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ISOLATION OF PHENOLICS COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *CITRULLUS COLOCYNTHIS* EXTRACTS FROM THE ALGERIAN FLORA

MeriemBireche[1], Boulanouar Bakchiche[1*], AbdelazizGherib[1], Ahmet Çakir[2], NazimŞekeroğlu3, Muhittin Kulak[2]

[1]Laboratory of Process Engineering, Amar Telidji University, 03000, Laghouat, Algeria.
[2]Deparment of Chemistry Faculty of Science and Literature, Kilis 7 Aralık University, KİLİS, Turkey.
[3] Plant and Animal Production Department, Kilis 7 Aralık University, KİLİS, Turkey.

*Corresponding author E-mail: **b.bakchiche@lagh-univ.dz**

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ABSTRACT

Citrullus Colocynthis is a spontaneous plant from *Cucurbitaceae* family, Very encountered in the Algerian deserts, It is used in traditional medicine against cancer and diabetes and other diseases. In the present work we quantified the phenolic compounds extracted from the fruits of *Citrullus Colocynthis* by two solvents, butanol and ethyl acetate. The polyphenol dosage shows that the ethyl acetate extract has the best values (23.06 mg / g) as well as the value of the flavonoids (9.19 mg / g).The percentage of inhibition of the free radical DPPH • for the ethyl acetate extract has a very high value (91.85%). This value has an IC_{50} (0.39 mg / ml).The 1H and 13C NMR identification of the isolated extracts of *CitrullusColocynthis* fruits shows that the plant studied contains two flavonoids, the first is "2-(4-hydroxyphenyl)-7-(3-methylbut-2-en-1-yl)-5-(((*2R,3S,4R,5R,6S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-*2H*-pyran-2-yl)oxy)chroman-4-one " And the second is "5,7-dihydroxy-2-(4-hydroxyphenyl)-6- ((*2S,3R,5S,6R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-*2H*-pyran-2-yl)-2H-4λ^5-chromen-4-one ".

INTRODUCTION

The Sahara, the largest and warmest desert in the world, possesses in its northern part, the northern Sahara,diffuse and sparse vegetation. The state of the spontaneous flora in this zone as well as the relations between man and the plant species, deserve special attention.Some species possess pharmacological properties which give them a medicinal interest ***(Ould El Hadj M. Didi et al ; 2003).***The present study is interested in to one of the spontaneous species,*Citrullus Colocynthis*, is a perennial herb of the family *Cucurbitaceae****(A .Chehma ; 2006).***

The *Citrullus Colocynthis*, originating from arid soils, is very common in humid or moderately dry tropics,***(Bruneton, J. 1996)***it occupies a very large region that stretches from The North African, **f**rom the Sahara, Egypt, Saudi Arabia to India,as well as the Mediterranean region ***(Batanouny, K.H, 1999).*** According to the bibliography,***(A.I. Hussain et al., 2013, Delazar et al., 2006*)**,** the *Citrullus Colocynthis* fruits are rich in flavonoids and polyphenols, this richness gives it a very important antioxidant power. Flavonoids and other phenolic compounds have been suggestedto play preventive role against the incidence of some common diseases like cancer, cardiovascular and neurodegenerative disorders ***(Hussain et al., 2008).***Some phenolic compounds including flavonols and hydroxycinnamic acids have been widely distributed in plants and among them flavonols are of particular importance in the human diet

as antioxidants. Due to large number of phenolic compounds in plant materials, quantitative determination of individual flavonoids and phenolic acids is a tedious job. Therefore, these compounds are normally hydrolyzed and the resulting aglycones are identified and quantified. Different methods for extraction, hydrolysis and analysis of phenolics have been published ***(Obmann et al., 2012; Wei et al., 2012).***Present study was carried out to investigate phenolics in the fruit extracts of *Citrullus Colocynthis.*

II.Material and Methods

I.1Plant material

The plant used in this study was collected in February 2013 in area "Al Mansura" in the Wilaya of Ghardaia, which is located in Algerian Sahara. The study station "Al Mansura", is located 70 km south of Ghardaia**(*Harrat .Z et al, 2009; Garni .R et al , 2014)*** The identification of this plant was confirmed with the contribution of the members of the laboratory of Process Engineering, University of Laghouat.

