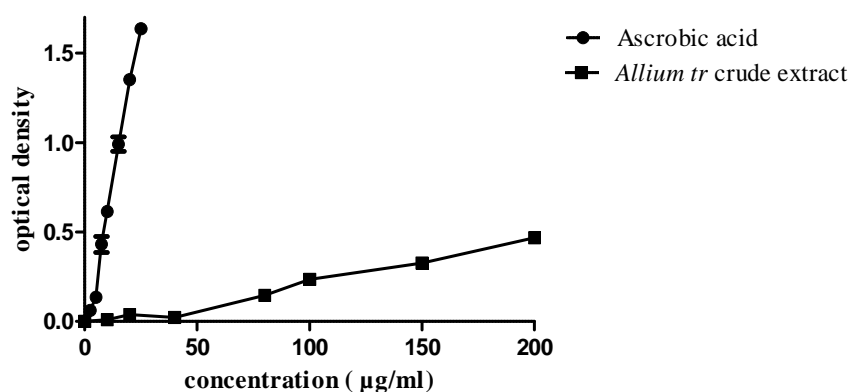


Figure 2: *Allium trichocnemis* methanol extract reducing power.

The reducing power of *Allium trichocnemis* methanol extract was dose dependent, exhibited a weak reducing power for  $Fe^{3+}$  in comparing with the standard. The  $EC_{50}$  of the extract was in range of  $209, 86 \pm 1, 65^{***}$  µg /mL against  $8.46 \pm 0.09$  µg /mL for Ascorbic acid.

Wangcharoen et al [27], found that heating causes decrease in antioxidant activity due to the decomposition of phenolic compounds and S – containing compounds, and the use of 45 °C in rotavapory may explain the weakness of *Allium tr* methanol extract.

*Allium tr* methanol extract was weak to inactive against the strains used, however, it present a totally mycelia growth inhibition against *Aspergillus niger* (table 2).

Table 1: Antifungal activity of methanolic extract of *Allium trichocnemis* standard and control

	<i>Aspergillus flavus</i> NRRL3357	<i>Aspergillus niger</i> 2CA936	<i>Candida albicans</i> ATCC1024
Nystatin	15,53±0,79	9,40±0,22 <sup>a</sup>	9,29±0,19
Clotrimazon	23,86±1,15	15,85±0,32 <sup>b</sup>	44,28±0,49
Amphotericin	16,20±1,19	17,55±0,14 <sup>c</sup>	15,58±0,12
Methanolic extract	No inhibition	100% inhibition of spores formation <sup>a, b, c</sup>	No inhibition
Control	No inhibition	No inhibition	No inhibition

The similar letters in different columns indicate a very significant difference.

In literature, the antibacterial activity of garlic which is another species of the genera *Allium* is mainly due to the presence of allicin produced by the enzymatic activity of allinase on alliin. Allicin is considered to be the most potent antibacterial agent in crushed garlic extracts, but it can be unstable, breaking down within 16 hours at 23°C [28]. However, the use of a water-based extract of allicin stabilizes the allicin molecule due to the hydrogen bonding of water to the reactive oxygen atom in allicin or there may be water soluble components in crushed garlic that destabilize the molecule [29]. Hence, the extraction method, the temperature during the incubation period of strains and the DMSO highly affect the anti-microbial activity, but *Aspergillus niger* present a sensibility to other molecules which need more investigation.

In the present investigation, the in vitro anti-inflammatory effect of *Allium trichocnemis* was evaluated against denaturation of egg albumin. The 800 µg/mL of methanol extract of *Allium trichocnemis* showed a maximum of

mean inhibition of protein denaturation  $22,58 \pm 3,63 \%$  \*\* and whereas for Diclofenac sodium, it was found to be  $90,13 \pm 3,71 \%$  (figure 3).

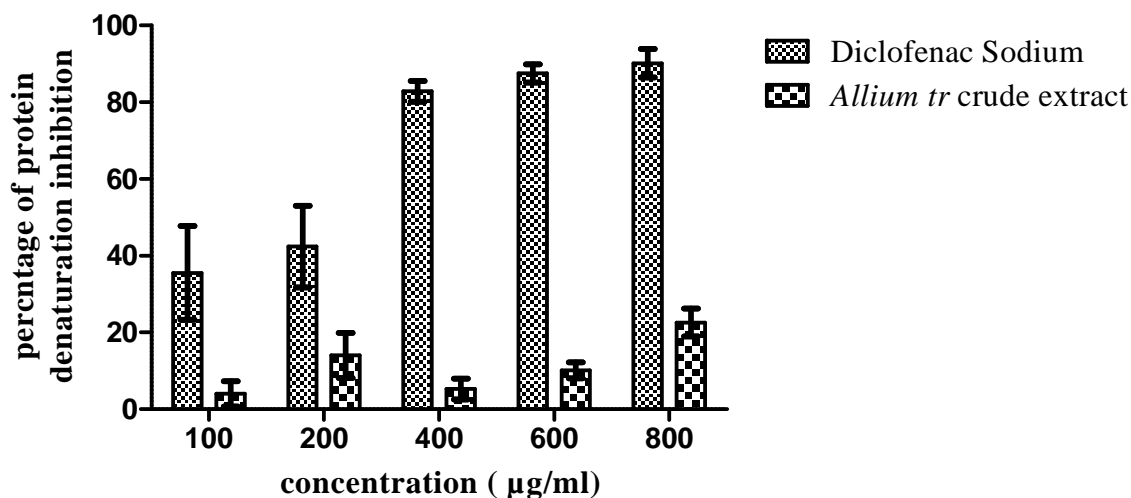


Figure 3: *Allium trichocnemis* methanol extract proteins denaturation test.

Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Methanol extract and standard have shown dose dependent ability to thermally induced protein denaturation (figure 3).

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play important role in the development of tissue damage during in inflammatory reactions and significant level of protection was provided by proteinase inhibitors [30]. Recent studies have shown that many flavonoids and related polyphenols contributed significantly to the antioxidant and anti-inflammatory activities of many plants. Hence, *Allium trichocnemis* polyphenols can weakly provide protection against leukocytes proteinase.

### CONCLUSION

In the present study, results indicate that the methanol extract of *Allium trichocnemis* possess a strong antifungal capacities, a moderate antioxidant activity and a weak anti-inflammatory properties. These activities may be due to the strong occurrence of secondary metabolites such as alkaloids, flavonoids, tannins, steroids, and phenols. Nevertheless, the extraction method compromise the integrity of bioactive molecules which can be extracted from this species, consequently, act upon the therapeutic capacities of the obtained extract. Further investigation are needed to improve and to detail the mechanism of action of every groups of molecules of this endemic species.

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