POTENT *IN VITRO* BIOACTIVITIES AND PHENOLIC CONTENTS OF *CRUCIANELLA ANGUSTIFOLIA* L. USING VARIOUS SOLVENT SYSTEMS

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Abstract. The aerial part and root extracts of *Crucianella angustifolia* grown in Tunisia were prepared using aqueous and organic solvents to determine the phenolic contents. Besides, the antioxidant activity was evaluated using the DPPH and ABTS radical scavenging and reduction power tests. The α-glucosidase inhibition activity was assessed using p-nitrophenyl-α-D-glucopyranoside. The antimicrobial activity of the extracts was evaluated on a set of medically important bacterial strains, the fungus *Candida albicans* and the virus *Coxsackivirus* B3. The results showed that methanolic and aqueous extractions yielded the highest quantities of polyphenols, flavonoids and flavonols. The obtained extracts exhibited a pronounced antioxidant activity. All methanolic extracts showed the greatest anti-α-glucosidase activity. The methanolic extract exhibited the highest antibacterial effect, while petroleum ether extract was the most effective against *C. albicans*. The various extracts turned out to be non-toxic on Hep-2 cell line with a CC_{50}>1000 µg/ml for all of them. Petroleum ether extract of aerial part was the most effective against virus with an IC_{50} equal to 5.92 µg/ml. The unraveled biological activities of *C. angustifolia* are likely to be attributed to the phenolic constituents contained in the plant. Further studies are needed to depict the components and mechanisms underlying the observed biological effects.
Keywords: *antioxidant. anti-α-glucosidase. antimicrobial. cytotoxicity. phenolics*

Introduction

Many alternative therapies are proposed such as phytotherapy, aromatherapy, oligotherapy, etc. and increasingly used by people who have become more aware of harmful effects of synthetic drugs. Nevertheless, hitherto the speed and the size of the discovered alternative medicines are not sufficient to get rid of synthetic therapies. *Crucianella angustifolia* L. species belongs to the genus *Crucianella*, the family of Rubiaceae and the order of Gentianales, is distributed worldwide including Spain, France, Italy, California, Iran, Turkey, Libya, etc. *C. angustifolia* also grows ~~also~~ in the north and center of Tunisia (Pottier-Alapetite, 1981). The family of Rubiaceae is traditionally known for its medicinal use and therefore represent a promising source of new bioactive substances. Rubiaceae family includes *Coffea* L. specie which figures among the most economically important global market for vegetable products. Rubiaceae family also encompasses the famous species *Gardenia jasminoides* used in monoi manufacturing known for its nice and intensive smell. Besides, this group of plants is medically important. Indeed, some studies have reported that plants belonging to Rubiaceae family were used in anti-inflammatory, analgesic, and anti-microbial purposes (De Moura et al., 2021). Rubiaceae specie are rich with phenolic compounds known for their biological activities including the reduction of oxidative stress implemented in inflammatory processes and the development of acute and chronic inflammatory diseases (Hussain et

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al., 2016), anti-bacterial activity (Bouarab-Chibane et al., 2019), anti-fungal activity (Elansary et al., 2020) and antiviral activity (Kamboj et al., 2012).

In this respect, our study aimed to determine in first instance the phenol contents of the aerial and root parts of *C. angustifolia* growing spontaneously in Tunisia and the antioxidant, the α-glucosidase inhibition, the anti-bacterial activity against a set of bacteria with medical concerns due to their resistance to antibiotics, the anti-fungal, cytotoxicity and the anti-viral activity against a medically important virus causing a wide range of diseases such as type 1 diabetes (Jaidane et al., 2010; Alhazmi et al., 2020), myocarditis (Fairweather et al., 2012; Daba et al., 2019) and a central nervous system pathology especially among young infants (Jmii et al., 2020) which is the Coxsackievirus B3, belonging to enteroviruses genus, that has remained hitherto with no available specific treatment.

Material and methods

Plant material

Crucianella angustifolia L. plant (*Figure 1*) in the flowering stage was collected in June 2020 from Monastir region situated in the East-center of Tunisia (35°46'N, 10°50'E) and was botanically identified by the botanist Pr. Fethia Skhiri (Laboratory of Genetic Biodiversity and Valorisation of Bioresources, Higher Institute of Biotechnology, University of Monastir, Monastir, Tunisia) and according to the description of morphology existing in Tunisian Flora (Pottier-Alapetie, 1981). A voucher specimen (CAABS2020) was deposited in the herbarium of the Laboratory of Transmissible Diseases and Biologically Active Substances, Faculty of Pharmacy, University of Monastir, Tunisia. Fifty different plant samples were cleaned and separated into aerial parts and roots, and then left to dry at room temperature in shadow for 10 days and powdered before to proceed with extractions.

Figure 1. *Crucianella angustifolia L.*

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Aqueous and organic extracts preparation and yields

The aqueous extracts were prepared by immersing the plant material (40 g) in distilled water (1 L). Then, the obtained extracts were sterilized by filtration through 0.45 µm filters (Ben Sassi et al., 2008). For organic extraction, 100 g of the powdered plant material were macerated in increasing polarity organic solvents (petroleum ether, ethyl acetate and methanol). Extractions were performed during 48 h and repeated thrice for each type of solvent. The obtained extracts were subjected to vacuum evaporation in a rotary evaporator (BUCHI, Germany), at 45°C to discard the residual solvent. Yields were determined on a dry-weight basis (*Eq.1*) by the following formula:

$$\text{Yield (\%)} = (W'/W) \quad \text{(Eq.1)}$$

With W' corresponds to the weight of the obtained extract and W represents the weight of plant material used in extraction.

Phenolic content determination

Total phenols were quantified by Folin-Ciocalteu method (Singleton and Rossi, 1965) and results are reported as mg of gallic acid equivalent/g of dry weight (mg GAE/g DW). The determination of flavonoid and flavonol contents was carried out using aluminum chloride as described by Lamaison and Carnat (1991) and Miliauskas et al. (2004), respectively. The amounts of tannin were determined by the method described by Sun et al. (1998). Flavonoid, flavonol and tannin contents were expressed as mg of catechin equivalent/g of dry weight (mg CE/g DW).

