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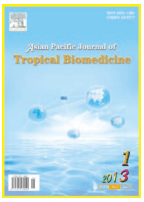

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Phytochemical screening and free radical scavenging activity of *Citrullus colocynthis* seeds extracts

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PEER REVIEW

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Comments

The work and results obtained are interesting. Its will certainly enrich research into valorisation of medicinal plants as antioxydants source.
(Details on Page 39)

ABSTRACT

Objective: To study the phytochemical screening of different extracts from *Citrullus colocynthis* (*C. colocynthis*) seeds extracts and to assess their antioxidant activity on the DPPH free radical scavenging. **Methods:** Phytochemical screening, total content of polyphenols and flavonoids of *C. colocynthis* seeds extracts, including a crude aqueous extract (E1), a defatted aqueous extract (E2), a hydromethanolic extract (HM), an ethyl acetate extract (EA) and a *n*-butanol extract (*n*-B) was carried out according to the standard methods and to assess their corresponding effect on the antioxidant activity of this plant. **Results:** None of these extracts contained detectable amount of alkaloid, quinone, antraquinone, or reducing sugar. Catechic tannins and flavonoids were abundant in E1, HM and EA, whilst terpenoids were abundantly present in E1 and *n*-B but only weekly in HM. Coumarins were found in E2, EA and *n*-B. Polyphenols, expressed as gallic acid equivalent, amounted, per 100 g plant matter, to 329, 1002 and 150 mg in EA, HM an E1 respectively. Flavonoids, expressed as catechin equivalent, amounted, per 100 g plant matter to 620, 241 and 94 mg in EA, HM and E1 respectively. Comparable values were found in *n*-B and E1, with lower values in E2. Quercetin, myricetin and gallic acid were found in the EA and HM extracts by thin layer chromatography, The antioxidative effect of these extracts yielded, when tested at a concentration of 2000 μg/mL in a 1,1-diphenyl-2-picrylhydrazyl assay, a reducing percentage of 88.8% with EA, 74.5% with HM and 66.2% with E1, and corresponding IC_{50} of 350, 580 and 500 μg/mL as compared to 1.1 μg/mL for ascorbic acid. **Conclusions:** These qualitative and quantitative analytical data document the presence in *C. colocynthis* extracts of such chemical compounds as flavonoids responsible for the antioxidant activity, as well as other biological activities of this plant.

KEYWORDS

Citrullus colocynthis, Polyphenols, Flavonoids, Free radical scavenging

1. Introduction

Medicinal plants contain several active principles with specific therapeutic effects. They represent a source of chemical compounds such as tannins, flavonoids, saponins, resins and alkaloids with curative properties, often not provided by synthetic chemical compounds[1]. In Africa, 80% of the population use medicinal plants for primary health care. In traditional medicine, several medicinal plants are used for the treatment of diabetes mellitus[2]. Ethnobotanic inquiries have recorded the plants used in such a perspective, among which *Citrullus colocynthis* (*C. colocynthis*) represent one of the most commonly used species[3,4].

Infusion prepared from the fruit and seeds of this plant from the cucurbitaceae family are indeed recommended to diabetic patients[5]. The chemical compounds identified in antidiabetic plants are often alkaloids and polyphenols, but also polysaccharides, gums and glycans[2].

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The major aim of the present work, since various extract of *C. colocynthis* affected insulin release in isolated rat pancreatic islets (data not shown), consists in a phytochemical screening of different extracts from *C. colocynthis* seeds in order to scrutinize the presence or absence of chemical compounds such as tannins, flavonoids, alkaloids and terpenoids, to determine the total content of polyphenols and flavonoids in such extracts, and to assess their antioxidant activity on the DPPH free radical scavenging.

2. Material and methods

2.1. Plant material

The fruits of *C. colocynthis* were collected at maturity between September and November in a west Algeria area, which stretches from Mechria to Aïn sefra. In the laboratory, seeds were salvaged, dried, sheltered from whilst light, and eventually stored up to the time of further use at ambient temperature.

2.2. Defatting

Certain extracts required a defatting of the vegetal material, in order to remove appreciable amount of lipids. For such a purpose, the dried seeds (100 g) were finely grounded and defatted in hexane (150 mL) over 3 h. Defatted sample then dried over 24 h and stored at ambient temperature whilst sheltered from light, and eventually stored up to the time of use.

2.3. Preparation of extract

2.3.1. Non-defatted crude aqueous extract (E1)

The non-defatted crude aqueous extract was prepared as follows. Non-grounded seeds were placed in hot water (225 mL) for one hour at 50 °C. Warm extraction under reflux was then conducted over 15 min, the cooled solution being eventually filtered and centrifuged and the supernatant recovered and evaporated to dryness.

2.3.2. Defatted crude aqueous extract (E2)

The seeds used during the preparation of the first aqueous extract (E1) were recovered, dried, and then used to prepare another aqueous extract, *i.e.* the defatted crude aqueous (E2). After extraction, the recovered seeds were dried over 24 h at 37 °C, then finely grounded and defatted with hexane (150 mL) over 3 h. This procedure allowed to remove fats and lipophilic matters. The defatted seeds were then placed in hot water for 1 hour at 50 °C, the further handling of the extract being the same as that for the preparation of extract E1.

2.3.3. Hydro-methanol extract (HM)

Grounded and defatted seeds (50 g) were extracted thrice under reflux in 100 mL of a H_2O/CH_3OH mixture (30/70; v/v). After filtration and centrifugation, the supernatant solution was recovered and evaporated to dryness.