I.2. Dosage of polyphenols and flavonoids

I.2.1. Preparation of samples

We used the same extraction and purification methodology written by ***(M.G.L. Hertog;1992).***The solvents used for the extraction are butanol and ethyl acetate.

I.2.2. Determination of total phenols

The total phenols determination was carried out by an adapted method ***(Slinkard and Singleton; 1977)***with the Folin-Ciocalteu reagent. 250 µl of each extract was added to 1 ml of Ciocateufolatereagent10 times diluted in bi-distilled water.The solutions were mixed and incubated for 5 minutes. After incubation 1 ml of the sodium carbonate solution Na_2CO_3 (20%) was added. The mixture is incubated for 30 minutes in the dark at room temperature. The absorbance of all the extracts was measured by the spectrophotometer (UV-Vis) at 760 nm. The concentration of the total polyphenols is calculated from the regression equation of the calibration range, established with the Gallic acid standard (0.01-0.1mg / ml) and expressed in milligrams of gallic acid equivalents per milligram of extract (mgGAE / mg).A calibration curve is then obtained.

I.2.3.Determination of flavonoids

The aluminum trichloride ($AlCl_3$) method is used to quantify the flavonoids in our extracts, using rutin as the standard ***(Boulanouaret al., 2013).*** 2 ml of each extract and standard (dissolved in methanol) with the appropriate dilutions was added to an equal volume of a solution of AlCl3 (2% in methanol).The mixture was vigorously stirred and the absorbance at 430 nm was measured after 15 minutes of incubation. The quantification of the flavonoids was performed as a function of a linear calibration curve,Which realized by rutin standard at different concentrations (0.01-0.1 mg / ml) under the same conditions as the sample.The results are expressed in milligrams of rutin equivalent per gram of extract (mg RE/ g).

I.3.Free radical scavenging activity (DPPH)

A methanolic stock solution (50 µL) of each sample at different concentrations was placed in a cuvette, and 2 mL of 60 µM methanolic solution of DPPH• (2,2-diphenyl-1-picrylhydrazyl) wasadded **(*Brand-Williams et al., 1995).***Absorbance measurements were made at 517 nm using a Shimadzu 160-UV spectrophotometer (Tokyo, Japan) after 60 min of reaction at room temperature. Butylated hydroxy toluene (BHT) and butylated hydroxyl anisole (BHA) were used as positive control for comparison and 90 µM DPPH• solution was taken as blank. The percent scavenging was calculated by following formula:

$$\text{Scavenging } (\%) = 100 \times \frac{\text{A blank} - \text{A sample}}{\text{A blank}}$$

Where Ablank is the absorbance of the DPPH solution and Asample is the absorbance of the extract solution. Extract concentration providing 50% scavenging (IC_{50}) was calculated from the graph-plotted inhibition percentage against extract concentration **(Molyneux Pet al ; 2005).**

II.5. Isolation of phenolic compounds

II.5.1. Hot Extraction

In a flask a mass (200 g) of well-ground fruit, add the methanol and carry out the assembly of soxlet. After 6 cycles, evaporate the solution obtained using a rotavapor.

II.5.2. Open column chromatography and flash (CC)

Column chromatography was carried out with several types of stationary phases in glass columns. The size and the diameter of the column is chosen according to the quantity of

sample to be purified and the desired resolution. The stationary phases used were silica gel 60 Å 40-63 µm (Merck) and 60 Å RP-18 40-60 µm silica gel (Merck).The amount of silica used is generally 30 to 40 times greater than the amount of sample deposited. The samples to be fractionated were deposited in solid form mixed with silica or in liquid form.The exclusion chromatographies are carried out on Sephadex® LH20 gel (Sigma Aldrich).

II.5.3. Thin layer chromatography (TLC)

The thin film analyzes are carried out on aluminum plates with two different supports: in normal phase, were used silica gel-coated plates60 F254 (Merck) and reverse phase, the plates were coated with silica gel 60 RP-18 F254S (Merck). The development of the plates was carried out in glass tanks saturated with the appropriate eluant.The observation of the CCM is carried out in visible light and under UV (254 and 365 nm).The reagent used for the disclosure of the plates was sulfuric vanillin which was prepared as described below.