Antioxidant activity assessment

The antioxidant activity of our extracts was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays in addition to the reducing power test.

The ability to reduce the DPPH and ABTS free radicals was determined according to the methods described by Huang et al. (2005) and Re et al. (1999), respectively. The percentage radical inhibition (*Eq.2*) was determined by the following formula:

$$\text{\% radical (DPPH or ABTS) inhibition} = [(\text{A control} - \text{A sample})/\text{A control}] \times 100 \quad \text{(Eq.2)}$$

where, A control is control reaction absorbance, and A sample is sample test absorbance. Thereafter, IC50 value (concentration responsible to inhibit DPPH or ABTS radicals to 50%) was determined.

To confirm the antioxidant activity of the tested extracts, their reducing power was assessed according to the method described by Sanja et al. (2009). The absorbance was measured at a wavelength of 700 nm and absorbance increase indicates a reducing power increase. Trolox was used as a reference product. Each treatment was replicated thrice for every concentration and test.

α-glucosidase inhibitory activity

Plant extracts were evaluated for their α-glucosidase inhibitory activity according to the method described by Tao et al. (2013). Plant extracts were mixed with the enzyme α-

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glucosidase (0.3 U/ml) and the substrate p-nitrophenyl-α-D-glucopyranoside (2.5 mmol/l). After incubation for 15 min at 37°C, the mixture absorbance was measured at 405 nm. Acarbose which inhibits specifically α-glucosidase enzyme was used as a reference control. The percentage α-glucosidase inhibition (*Eq.3*) was calculated using the following formula:

$$\alpha\text{-glucosidase inhibition (\%)} = [1- (\text{A sample}/\text{A control})] \times 100 \qquad \text{(Eq.3)}$$

where A sample is the absorbance of the sample and A control is the absorbance of the control. IC_{50} (concentration required to inhibit 50% of the enzyme activity) was calculated for each extract using linear regression analysis. Each treatment was replicated thrice for every concentration.

Evaluation of the anti-bacterial and anti-fungal activities

The anti-bacterial activity of the various obtained extracts was evaluated on 14 bacterial strains: *Escherichia coli* ATCC 8739, *Bacillus anthracis* (clinical strain), *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* (clinical strain), *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus simulans* CIP 8164, *Staphylococcus hominis* CIP 8157, *Staphylococcus haemolyticus* CIP 8156, *Escherichia coli* CIP 54117, *Proteus mirabilis* CIP 6450, *Hafnia alvei* CIP 5731, *Bacillus subtilis* CIP 5265, *Staphylococcus epidermidis* CIP 53124, while the anti-fungal activity was assessed on the unicellular fungus *Candida albicans* (clinical strain). The American Type Culture Collection (ATCC) and clinical strains were provided from the Laboratory LR99ES27, Monastir, Tunisia. Pasteur Institute Collection strains were acquired from the Laboratory of Marine Biotechnology and Chemistry, Bretagne Sud University, Vannes, France. The selected microorganisms were chosen for their medical interest. Two types of tests were carried out.

Agar disc diffusion method

Bacterial strains and *C. albicans* (10^6 UFC/mL) were seeded to Mueller Hinton (MH) or Sabouraud agar medium, respectively for bacteria or yeast. Then, paper discs with 6 mm in diameter were impregnated with 10 µl of the extract for testing at a concentration of 5 mg/mL (Bel Hadj Salah et al., 2022). Negative and positive control discs were also included in the assay and were impregnated respectively by the 10 µl of DMSO (1%) or ampicillin (10 µg/disc) for bacterial cultures and amphothericin B (10 µg/disc) for yeast cultures. Thereafter, Petri dishes were incubated at 37°C for 24 h. All experiments were done in triplicate. The activity was evaluated by measuring the radius of the inhibition zone (mm) and results are interpreted as per Ponce et al. (2003) as follows:

Non-sensitive or resistant strain (-): measured diameter of inhibition (including disc) < 8 mm; sensitive strain (+): 8 mm < diameter < 14 mm; very sensitive strain (++): 15 < diameter < 19 mm; highly sensitive strain (+++): diameter > 20 mm.

Micro-well plate dilution method

Resazurin reagent was used to assess the viability and the growth of the tested bacteria or yeast following the treatment with the obtained extracts. 100 µl of the extract to test were serially diluted in micro-well plates (1:2 dilutions in medium), the obtained concentrations after dilution ranged from 5 to 0.001 mg/ml. Then, 20 µl of the bacterial

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or yeast suspension and 10 µl of Resazurin reagent were added to each well. For the negative control wells (MH or Sabouraud) medium was added, while for positive control ones, 10 µl of diluted ampicillin or amphotericin B solution were added instead of diluted extracts. Afterwards, microplates were maintained at 37°C for 24 h before assessing the antimicrobial activity. Indeed, growing bacteria or yeast metabolize (reduction reaction) the Resazurin reagent leading to a change in its color from blue to pink. Thus, when microbial growth is inhibited, the color of Resazurin reagent remains blue and this characteristic was exploited to evaluate the minimal inhibitory concentration (MIC) (Yajko et al., 1995; Lozano-Chui et al*.,* 1998). Thereafter, the minimal bactericide or fungicide concentrations were determined for the various tested extracts as follows: the content of microplate wells having a concentration ≥ MIC was used to inoculate agar medium MH or Sabouraud respectively for bacteria or yeast. The microorganisms were allowed to grow for 24 h at 37°C and the extract was considered bactericide or fungicide when it was able to reduce the microorganisms number by up to 1000-fold (Scott, 2009). Hence, the minimal bactericide (CMB) or fungicide concentrations were determined and the CMB/CMI quotient was calculated and interpreted according to Berche et al. (1991). Thus, if CMB/CMI = 1 to 2, the tested extract has a bactericide or fungicide effect; if CMB/CMI = 4 to 16, the tested extract has a bacteriostatic or fungistatic effect. All experiments were done in triplicate.

