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Evaluation of Antioxidant and Antimicrobial Activities of Flowers Extracts of *Chrysanthemum Segetum* L.

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ABSTRACT

The present study deals with the assessment of the total phenolics and flavonoids contents of different extracts of Chrysanthemum segetum namely hexane, chloroform (CHCl3), ethyl acetate (EtOAc) and n-butanol (n-BuOH) extracts. N-BuOH extract displayed the maximum phenolics content (185.50 ± 0.09 µg GAE/ mg of extract) followed by EtOAc extract (140.25±0.06). Conversely, the flavonoids content was found to be maximum in EtOAc extract (40.30 ±0.90 µg of QE/ mg extract) followed by n-BuOH extract (27.00 ± 0.30). The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect study reveals that the inhibition percentage (I %) increases with time and concentration where the values were found to be 76.70, 79.50, 73.65, and 36.50% for n-BuOH, EtOAc, CHCl3 and hexane extracts, respectively. IC50 of DPPH scavenging activity of the four extracts was found to be 26.50, 29.25, 34.70 and 59.20 µg/mL for n-BuOH, EtOAc, and CHCl3, respectively. The antimicrobial activity was assessed against a set of Gram-positive and Gram-negative bacteria as well as against the fungus Candida albicans to find out that the n-BuOH and EtOAc extracts exhibit potent activities against Gram-positive bacteria together with the fungus compared to the other extracts. In addition, Staphylococcus aureus was found to be more sensitive and Pseudomonas aeruginosa more resistant to the tested extracts.

Keywords: *Chrysanthemum Segetum, Polyphenols, Flavonoïds, Anti-Microbial Activity, Antioxidant Activity.*

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1. INTRODUCTION

Algeria involves very assorted flora especially medicinal and aromatic plants because of its vast surface area and varied environments. It contains many native plants which have not been subjected to any study yet. Some reports have revealed that there are at least 500 herbs belonging to 109 genera of Asteraceae family used by people in traditional medicine, including 15 species of the genus *Chrysanthemum* (Quezel and Santa, 1963).

Phytochemical and biological researches have shown that many natural compounds displayed important therapeutic effects namely coumarins, alkaloids, flavonoids, steroids and so forth (Ochocka et al. 1995, Hu et al., 1994; Valant et al., 2003).

The species of the genus *Chrysanthemum* are rich in secondary metabolites that engender a variety of biological activities such as antimicrobial and antiviral actions. *Chrysanthemum segetum* is widely distributed in Algeria. Some studies have reported the isolation and characterization of flavonoids, coumarins and polyacetylenes from this species (Kennouche et al, 2017, Park et al, 2009, Teps et al., 2005, Wang et al., 2012, Kim et al, 2005, Nguyen et al, 2006, Ben Sassi et al, 2008, Zając, et al, 2012).

Our work mainly aimed at:

1) Evaluation of phenolic content and flavonoid content of Hexane, CHCl3, EtOAc and n-BuOH extracts of *C. segetum* L. flowers;
2) Evaluation of the scavenging activity of these extracts against DPPH radical;
3) Estimation of the antimicrobial activity of the extracts against five bacterial strains and one fungal strain at different concentrations.

2. MATERIALS AND METHODS

2.1. Plant material

Chrysanthemum segetum L plants were collected in Beni belaid not far from Jijel and was identified by Prof Zellagui, Larbi ben M'hidi University, Oum Elbouaghi. A voucher specimen was deposited at the biology Department, University of Oum Elbouaghi under the code number ZA 118.

1300 g of *Chrysanthemum segetum* flowers was finely grounded, soaked in in a mixture of methanol and water (70: 30) and left for 24 hours. The solution was then filtered to obtain the hydro-alcoholic extract. The extraction was repeated 3 times by replenishing the solvent each time. The evaporation was done under decreased pressure and the concentrates were put in 1500 ml of boiled distilled water and left overnight in a cool place and then filtered to get rid of dust and impurities. The residue (102 g) was extracted in a

separating funnel, using organic solvents namely hexane, chloroform, ethyl acetate and n-butanol respectively (Markham, 1982).