II.6. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectroscopy is always the best method for determining the structure. The interpretation of the 1H, 13C, DEPT (90-135), COSY, HMQC, HSQC, HMBC and NOESY spectra generally leads not only to the determination of the structure of the molecule but also to the determination of its relative configuration. NMR spectra of the ligands and complexes were recorded at 25 ° C in DMSO-d6 using a Bruker apparatus400 MHz. The mass spectra of the ligands were recorded on a Zivak Tandem Gold LC-MSMS (ESI) spectrometer. The positive and negative ion modes were used simultaneously in the MS analyzes ***(Aydin et al., 2014).***

III.Result and discussion

III.1. Extract yield

The yields of components extracted from C. colocynthis fruit was 4.66 g / 100 g for the ethyl acetate extract and 3.57 g / 100 g for the butanol extract, the total percentage of extraction was 8,41%.According to the findings, ethyl acetate is the most favored solvent for extracting phenolic compounds from our studied plant.`

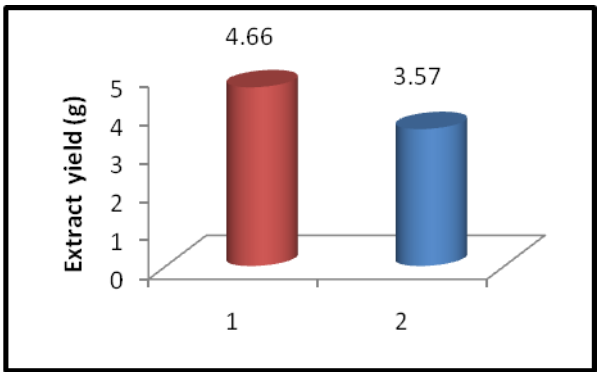

Figure 1 shows the yield of the two extracts.

2. Total phenolic, total flavonoids and antioxidant activity

The results of the polyphenol, flavonoid, percentage free radical DPPH inhibition and EC_{50} values are summarized in Table 1.

The results of total phenols assays as well as flavonoids from the calibration curves found show that the extract of ethyl acetate has better polyphenol contents (23.06 mg / g)compared to that of the butanol extract (11.86 mg / g), the same remark for flavonoid contents, where the butanol extract has a value of (4.51 mg / g) while the ethyl acetate extract has a value of (9.19 mg / g). The Ciocateu folate reagent is extremely sensitive to the reduction of all hydroxyl groups, not only those of the phenolic compounds, but also certain sugars and proteins etc ... [62].Dosage by this reagent therefore gives a raw evaluation of all the phenolic compounds of an extract. It is not specific to polyphenols, but many compounds can react with the reagent, giving a high apparent phenolic ratio [63]. Polyphenols are a class of natural compounds that exhibited antioxidants activity and act as freeradical terminators ***(Huang et al., 2005).***Many studies confirmed that C. colocynthis extracts are rich source of phenolic antioxidants and exhibited good antioxidant activity ***(Dallak, 2011; Gill et al., 2011; Kumar et al., 2008; Sebbagh et al., 2009).*** For the evaluation of the antioxidant activity, two approaches are applied: on the one hand, the determination of the relative reduction of the DPPH radical at a reference time, and determining the amount of antioxidant needed to reduce 50% DPPH •***(Sanchez-Moreno et al., 1998; Scherer, R., et al., 2009).***The inhibition percentage of the free radical DPPH • for the ethyl acetate extract has

a very high value (91.85%) followed by the value of the butanol extract which also present significant inhibition (85.60%) comparing by the value of the standard, ascorbic acid, which inhibits (96.38%) of free radical DPPH ˙.

To overcome the influence of concentration, the reactivity is estimated by the effective concentration IC_{50} (or the reverse 1 / IC_{50}) of the antioxidant, which corresponds to a 50% reduction in the activity (of the absorbance) of the DPPH in the reaction medium.The antioxidant capacity of a compound is even higher than its IC_{50} is small. The IC_{50} index shows the concentrations of the antioxidant which are necessary to decrease the initial concentration of DPPH with 50% ***(Popovici et al., 2009).***For our studied extracts, the IC_{50} value of the ethyl acetate extract has the lowest value (0.39 mg / ml)compared to the butanol extract valuewhich has an IC_{50} (0.62 mg / ml). This confirms that the first extract (ethyl acetate) has the power to decrease the initial concentration of the free radical DPPH˙.