Cytotoxicity and anti-viral activity

Cytotoxicity

The cytotoxicity of the various extracts obtained from *Crucianella angustifolia* was assessed on the Hep-2 cells by the MTT salt reduction method. Thus, 5.10^4 cells were seeded in 96-well plates and allowed to grow for 24 h to reach 90% of confluence. Cells were cultured in MEM medium supplemented with 1% penicillin/Streptomycin antibiotics, 10% FCS, 1% non-essential amino acids, and 2.5 µg/ml of Amphotericin B. Thereafter, culture medium was removed from wells and cells were washed with PBS before adding 200 µl of the extract solution (two-fold dilutions, ranging from 1000 to 3.9 µg/ml). Cells were incubated for 72 h at 37°C and 5% CO_2. For control cells, culture medium was added to wells instead of diluted extracts. Subsequently, cells were washed with PBS after removing the culture medium and MTT solution (20 µl; 5 mg/ml) was added to each well. Plates were incubated at 37°C and 5% CO_2 for 4 h and formazan crystals formed by mitochondrial metabolism were solubilized in DMSO. The absorbance was measured on a microplate reader (ThermoFisher Scientific, USA) at a wavelength of 540 nm (Armania et al., 2013). Cell viability was determined by comparing the treated cells absorbance with the absorbance of the control cells using the following formula (*Eq.4*):

$$\text{Cell viability (\%)} = \text{Abs}_{\text{treated}} / \text{Abs}_{\text{control}} \times 100 \qquad \text{(Eq.4)}$$

where $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{treated}}$ are the absorbance for the wells without and with extract, respectively. The CC50 (extract concentration that reduced cell viability by 50%) and CC10 (extract concentration that leaves 90% viable cells) values were determined. Concentrations that were equal or bellow CC10 were used in anti-viral activity assessment. All experiments were done in triplicate.

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Anti-viral activity

The various extracts obtained from *Crucianella angustifolia* were tested for the capacity to suppress or at least reduce cythopathic effect caused by virus infection and the ensuing cell death. Anti-viral activity was evaluated by monitoring cythopathic effect appearance and the determination of cell viability after a period of incubation with the virus. To this end, cell culture (5 10^4 cells/100 µL/well) was done in 96 well plates at 37°C and 5% CO_2 for 24 h. The medium was removed, and cells were washed twice with Phosphate-buffered saline (PBS). Then, extract (50 µl) diluted in MEM 2% FCS (concentration ranging from 125 µg to 3.9 µg/ml) was added. Shortly after, cells were inoculated with 10^4 $TCID_{50}$ CVB3 suspension (50 µl). In the same way, cell death induced by virus infection was evaluated by MTT assay. The percentage of virus inhibition (*Eq.5*) was measured by the following formula (Schmidtke et al., 2001):

$$\text{Virus inhibition (\%)} = \text{T-VC/CC-VC} \quad \text{(Eq.5)}$$

where T, VC and CC are the optical densities of compound treated cells, virus control and cell control, respectively. The IC50 (50% inhibitory concentration of the virus) value was determined from the dose-response curve obtained from the results. The selectivity index (SI) was evaluated as the CC50/IC50 (Ellithey et al., 2014). Moreover, cells were daily examined for the cytopathic effect with an inverted microscope (TCM 400, Labomed, USA). All experiments were done in triplicate.

Statistical analysis

Data means and standard deviation were estimated from independent experiments. All tests were performed in triplicate. Analysis of data was carried out by one-way analysis of variance (ANOVA) in the SPSS program Ver. 20., followed by multi-range post hoc of Duncan's test and $p<0.05$ was considered significant.

Results

Extraction yields and phenolic contents

Extractions were carried out from the aerial part and roots of *Crucianella angustifolia* using solvents with various polarities. The highest yield (13.58%) was obtained by methanolic extract, followed by petroleum ether one (1.81%) from the plant aerial part (*Table 1*). The amounts of total phenols, flavonoids, flavonols and tannins extracted from *C. angustifolia* varied depending on the plant part and the type of solvent used in extraction (*Table 1*). Methanolic extract yielded the highest quantities of phenols for both roots and aerial part (6.67 and 4.64 mg GAE/g DW, respectively). The highest quantity of flavonoid was recovered from roots of the plant using methanol (240.6 mg CE/g DW) followed by ethyl acetate extract from roots alike. Aqueous extract from the plant aerial part yielded the highest quantity of flavonols (77.21 mg CE/g DW) followed by ethyl acetate extract from roots (27.55 mg CE/g DW). Aqueous extract from aerial part and roots of the studied plant yielded the highest amounts of tannin (4.56 and 1.5 mg CE/g DW, respectively) followed by the methanolic extract from the plant aerial part (1.38 mg CE/g DW) (*Table 1*).

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Table 1. *Yields, total phenol, flavonoid, flavonol and tannin contents of aqueous and organic extracts of aerial part and roots of Crucianella angustifolia*

Organ	Solvent	Yield (%)	Total phenols (mg GAE/g DW)	Flavonoids (mg CE/g DW)	Flavonols (mg CE/g DW)	Tannins (mg CE/g MS)
Aerial part	Petroleum ether	1.81^{d}	0.29 ± 0.02^{a}	8.57 ± 0.34^{b}	3.38 ± 0.12^{b}	0.1 ± 0.004^{c}
	Ethyl acetate	0.79^{a}	1.55 ± 0.03^{d}	28.71 ± 0.05^{c}	2.05 ± 0.08^{a}	0.045 ± 0.002^{b}
	Methanol	13.58^{e}	4.64 ± 0.1^{e}	67.21 ± 0.2^{d}	5.33 ± 0.1^{c}	1.38 ± 0.1^{d}
	Aqueous		1.06 ± 0.05^{c}	90.0 ± 0.88^{e}	77.21 ± 0.23^{g}	4.56 ± 0.08^{f}
Roots	Ethyl acetate	0.95^{b}	5.18 ± 0.19^{f}	129.85 ± 1.12^{f}	27.55 ± 0.12^{f}	0.035 ± 0.004^{a}
	Methanol	1.39^{c}	6.67 ± 0.07^{g}	240.6 ± 7.8^{g}	12.81 ± 0.16^{e}	0.04 ± 0.005^{ab}
	Aqueous		0.53 ± 0.04^{b}	7.42 ± 0.06^{a}	6.44 ± 0.37^{d}	1.5 ± 0.09^{e}