2.2. Total phenolic content (TPC)

The polyphenols content of the methanolic extract was measured by the Folin-Ciocalteu method **(**Slinkard and Singleton, 1977**).** 300 µL of the samples were added to 1500 µl of Folin-Ciocalteu and 1200 µl aqueous sodium carbonate Na_2CO_3 (7.5%). The mixture was incubated for 1 hour and the absorbance of total phenols was determined at 760 nm by a spectrophotometer (SHIMADZU UV1650PC). The standard curve was prepared using 0.02, 0.05, 0.1, 015, 0.5mg/mL of gallic acid solutions in methanol. TPC was measured from standard gallic acid curve, displayed as milligrams of Gallic acid equivalents per g of dry weight plant material (GAE/ g DW).

2.3. Total flavonoids (TFC)

The total flavonoids content was calculated based on the aluminum chloride colorimetric method according to Chang **(**Chang et al, 2002**)** with few modifications. The results are displayed as mg of quercetin equivalents per 100 g of dry weight (QE/100 g DW). This method is according to the quantification of yellow color produced by the interaction of flavonoids with $AlCl_3$ reagent. 1500 µL of $AlCl_3$ (2% w/v) was added to 1500 µL of each sample in methanol solution and incubated for 10 min in the shade at the room temperature. The absorbance was determined at 430 nm by SHIMADZU UV1650PC-UV-visible spectrophotometer. TFC was calculated from standard quercetin curve and displayed as mg of quercetin equivalents per g of dry weight of plant material (QE/g DW).

2.4. Antioxidant Activity

Evaluation of the free radical scavenging activity of the various extracts was performed by a modified quantitative DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. Different concentrations of extracts in methanol were provided (50, 100, 150, and 200 $\mu g \cdot ml^{-1}$). Blank samples were run by 1 mL methanol instead of the sample. 1 mL of 0.004% DPPH in methanol was mixed with 1 mL of the test solution, or standard, and diluted with 1 ml of methanol. The mixture was allowed to stand at ambient temperature in dark for 30 min. The change in color from deep violet to light yellow was then evaluated at 517 nm. Inhibition of free radicals in percentage (I %) was determined based on the following equation: $I\%=((A_0-A_1)/A_0)$ x100, with A_0 being the absorbance of the control reaction (comprising all reagents except the sample) and A_1 the absorbance of the extract. Extract concentration providing 50% inhibition (IC_{50}) was measured from the graph plotted of inhibition percentage against extract concentration. Measurements were done in triplicates.

2.5. Antimicrobial activity assay

a. Microorganisms Incubation conditions

In total four bacterial cells were used in this study which was composed of one gram-positive bacterium namely *Staphylococcus* sp., two gram-positive bacteria, *Klebsiella pneumonia* and *Escherichia coli*, as well as a fungus, *Candida albicans*. Bacterial strains were grown and maintained on Nutrient Agar slants and on Sabouraud Glucose Agar slants, and then stored at 4°C. All tested strains were prepared and tested against the extracts for estimating the minimum inhibitory concentration (MIC). Each isolate was tested three times to calculate the mean reading.

b. Disc diffusion assay

The plant extracts were dissolved in DMSO for final concentrations (0.25, 0.5, 1, 2, 3 mg/mL) and filter-sterilized through a 0.45 membrane filter. The antimicrobial activity was estimated by the method of disc diffusion. Sterilized filter paper discs (7 mm in diameter) were saturated with different concentrations of butanol extracts (with control disk saturated with methanol 50%) and placed on petri plates containing 20 ml of nutrient agar (for bacteria). These plates were inoculated with 100 µl of suspension with a concentration of 10^8 colony-forming units (CFU)/mL for each microorganism.

Then, the plates were let in an inverted position in the incubator under the temperature of 37 °C for 24 hours. This operation was replicated with an industrial antibiotic. The diameter of the inhibition zone around each disc was determined for three replicates.

c. Determination of the minimum inhibitory concentration (MIC)

The broth micro-dilution method was utilized to calculate the MIC based on Carbonnelle et al. (1987). Serial dilutions (0.25, 0.5, 1, 2, 3 mg/mL) of extracts were prepared directly in a microliter plate containing Mueller Hinton broth. The bacterial inoculum was added to get a final concentration of 5×10^5 CFU/mL in each well. The positive control was utilized with Gentamicin as standard drug at final concentrations of 0.125-128 mg/mL. The petri dish was covered with a sterile sealer and incubated for 24 h at a temperature of 37°C. The MIC was shown as the minimum concentration of the extract which completely inhibits the bacterial growth. The lower is the MIC, the higher is the activity of the extract.