2. Isolation of phenolic compounds

After evaporation of the isolated and recovered fractions by open column chromatography we have obtain two major compounds called CC1 (5,218g) and CC2 (2,071g).To determine the chemical composition of total phenols of these two raw productsNMR analyzes were carried out. Tables 2 and 3 summarize the chemical shifts of 1H, 13C, DEPT (90-135) NMR and the proposed assignment of each peak of the isolated compounds CC1 and CC2.

Using the 1 H and 13 C NMR spectra with the interpretations tables, it was possible to determine the supposed structure of our molecule studied CC1 and its chemical shifts, the DEPT spectra at 13C NMR make it possible to differentiate C, CH, CH_2 and CH_3 groups the DEPT 90 shows only the CH groups, the DEPT 135 shows the CH, CH_2 and CH_3 groups. This latter exists only at the chemical shift between (160-100 ppm).

The CH and CH_3 groups show positive peaks, whereas the CH_2 groups show negative peaks.

From Table 2, there is a quadrupled peak at δ (7.01-6.84 ppm) for 1H and at δ (122.14 - 99.75ppm) for 13C which corresponds to four parallel molecules H of benzene.

The peaks at 1H chemical shift (4.67 - 4.04 ppm) and between δ (26.91 - 23.28 ppm) at 13C represent a doubled and tripled multiplicity which corresponds to a phenol.
The peaks of 1H δ (3.65 - 3.33 ppm) belong to protons of glucose (sugar). These chemical shifts are associated with the others between (76.71 - 70.46 ppm) for 13C.Similarly,using 1 H and 13 C NMR spectra with the interpretations tables, it was possible to determine the supposed structure of our molecule CC 2 and its chemical shifts.

According to Table 3, the presence of phenol at δ (122.27 - 99.71 ppm) for 13C and δ (4.69 - 4.07 ppm) for 1H, the presence of two molecules of benzene, one at δ (28.14 - 19.50 ppm) for 13C and at (6.98 - 6.83 ppm) for 1H and the other at a chemical shift δ (203.58 - 198.35 ppm) for 13 C and a chemical shift between (3.90 -3, 86 ppm) for 1 H.

The results confirm the presence of a sugar molecule at δ (76.68 - 69.49 ppm) for 13C and between (3.57 - 2.58 ppm) for 1H.The table also presents several double bonds, CH, CH 2, CH 3 bonds and OH bonds which belong to alcohols or phenols. After the interpretation of the 1 H and 13 C NMR spectrum , it is confirmed that the two molecules studied are composed of several protons which belong to aromatic cycles, sugars, alcohols or phenols, methyl, methylene and methynes, and using these results the proposed structures of CC1 and CC2 can be determined.

The NMR spectroscopic analyses of the fraction extract from *C. Colocynthis* fruits afforded tow flavonoid glycosides, 2-(4-hydroxyphenyl)-7-(3-methylbut-2-en-1-yl)-5-(((*2R,3S,4R,5R,6S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-*2H*-pyran-2-yl)oxy)chroman-4-one[1] and 5,7-dihydroxy-2-(4-hydroxyphenyl)-6- ((*2S,3R,5S, 6R*)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl)-*2H*-pyran-2-yl)-2H-4λ^5-chromen-4-one [2] (Figure 2) ,and also by comparing experimental data with respective literature data (4, 18-25).

Table1 :Total phenolic contents, total flavonoid contents, DPPH free radical scavenging capacity and antioxidant activity of *C. Colocynthis* fruits.

C.ColocynthisEctract	Antioxidanteessay			
	Total phenolic contents (mg/g)	Total flavonoid contents (mg/g)	DPPH radical scavenging (%)	DPPH, IC_{50}(mg/ml)
Ethylacetateextract	23.06 ± 0.101	9.192 ± 0.348	91,851± 0,421	0,392±0,771
Butanol extract	11,86±0, 367	4,517±0,683	85,603±0,221	0,624±0,403
Ascorbic acid	-	-	96,385±0,615	0,00687±0,365