Means in the same column (a-g) bearing different superscripts in sample are significantly different by Duncan's multiple range test ($p<0.05$) and All tests were performed in triplicates (n = 3); mg GAE/g DW: mg gallic acid equivalents per gram of dry weight of the plant material; mg CE/g DW: mg catechin equivalents per gram of dry weight of the plant material

Antioxidant activity

As detailed above in the material and methods section, the DPPH and ABTS radical scavenging and reducing power tests were used to evaluate the antioxidant capacity of the various extracts obtained from the aerial part and roots of *Crucianella angustifolia*. By the DPPH test, the plant extracts exhibited remarkable free radical scavenging activity. The highest rates were recorded for methanolic extracts for both aerial part and roots of the plant (IC50 of 3.74 and 4.91 mg/ml, respectively) (*Table 2*). As shown in the *Table 2*, roots ethyl acetate extract exhibited the second highest free radical scavenging effect (IC50 of 10 mg/ml). ABTS test was also used to confirm results, with respect to antiradical activity, obtained by the DPPH test. Similarly to DPPH test, the methanolic extracts obtained from roots and aerial part of the studied plant showed the highest antioxidant activity (IC50 of 4.91 and 6.09 mg/ml, respectively) (*Table 2*). The ethyl acetate extract obtained from roots presents an IC50 of 7.81 mg/ml (*Table 2*). Reducing power test demonstrated that aqueous and organic extracts from both roots and aerial part enhance iron reduction in solvent-dependent manner. All tested extracts showed a lower reducing activity than the antioxidant standard Trolox (*Table 2*).

α-glucosidase inhibitory activity

The anti-α-glucosidase activity of *Crucianella angustifolia* extracts are presented in *Table 3*. The highest activity was obtained by methanolic extracts of the plant's aerial part and roots with IC50 of 0.03 and 0.04 mg/ml, respectively. This inhibitory activity was superior to that of acarbose (IC50=0.07 mg/ml).

Anti-bacterial and anti-fungal activities

Agar disc diffusion assay

For the extracts obtained from the *Crucianella angustifolia* aerial part, methanol turned out to be the most active extract (*Table 4*). It inhibited the diffusion of most of the bacteria strains tested. Among these strains, *Pseudomonas aeruginosa* was the most sensible to methanolic extract with a diameter of the inhibition zone equal to 12 mm followed by *Bacillus subtilis* CIP 5265 (11 mm). Regarding the anti-fungal activity, the petroleum

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ether extract was the most active against *Candida albicans* with an inhibitory zone diameter of 12 mm (*Table 4*). For the roots extracts of *C. angustifolia*, similarly to the aerial part the methanolic extract was the most active one which turned out to be effective against many tested bacteria strains. The roots methanol extract applied to *Escherichia coli ATCC* 8739 showed the highest diameter of inhibition zone which was equal to 13 mm. The roots organic extracts were active also against *C. albicans* yeast and the ethyl acetate extract was the most effective among the tested extracts (*Table 4*).

Table 2. *Antioxidant capacity assessed by DPPH, ABTS and reducing power tests of aqueous and organic extracts of aerial part and roots of Crucianella angustifolia*

Organ	Solvent	DPPH test		ABTS test		Reducing power test	
		Scavenging activity at 10 mg/ml (%)	IC50 (mg/ml)	Scavenging activity at 10 mg/ml (%)	IC50 (mg/ml)	Absorbance at 10 mg/ml	IC50 (mg/ml)
Aerial part	Petroleum ether	11.1 ± 1.2^{b}	> 10	14.93 ± 1.1^{c}	> 10	0.13 ± 0.09^{b}	> 10
	Ethyl acetate	24.9 ± 1.65^{c}	> 10	40.74 ± 1.4^{d}	> 10	0.25 ± 0.05^{c}	> 10
	Methanol	75.0 ± 1.78^{e}	3.74	76.11 ± 1.5^{g}	4.91	0.32 ± 0.07^{d}	> 10
	Aqueous	8.14 ± 0.85^{a}	> 10	5.48 ± 0.45^{a}	> 10	0.12 ± 0.03^{b}	> 10
Roots	Ethyl acetate	50.0 ± 1.1^{d}	10	58.79 ± 1.6^{e}	7.81	0.27 ± 0.03^{c}	> 10
	Methanol	79.94 ± 1.5^{f}	4.91	72.24 ± 1.8^{f}	6.09	0.57 ± 0.04^{e}	7.48
	Aqueous	7.55 ± 0.72^{a}	> 10	8.26 ± 0.42^{b}	> 10	0.08 ± 0.01^{a}	> 10
Trolox (250 µg/ml)		91.26 ± 1.5^{g}	0.136	86.44 ± 1.2^{h}	0.145	0.68 ± 0.05^{f}	0.166

Means in the same column (a-h) bearing different superscripts in sample are significantly different by Duncan's multiple range test ($p<0.05$) and All tests were performed in triplicates (n = 3); IC50: Inhibitory concentration at 50%

Table 3. *α-glucosidase inhibition of Crucianella angustifolia extracts*

Organ	Extract	IC$_{50}$ (mg/mL)
Aerial part	Petroleum ether	0.099 ± 0.02^{c}
	Ethyl acetate	0.3 ± 0.01^{e}
	Methanol	0.03 ± 0.01^{a}
Roots	Petroleum ether	0.14 ± 0.01^{d}
	Ethyl acetate	0.39 ± 0.02^{e}
	Methanol	0.04 ± 0.01^{a}
Acarbose		0.07 ± 0.01^{b}

IC50 values are shown as mean±SD of three independent experiments. Different letters show significant differences by Duncan's multiple range test ($p<0.05$) and All tests were performed in triplicates (n = 3); IC50: Inhibitory concentration at 50%

Micro-well plate dilution assay

Micro-well plate dilution assay was performed for *Crucianella angustifolia* extracts to determine the MIC, MBC, and MFC of extracts proven with anti-bacterial and anti-fungal activities as witnessed by the disc-diffusion inhibition tests. Results demonstrated that the totality of extracts from the aerial part of *C. angustifolia* had important anti-bacterial and anti-fungal activities with MIC ranging from 5 to 0.001 mg/ml, MBC ranging from 5 to 0.004 mg/ml and MFC varying from 0.156 to 0.625 mg/ml (*Table 5*).