3. RESULTS AND DISCUSSION

3.1. Polyphenols content:

Polyphenols are important plant secondary metabolites with antioxidant activity in chelating redox active metal ions, inactivating lipid free radical chains and preventing hydro-peroxide conversions into reactive oxy-radicals.

The total phenol content (TPC) and flavonoids content (TF) in plant extracts were assessed using the techniques mentioned previously. TPC and TF were respectively expressed as mg of Gallic acid equivalent (GAE) and as g of quercetin equivalent per g of dry matter (DM) (Table 1).

Table 1. Total phenolics and flavonoids of the four extracts.

extract	TPC in µg AGE/ mg extract	TF in µg QE/ mg extract
Hexane	57.30 ± 0.12	-
$CHCl_3$	62.20 ± 0.15	0.90 ± 0.08
EtOAc	140.25 ± 0.06	40.30 ± 0.90
BuOH	185.50 ± 0.09	27.00 ± 0.30

The TPC and TF yield of the extracts (Hexane, $CHCl_3$, EtOAc and n-BuOH) vary considerably according to the polarity of the solvent used. For TPC, the n-BuOH extract gave a greater yield than all extracts followed by EtOAc, whereas the $CHCl_3$ extract contained the least found amount. For TF, the results show the maximum content in EtOAc extract. Hence, the results showed that the extracts of EtOAc and BuOH contained significant amounts of total phenols and total flavonoids due to their high polarity compared with Hexane and $CHCl_3$. This is in concordance with what was reported in other plant species such as *S. pinata* (Flamini et al. 1994) and *S. ligustica* (Flamini et al., 1999), as well as *Chrysanthemum* species, including *C. coronarium* L. (Yuan Yuan et al., 2009; Ibrahim et al., 2007), *C. segetum* L., and *C. morifolium* Ramat (Hu et al., 1994).

3.2. DPPH scavenging effect:

Table 2: inhibition percentage at different times and concentrations for each solvent extract of *C. segetum* flowers

Temps (s)	Solvent	Crude extract	1/10	$1/10^2$	$1/10^3$	$1/10^4$
30″	Hexane	13	11	10	8	7.20
	$CHCl_3$	27.25	22	9	7	6
	EtOAc	29.50	28.50	21.50	15.50	14.50
	BuOH	24.33	20.50	18.50	13.50	10.25
60″	Hexane	15.20	12.70	12	9.30	7.90
	$CHCl_3$	31	24.35	10.65	8	7.15
	EtOAc	33	30	23.25	18.50	16.70
	BuOH	31.83	27.50	22.10	17.90	13.50
90″	Hexane	17	14.15	14	10	8.40
	$CHCl_3$	35	28	11.70	8.70	7.70
	EtOAc	34.40	31.80	25	20	18.20
	BuOH	39.50	34.50	28.75	23.50	21.50
120″	Hexane	19	15	13	11	9.20
	$CHCl_3$	38	31	12	10	8.15
	EtOAc	41.50	35.50	29.50	21.50	19.65
	BuOH	47.50	39.50	35.90	30.50	27.15
180″	Hexane	23	16.45	14	11.90	9.50
	$CHCl_3$	47	38.65	2.70	10.50	9
	EtOAc	52.50	40.50	35.50	24.50	21.50
	BuOH	51.50	45.50	40.50	34.50	31.50
240″	Hexane	28	18	14.40	13	10
	$CHCl_3$	55.30	40	14.20	12	9.50
	EtOAc	57.50	50.50	38.50	26.50	22.50
	BuOH	58.50	51.50	46.50	38.50	33.50
300″	Hexane	36.50	23	19	14	11
	$CHCl_3$	73.65	47	18	13.25	9.50
	EtOAc	79.50	54.50	44.50	31.50	24.50
	BuOH	76.70	62	49.50	43	36.37

The results of the table 2 show that the inhibition percentages I (%) increased by increasing the time and/or concentrations up to maximum after 5 min for all samples. The percentage of crude EtOAc extract was 29.50% at 30 seconds and increased until 79.50% at 5 min followed by n-BuOH with a maximum value of 76.70% and $CHCl_3$ with 73.65 and Hexane with 36.50%. The dilutions are somewhat acceptable for EtOAc and n-BuOH extracts increasing from 24.50 to 54.50% and 36.37 to 62.00% respectively, while Hexane and CHCl3 showed weak effects.