Table 2. 1H and 13C NMR spectra interpretation of compound CC1

N° pic	δ13C(ppm)	DEPT	δ1H(ppm)	multiplicity	attribution
1	215,16	C	-	-	-
2	198,35	C	-	-	-
3	150,16	CH	3,70	s	$OHCH=CH_2$
4	145,80	C	-	-	-
5	122,14	CH	7,01	q	Cycle benzène
6	121,28	CH	6 ,97	q	
7	120,94	CH	6,88	q	
8	99,75	CH	6,84	q	
9	79,67	C	2,08	d	$CH_2=C=CH_2$
10	76,71	CH	3,65	q	Glucose
11	76,17	CH	3,51	q	
12	72,91	CH	3,47	t	
13	70,46	CH	3,33	m	
14	60,54	CH_2	1,94	s	CH_2-CO-R
15	45,30	CH_2	4,61	d	Me_2CHOH
16	41,75	CH	4,07	t	
17	26,91	CH_3	4,67	d	phénol
18	25,48	CH_3	4,65	d	
19	24,04	CH_3	4,07	t	
20	23,28	CH_3	4,04	t	
21	19,41	CH_3	1,29	s	CH_3-C
22	19,21	CH_3	1,24	s	R-CH_3
23	17,33	CH_3	0,84	s	R-CH_3

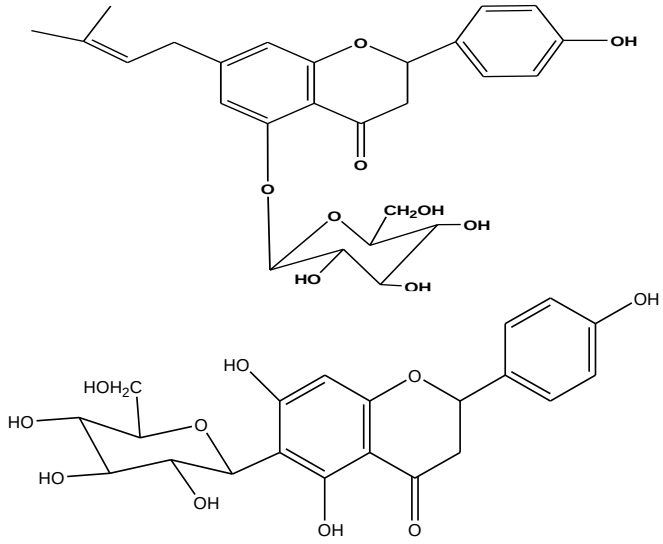

Figure 2.Structure of compounds [1-2] isolated from *C. Colocynthis*

Table 3. 1H and 13C NMR spectra interpretation of Compound CC2

N° pic	δ13C(ppm)	DEPT	δ1H(ppm)	multiplicity	attribution
1	215,83	C	1,318	m	CH_3-CH=O
2	203,58	C	3,90	q	CH-O- Ar
3	198,38	C	3,86	q	
4	145,76	C	-	-	-
5	122,27	CH	4,69	d	phénol
6	120,96	CH	4 ,54	m	
7	119 ,86	CH	4,45	m	
8	99,71	CH	4,07	s	
9	78,53	C	2,02	d	CH_2-C-O-R
10	76,68	CH	3,57	m	Glucose
11	76,15	CH	3,32	m	
12	72,91	CH	3,15	m	
13	70,28	CH	2,93	m	
14	70,17	CH	2,70	m	
15	69,49	CH	2,58	m	
16	69,26	C	3,70	s	CH_3-O-COR
17	48,14	CH_2	3,62	s	-CH_2-OH
18	45,44	CH_2	2,08	d	-CH_2-C=C
19	36,75	CH	7,02	d	
20	28,14	CH_3	6,98	q	Cycle benzène
21	27,94	CH_3	6,95	q	
22	24,13	CH_3	6,92	q	
23	23,30	CH_3	6,88	q	
24	19,50	CH_3	6,83	q	
25	19,30	CH	1,21	d	R-CH_3
26	17,42	CH	1,20	d	

CONCLUSION

In conclusion, this study first time reports the composition of phenolic acids and flavonoids in different parts of *C.Colocynthis* along with total phenolic, total flavonoid contents and in vitro antioxidant activity. The antioxidant activity results of the *C.Colocynthis* plant can improve the traditional use of this spontaneous plant and make it more usable.The prospects of our work in the future is isolated all the majority and minority molecules existing in this plant and perform tests of several activities such as antibacterial, anti-cancer and other activities.

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