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Table 4. *Anti-bacterial and anti-candidal activities (IZD in mm) of C. angustifolia aerial part and root extracts*

	Aerial part				**Roots**			**Ampicillin (10 µg/disc)**	**Amphotéricine B (10 µg/disk)**
Strain / **Extract**	**Petroleum ether**	**Ethyl acetate**	**Methanol**	**Aqueous**	**Ethyl acetate**	**Methanol**	**Aqueous**		
Escherichia coli ATCC 8739	9	7	10	7	6	13	6	20	
Bacillus anthracis (clinical strain)	7	7	10	7	6	12	6	25	
Bacillus subtilis ATCC 6633	7	8	7	7	7	8	6	22	
Staphylococcus epidermidis (clinical strain)	8	7	7	7	10	6	6	30	
Staphylococcus aureus ATCC 6538	8	8	8	7	7	8	6	28	
Pseudomonas aeruginosa ATCC 9027	7	9	12	7	7	8	6	30	
Staphylococcus simulans CIP 8164	9	7	7	8	6	7	6	38	
Staphylococcus hominis CIP 8157	7	7	7	7	6	12	6	40	
Staphylococcus haemolyticus CIP 8156	8	8	10	8	6	10	6	30	
Escherichia coli CIP 54117	9	10	8	9	6	6	6	30	
Proteus mirabilis CIP 6450	8	9	8	9	6	10	6	40	
Hafnia alvei CIP 5731	7	7	7	9	6	10	6	37	
Bacillus subtilis CIP 5265	7	7	11	8	6	10	6	35	
Staphylococcus epidermidis CIP 53124	7	7	9	7	6	8	6	34	
Candida albicans (clinical strain)	12	8	8	7	9	8	6		18

IZD: Inhibition Zone Diameter; CIP: strains from the Collection of Pasteur Institute; ATCC: American Type Culture Collection

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Table 5. *Anti-bacterial and anti-candidal activities (MIC, MBC and MFC in mg/ml) of C. angustifolia aerial part extracts*

Extract	**Aqueous**			**Petroleum ether**			**Ethyle acetate**			**Methanol**			**Ampicillin**		**Amphotericin B**	
Strain	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
Escherichia coli ATCC 8739	0.312	2.5	8	0.078	0.156	2	0.078	0.156	2	0.312	5	16	0.001	0.002		
Bacillus anthracis (clinical strain)	0.019	0.019	1	2.5	2.5	1	1.25	1.25	1	0.039	0.625	16	0.078	0.625		
Bacillus subtilis ATCC 6633	0.039	0.039	1	2.5	2.5	1	0.156	1.25	8	0.312	0.312	1	0.019	0.039		
Staphylococcus epidermidis (clinical strain)	0.019	0.312	16	0.312	1.25	4	0.156	2.5	16	0.019	0.312	16	0.002	0.004		
Staphylococcus aureus ATCC 6538	0.009	0.078	8	1.25	2.5	2	0.625	1.25	2	0.312	0.312	1	0.001	0.001		
Pseudomonas aeruginosa ATCC 9027	0.019	0.312	16	5	5	1	0.039	0.625	16	0.009	0.156	16	0.002	0.002		
Staphylococcus simulans CIP 8164	0.009	0.009	1	0.039	0.625	16	0.019	0.312	16	1.25	>5	-	0.001	0.002		
Staphylococcus hominis CIP 8157	0.019	0.312	16	0.009	0.156	16	0.009	0.156	16	>5	>5	-	0.009	0.078		
Staphylococcus haemolyticus CIP 8156	0.009	0.039	4	0.009	0.039	4	0.078	0.312	4	0.156	2.5	16	0.001	0.001		
Escherichia coli CIP 54117	0.078	0.312	4	0.078	0.078	1	0.004	0.009	2	0.039	0.625	16	0.019	0.078		
Proteus mirabilis CIP 6450	0.009	0.009	1	0.039	0.039	1	0.001	0.004	4	0.156	2.5	16	0.019	0.019		

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Extract / Strain	Aqueous			Petroleum ether			Ethyle acetate			Methanol			Ampicillin		Amphotericin B	
	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
Hafnia alvei CIP 5731	0.019	0.312	16	0.078	0.078	1	0.019	0.312	16	0.156	2.5	16	0.039	0.312		
Bacillus subtilis CIP 5265	0.019	0.312	16	0.078	0.312	4	0.019	0.312	16	0.625	0.625	1	0.039	0.039		
Staphylococcus epidermidis CIP 53124	2.5	>5	-	0.039	0.625	16	0.156	2.5	16	0.625	0.625	1	0.002	0.004		
Candida albicans (clinical strain)	0.019	0.156	16	0.019	0.312	16	0.039	0.625	16	0.019	0.312	16	-	-	0.012	0.015

MIC: Minimum Inhibitory Concentration (mg/ml); MBC: Minimum Bactericidal Concentration (mg/ml); MFC: Minimum Fungicidal Concentration (mg/ml); CIP: strains from the Collection of Pasteur Institute; ATCC: American Type Culture Collection; -: not determined

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Several extracts showed MICs and MBCs close to or lower than those of the antibiotic against several bacterial strains. Some of the tested extracts turned out to be bacteriostatic (MBC/MIC≥16) such as the aerial part methonolic extract which was bacteriostatic against *Escherichia coli* ATCC 8739, *E. coli* CIP 54117, *Bacillus anthracis* (clinical strain), *Staphylococcus epidermidis* (clinical strain), *S. haemolyticus* CIP 8156, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* CIP 6450 and *Hafnia alvei* CIP 5731, while other extracts exhibited a bactericide effect (MBC/MIC ≤ 4), for example the petroleum ether was bactericide against 11 bacterial strains (*Table 6*). The root methanolic extract was the most active among all root extracts and it showed important activity against *E. coli* ATCC 8739 (MIC = 0.004 mg/ml and MBC = 0.009 mg/ml), *B. anthracis* (clinical strain) (MIC = 0.009 mg/ml and MBC = 0.019 mg/ml) and *S. hominis* CIP 8157 (MIC = 0.009 mg/ml and MBC = 0.039 mg/ml) (*Table 6*). Methanol and ethyl acetate extracts were active against *C. albicans* with MIC of 0.625 and 0.156 mg/ml, respectively (*Table 6*).