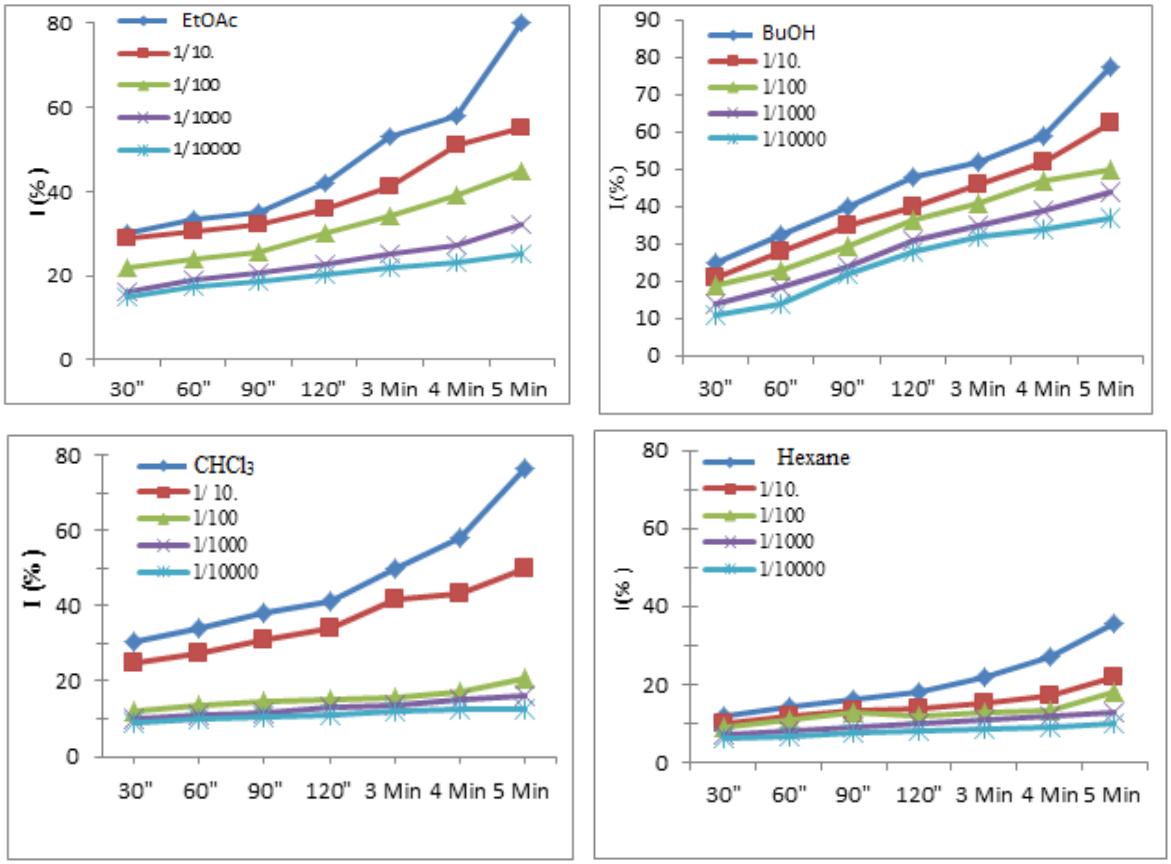

Figure 1: DPPH Inhibition effect of different extracts and dilutions of *C. segetum* L.

3.3. Antibacterial Activity

The results of Antimicrobial activity are shown in Table 3. According to the results, *C. segetum* was effective against some of the common bacteria (*S. aureus* and *C. albicans*).