Table 6. *Anti-bacterial and anti-candidal activities (MIC, MBC and MFC in mg/ml) of C. angustifolia root extracts*

Extract / Strain	Ethyle acetate			Methanol			Aqueous		
	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC	MIC (mg/ml)	MBC or MFC (mg/ml)	MBC /MIC
Escherichia coli ATCC 8739	>5	>5	-	0.004	0.019	4	>5	>5	-
Bacillus anthracis (clinical strain)	>5	>5	-	0.009	0.019	2	>5	>5	-
Bacillus subtilis ATCC 6633	2.5	>5	-	0.156	0.625	4	>5	>5	-
Staphylococcus epidermidis (clinical strain)	0.312	0.625	2	>5	>5	-	>5	>5	-
Staphylococcus aureus ATCC 6538	1.25	2.5	2	0.312	0.625	2	>5	>5	-
Pseudomonas aeruginosa ATCC 9027	1.25	2.5	2	0.312	0.625	2	>5	>5	-
Staphylococcus simulans CIP 8164	>5	>5	-	0.625	2.5	4	>5	>5	-
Staphylococcus hominis CIP 8157	>5	>5	-	0.009	0.039	4	>5	>5	-
Staphylococcus haemolyticus CIP 8156	>5	>5	-	0.039	0.078	2	>5	>5	-
Escherichia coli CIP 54117	>5	>5	-	>5	>5	-	>5	>5	-
Proteus mirabilis CIP 6450	>5	>5	-	0.019	0.078	4	>5	>5	-
Hafnia alvei CIP 5731	>5	>5	-	0.019	0.078	4	>5	>5	-
Bacillus subtilis CIP 5265	>5	>5	-	0.039	0.078	2	>5	>5	-
Staphylococcus epidermidis CIP 53124	>5	>5	-	0.312	1.25	4	>5	>5	-
Candida albicans (clinical strain)	0.156	0.312	2	0.625	1.25	2	>5	>5	-

MIC: Minimum Inhibitory Concentration (mg/mL); MBC: Minimum Bactericidal Concentration (mg/mL); MFC: Minimum Fungicidal Concentration (mg/mL); CIP: strains from the Collection of Pasteur Institute; ATCC: American Type Culture Collection; -: not determined

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Cytotoxicity and anti-viral activity

Cytotoxicity

The cytotoxicity of the various extracts from *Crucianella angustifolia* aerial part and roots was assessed using the MTT assay on Hep-2 cell line for concentrations ranging from 1000 to 3.9 µg/ml. All extracts obtained from the plant turned out to be not toxic for Hep-2 cells with a CC50>1000 µg/ml (*Figure 2* and *Table 7*). For the CC10, which is the concentration tolerated to be used in biological assays, all extracts were well tolerated by Hep-2 cells at high concentrations ranging from 500 to >1000 µg/ml which opens perspective for their use in therapies development (*Figure 2* and *Table 7*). Furthermore, the addition of plant extracts enhanced cell proliferation with an increase of cell viability that can attain, for example, 169.32 % when cultivated cells were treated with a low concentration (62.5 µg/ml) of the aqueous extract obtained from the plant roots (*Figure 2* and *Table 7*).

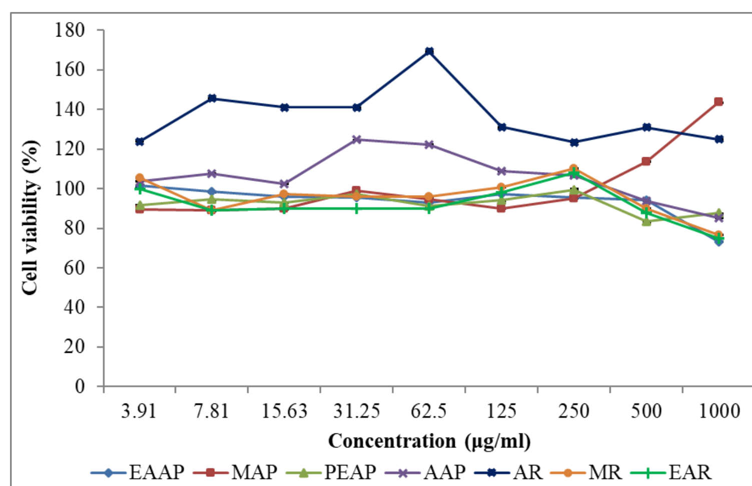

Figure 2. *Viability rates of Hep-2 cells treated with different concentrations of C. angustifolia extracts. EAAP: Ethyl acetate extract from the aerial part of the plant; MAP: Methanol extract from the aerial part of the plant; PEAP: Petroleum ether extract from the aerial part of the plant; AAP: Aqueous extract from the aerial part of the plant; AR: Aqueous extract from the roots of the plant; MR: Methanol extract from the roots of the plant; EAR: Ethyl acetate extract from the roots of the plant*

Table 7. *Cytotoxicity (CC_{10} and CC_{50}) and anti-CVB3 activity (IC_{50}, and SI) of the various extracts obtained from C. angustifolia*

Organ	Solvent	**CC50** (µg/ml)	**CC10** (µg/ml)	**IC50** (µg/ml)	**SI**
Aerial part	Petroleum ether	>1000	841,66±0,91	5.92±0,07	>168.92
	Ethyl acetate	>1000	610,73±0.63	-	-
	Methanol	>1000	>1000	-	-
	Aqueous	>1000	725,88±0.85	-	-
Roots	Ethyl acetate	>1000	543.18±0.74	-	-
	Methanol	>1000	500.00±0.67	-	-
	Aqueous	>1000	>1000	8.93±0.09	>111.98

CC50: extract concentration that reduced cell viability by 50%; CC10: extract concentration that leaves 90% viable cells; IC50: extract concentration that inhibited virus by 50%; SI: selective index; -: inactive. All tests were performed in triplicates (n = 3) and are significant at $p < 0.05$ when compared to control

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Anti-viral activity

The anti-viral activity of *Crucianella angustifolia* extracts was evaluated at a concentration ranging from 125 to 3.9 µg/ml chosen on the basis of cytotoxicity results. This concentration range enables to obtain 90% of cell viability for all tested extracts. Microscopic observations demonstrated that plant extracts reduced the cytopathic effect that CVB3 infection could cause (*Figure 3*). This protection was reflected in the maintenance of cell viability as determined by MTT assay. Anti-viral activity varied according to the type and the concentration of the used extract (*Figure 4*). Petroleum ether extract from the plant aerial part was the most effective with an IC_{50} equal to 5.92 µg/ml and a CC_{50}>1000 µg/ml. The high selectivity index (>168.92) (*Table 7*) suggests the anti-coxsackievirus B3 potential of the extract.

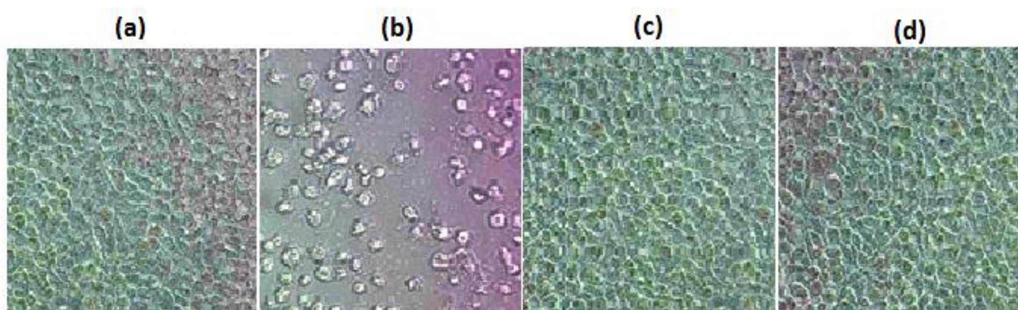

Figure 3. *An Illustration of CVB3 cythopathic effect inhibition in Hep-2 cells by extracts of C. angustifolia. (a): control cells with no treatment or infection, no cythopathic effect was observed and cells appeared connected and expanded; (b): Virus control with induced cytopathic effect featured by lysis plaques formation and cell shrinking; (c): Inhibition of CVB3 cytopathic effect by aqueous extract from plant roots used at a concentration of 125µg/ml, no cellular abnormalities were noticed and cells appeared similar to control cells; (d): Likewise when cells were treated by petroleum ether extract from the plant aerial part at a concentration of 125µg/ml, no cythopathic effect was observed and cells appeared normal*

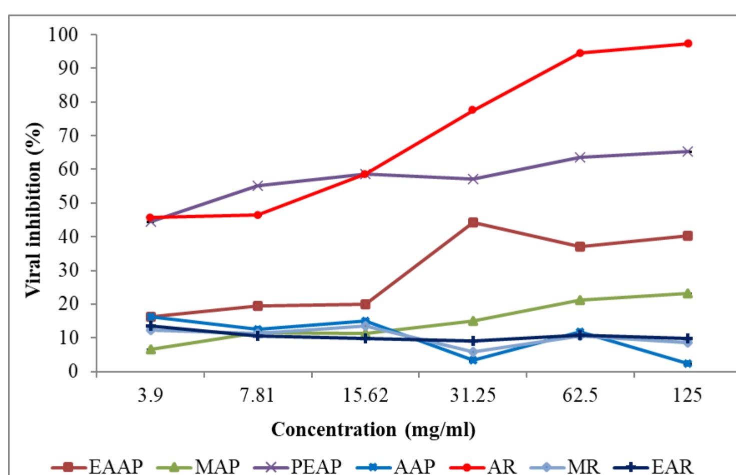

Figure 4. *CVB3 inhibition activity of C. angustifolia extracts. EAAP: Ethyl acetate extract from the aerial part of the plant; MAP: Methanol extract from the aerial part of the plant; PEAP: Petroleum ether extract from the aerial part of the plant; AAP: Aqueous extract from the aerial part of the plant; AR: Aqueous extract from the roots of the plant; MR: Methanol extract from the roots of the plant; EAR: Ethyl acetate extract from the roots of the plant*

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Discussion

The quantities of phenolic compounds extracted from *Crucianella angustifolia* were variable depending on the plant part and the solvent used in extraction. Indeed, the accumulation of phenolic compounds in plant organs is linked to the presence of specific tissues such as mesophyll, epidermis, chloroplast which explains the variation between plant organs in terms of phenols production (Hudaib et al., 2002). In addition, the synthesis of phenolic compounds is related to other factors including genetic mutations, infections, climate change, soil type, and plant age (Luciano et al., 2004; Chen et al., 2009). On the other hand, the amounts of extracted phenols also depend on the type of solvent used in extraction; for example, in our study, methonolic extraction yielded the highest quantities of flavonoids from the plant roots (240.6 mg EC/g MS) while, when aerial part was used, it was water that yielded the highest quantities of flavonoid (90.0 mg EC/g MS). In general, the Rubiaceae family is known for its richness in several chemical compounds like iridoids, indole alkaloids, anthraquinones, terpenoids, flavonoids and phenolic compounds (Adamski et al., 2020; Malihe et al., 2023). Moreover, the secondary metabolites profile is even taken into account in the classification of plants belonging to Rubiaceae family (Martins and Nunez, 2015).