Table 3: Antimicrobial activity of different extracts of *C. segetum*

microorganism		Concentrations (µg/mL)					
		0.25	**0.5**	**1**	**2**	**4**	**8**
Gram (+)							
Staphylococcus sp.	***Hex.***	-	-	-	-	-	-
	Chl.	-	-	-	7.00±0.35	9.20±0.60	11.50±0.90
	Acet.	-	-	-	7.00±0.35	11.20±0.60	**13.50±0.90**
	But.	-	-	-	6.00±0.30	8.00±0.30	11.70±0.40
Staphylococcus aureus ATCC 25923	***Hex.***				5.50±0.35	8.20±0.60	10.50±0.90
	Chl	-	-	6.00±0.90	7.50±0.35	10.20±0.60	13.50±0.90
	Acet.			**7.50±0.90**	**9.00±0.35**	**12.20±0.60**	**20.50±0.90**
	But.			6.50±0.90	7.50±0.35	10.20±0.60	15.50±0.90
Gram (-)							
Escherichia coli	***Hex.***	-	-	-	-	-	-
	Chl.	-	-	-	-	-	6.00±0.20
	Acet.	-	-	-	-		7.00±0.50
	But.	-	-	-	-		7.00±0.10
Klebsiella pneumoniae	***Hex.***	-	-	-	-	6.00±0.33	**8.50±0.50**
	Chl	-	-	-	-	-	08.20±0.50
	Acet.	-	-	-	-	-	8.00±0.30
	But.	-	-	-	-	5.50±0.20	**9.20±0.30**
Pseudomonas aeruginosa ATCC27853	***Hex.***	-	-	-	-	-	-
	Chl	-	-	-	-	-	-
	Acet.	-	-	-	-	-	-

	But.	-	-	-	-	-	-
Fungi							
Candida albicans	***Hex.***	-	-	-	-	-	-
	Chl.	-	-	-	-	6.20±0.70	10.30±0.50
	Acet.	-	-	-	**9.70±0.70**	**12.20±0.70**	**17.30±0.50**
	But.	-	-	-	6.70±0.70	10.20±0.70	13.80±0.50

The Gram-positive *Staphylococcus sp.* and the two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa ATCC27853* and the fungus *Candida albicans* were resistant to all Hexane concentrations of the C. *segetum* as shown in Table 3. The Gram-negative *Klebsiella pneumoniae* strain showed a cell growth at concentrations 0.25, 0.5, 1, and 2 µg/mL and low inhibition at concentrations of 4 and 8 µg/mL of hexane extract with a diameter of 6 and 8.50 mm, respectively.

The EtOAc extract gave the best results against *Staphylococcus aureus* and *Candida albicans* at 8 µg/mL, with the inhibition zone diameters of 20.50 and 17.30 mm, respectively. The BuOH extract behaved moderately with a maximum inhibition diameter of 15.50 mm at 8 µg/mL against *Staphylococcus aureus.*

3.4. Minimum inhibiting and Minimum bactericide Concentrations (MIC and MBC)

The results of minimum inhibiting and Minimum Bactericide Concentrations (MIC and CMB) for the Hexane, $CHCl_3$, EtOAc and BuOH extracts of *C. segetum* flowers on Gram-positive and Gram-negative strains are recorded in the table 4:

Table 4: MIC, MBC and MBC/ CMI for the four extracts.

microorganism		MIC				MBC				MBC/ CMI			
		Hexane	***CHCl3***	***EtOAc***	***n-Butanol***	***Hexane***	***CHCl3***	***EtOAc***	***n-Butanol***	***Hexane***	***CHCl3***	***EtOAc***	***n-Butanol***
*Staphylococcus sp.**	**Gram+**	+	1	1	1	8	8	4	8	-	8	4	8
Staphylococcus aureus ATCC 25923	**Gram+**	1	1	1	2	8	8	2	8	8	8	2	4
*Escherichia coli**	**Gram-**	+	ND	ND	ND	-	ND	ND	ND	-	-	-	-
*Klebsiella pneumoniae**	**Gram-**	2	ND	ND	ND	8	ND	ND	ND	4	-	-	-
Pseudomonas aeruginosa ATCC27853	**Gram-**	+	+	+	+	+	+	+	+	+	+	+	+
Candida albicans	**Fungi**	+	2	4	4	8	8	8	8	-	4	2	4

ND: Non defined , ATCC: American type culture collection

The value of **MBC/MIC** of the different extracts show that there is a significant bactericide effect on *Staphylococcus aureus* and *Staphylococcus sp.* and a fungicide effect on *Candida albicans* for most extracts.

4. CONCLUSION

Antioxidant and antimicrobial capacity of *Chrysanthemum segetum* were evaluated using appropriate methods. The findings seem to be interesting particularly when dealing with the ethyl acetate and butanol extracts. The results showed a resilient correlation between TPC and antioxidant activity with a good promise with literature reports. More studies about other biological activities and toxicity could be of a great importance so as to valorize and validate the traditional use of the species.

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