In the current study, *C. angustifolia* turned out to be rich in flavonoids and at lesser extent flavonols and it was low in Tannin. Our results are in accordance with those obtained by Vahedi et al. (2013) who found out that *Crucianella sintenisii*, which belongs to the *Crucianella* gener was rich in flavonoids, alkaloids, terpenes and steroids but poor in saponins and tannins. The only study carried out on the species *C. angustifolia* dates from 1938 (Juillet et al., 1938). These authors have shown that this species as well as *Crucianella maritima* are rich in iridoids. Likewise, Mitova et al. (1996) have shown that the *Crucianella graeca* species is rich in coumarins and iridoids. El-Lakany et al. (2004) have shown that *C. maritima* is rich in anthraquinones, iridoids, triterpenes, sterols and flavonoids and that the root part is rich especially in anthraquinones. In the same context, Sabri et al. (1988) were able to isolate the following flavonoids from the aerial part of *C. maritima*: quercetin, rhamnetin, isorhamnetin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-neohesperidoside. The methylphenylindole alkaloid was isolated from *Crucianella sintenisii* growing in Iran (Malihe et al., 2023).

The obtained extracts from *C. angustifolia* exhibited an antioxidant activity eventhough the latter was lower than the reference product (Trolox). To our knowledge, our study is the first to be interested in assessing the antioxidant power of *C. angustifolia*. Nevertheless, the antioxidant activity of other species belonging to the Rubiaceae family has well been described in the literature. For example, the species *C. maritima* has an antioxidant activity as determined by the DPPH radical scavenging test (Badr, 2008), *Capparis spinosa* has also exhibited an antioxidant activity which can reach (78,34%) for the methnolic extract tested for its DPPH radical scavenging power (Meddour et al., 2013), and the specie *Massularia acuminata* that showed a considerable antioxidant activity exerted principally by its phenolic compounds (Maloueki et al., 2015).

To our knowledge, no studies have previously been interested in evaluating the capacity of *C. angustifolia* extracts in the inhibition of the enzyme α-glucosidase. Nevertheless, some studies have been interested in assessing the potential of extracts from plants belonging to the family of Rubiaceae such as *Amaracarpus pubescens* extracts that have an inhibitory activity of α-glucosidase (Elya et al., 2012); the leaf extract of *Mussaenda roxburghii* exhibited also a capacity to inhibit *α*-glucosidase activity (Maiti

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et al., 2013) and different solvent extracts of *Morinda citrifolia* fruit pulp inhibit α-glucosidase enzyme in an *in vitro* study (Mahadeva, 2018).

Similarly to the antioxidant and α-glucosidase inhibition activities, the anti-bacterial and anti-fungal activities of *C. angustifolia* have not been previously evaluated. Nonetheless, several studies have been interested in evaluating the anti-bacterial activity of some species belonging to the genus *Crucianella*. Badr (2008) demonstrated that the *C. maritima* species possessed antibacterial and antifungal activity towards the same strains tested in the current study, namely *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and *Candida albicans* (ATCC 2091); this author used the same method that we adopted, which is the agar disk diffusion method. Likewise, El-Lakany et al. (2004) have demonstrated that *C. maritima* has an anti-bacterial activity especially towards *Staphylococcus aureus* and *Proteus mirabilis* strains which is in line with our results.

The various extracts of *C. angustifolia* were non-toxic towards Hep-2 cell line according to the criteria fixed by Prayong et al. (2008) indicating that an extract is to be considered non-toxic when CC_{50}>1000 µg/ml, which is the case for all extracts obtained in our study. This makes them promising candidates for therapeutics research studies. In this context, we have tried to evaluate the antiviral activity of extracts obtained from *C. angustifolia* towards the enterovirus CVB3. So far, no study has been conducted to evaluate the anti-viral activity of extracts or compounds derived from *C. angustifolia*. Nevertheless, some studies have been interested in studying the antiviral activity of extracts obtained from other species belonging to Rubiaceae family. For examples, Prajapati and Parmar (2011) demonstrated that *Rubia cordifolia* extracts were active against *Herpes simplex* viruses and *Vaccina* virus, extracts from *Nauclea latifolia* turned out to be active on *Herpes simplex* virus 2 (Donalisio et al., 2013), and similarly for Konigheim et al. (2012) who proved that extracts from *Heterophyllaea pustulata* were active toward on *Herpes simplex* virus 2.

As noted in the introduction, CVB3 and generally enteroviruses are of medical interest; yet no antiviral medications are currently approved for the treatment of enterovirus infections and usually ribavirin, a broad-spectrum antiviral drug, is prescribed in the case of such infections (Kirsi et al., 1983; Zhao-Hong et al., 2008). Extracts from the studied plant exhibited a relevant activity against CVB3 according to the criteria indicated by Cos et al. (2006) demonstrating that extracts with an IC50 below 100 µg/ml should be considered active against the tested virus. Most importantly, the selectivity index was high (168.92 and 111.98 µg/ml for aerial part petroleum ether and root aqueous extracts, respectively) which combines efficacy and safety. It is generally considered that a drug has a good safety profile if its SI exceeds the value of 10 (Tamargo et al., 2015). The antiviral activity of extracts from *C. angustifolia* is likely to be attributed to the presence of polyphenols such as flavonoids or flavonols known for their antiviral properties (Arora et al., 2023; Morimoto et al., 2023). So, the compounds responsible for the anti-viral activity of the tested extracts and their mode of action needs to be determined.

Conclusion

In the current study, the phenol content, the antioxidant, the inhibition of the α-glucosidase, the antibacterial, the antifungal, the cytotoxicity, and the antiviral activities of *Crucianella angustifolia* growing in Tunisia were studied for the first time in the present work. The results have shown that the extracts of *C. angustifolia* are rich in

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polyphenols especially in flavonoids. The biological studies of the obtained extracts have demonstrated a significant antioxidant activity, a power to hamper the activity of the α-glucosidase enzyme, an antibacterial and an antifungal activity, low cytotoxicity and a relevant activity against the virus CVB3. Results with respect to biological activities obtained in the present study are promising. Nevertheless, future studies are necessary to identify the major components that are responsible for the observed biological activities and their mechanisms of action.

Conflict of interests. Authors do not have any conflict of interests.

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