2.3.4. Ethyl acetate extract (EA)

Grounded and defatted seeds (50 g) were extracted thrice under reflux in 100 mL of a H_2O/CH_3OH mixture (30/70; v/v). After centrifugation and concentration the solution underwent extraction of the aqueous phase with hexane, followed by a 3-fold extraction with ethyl acetate, the organic phase being eventually evaporated to dryness.

2.3.5. n-Butanol extract (n-B)

The procedure was the same as that just described for the EA extract, except that, after exposure to hexane and ethyl acetate, the solution was extracted thrice with *n*-butanol, before being evaporated to dryness.

2.4. Phytochemical screening

The crude aqueous extracts E1 and E2, as well as the HM, EA and *n*-B extracts, underwent phytochemical screening in order to detect the presence (or absence) of alkaloids (Dragendorff and Mayer reagent), reducing sugar (Fehling reagent), flavonoids (cyanidine reaction), tannins (iron chloride), and terpenoids (Liebermann Burchard reaction)[6].

2.5. Total polyphenol content

The total polyphenol content of the extracts was assessed by the method of Folin-Ciocalteu. Aliquot parts (0.1 mL) containing either 1.0 mg/mL of each extract or gallic acid standard (4.5 to 22.7 μg/mL) were mixed with 2.0 mL of a 20 g/L sodium carbonate solution and 0.1 mL of the Folin-Ciocalteu reagent (0.2 N). After 30 min incubation, absorbance (750 nm) was measured at ambient temperature. The results being expressed as gallic acid equivalent[7,8].

2.6. Total flavonoid content

The total flavonoid content of extracts was estimated by a colorimetric procedure using aluminum trichloride ($AlCl_3$) and sodium hydroxide (NaOH). Aliquot parts (0.5 mL) containing either 1.0 mg/mL of each extract or catechin standards (5 to 60 μg/mL) were mixed with 2.0 mL of distilled water, 0.15 mL of a 150 g/L solution of sodium nitrite ($NaNO_2$), 0.15 mL of a 100 g/L solution of aluminum trichloride ($AlCl_3 \cdot 6H_2O$). After 6 min incubation at ambient temperature and addition of 2.0 mL NaOH (1 mol/L), the volume was brought to 5.0 mL distilled water, and after a further incubation of 15 min, the absorbance measured at 510 nm[7,9].

2.7.Thin layer chromatography

The five extracts were examined by thin layer chromatography. About 5 μL of each extract (100 mg/mL) were deposited on fluorescence silica gel plates (0.2 cm thickness, 60 F254) and migration conducted with various mixture of methanol/H_2O (60/40, 70/30, 80/20, 90/10); chloroform/methanol/H_2O (40.0/60.0/0.1) and ethyl acetate/methanol/H_2O (100.0/13.5/10.0). The standards included gallic acid, catechin, quercetin and myricetin. After drying the plate, revelation was conducted using an UV lamp at 254 and 366 nm.

2.8.Column chromatography

After thin layer chromatography, the HM, EA and *n*-B extracts revealed the presence of a single spot with a 0.9 R_f. Separation on silica gel column using a methanol/H_2O (80/20)

solution for elution yielded with UV lamp at 254 and 366 nm a majority spot with the same R_f (0.9). The collection of such a majority fraction yielded after evaporation to dryness a fraction A, which further underwent some phytochemical screening (tannins, alkaloids, flavonoids and terpenoids), as well *in vitro* testing for its insulinotropic action.

2.9. DPPH free radical scavenging activity

The free radical scavenging activity of extracts was tested by measuring absorbance at 517 nm after 30 min incubation of 5.0 mL of a DPPH (1,1-diphenyl-2-picrylhydrazyl) methanolic solution mixed with 1.0 mL of the E1, HM and EA extracts (tested at concentrations ranging from 500 to 2 000 μg/mL). The reducing power was expressed as percentage inhibition by comparison with ascorbic acid standards (1.0 to 8.0 μg/mL)[10].

3. Results

3.1. Phytochemical screening

Table 1 provides some characteristics of either the solid extracts obtained after evaporation to dryness as the hygroscopic paste obtained for the HM extract and fraction A. The percent extracts yielded from 0.20%, 0.90% and 4.2% for E2, E1 and HM respectively. As described in Table 2, the phytochemical screening of the six extracts and fraction A revealed the presence in large amount of catechic tannins and flavonoids in HM, EA and fraction A, but not so in E2 or *n*-B. Terpenoids were obtained in E1 and *n*-B, less so in HM, and absent in E2 and EA. Coumarins were found in E2, EA and *n*-B. Last, alkaloids, reducing sugars, anthraquinones, and quinones were not detected in any extract.

3.2. Total polyphenol and flavonoid content

Table 3 informs on the total content of polyphenols and flavonoids in the five extracts considered in this study. The results either as mg of gallic acid (polyphenols) or catechin (flavonoids) equivalent per g extract or as mg/100 g dry matter. The highest values were recorded in the HM and EA extracts, amounted to 329, 1 002 and 150 mg in EA, HM an E1 respectively. Flavonoids amounted to 620, 241 and 94 mg in EA, HM and E1 respectively.

3.3. Thin layer and column chromatography

Thin layer chromatography with water/methanol revealed in the HM, EA and *n*-B extracts, but so in the EI extract, the same spot with a R_f (0.82) corresponding to that of gallic acid. In the same system, myricetin and quercetin standards yield a spot with a 0.88 R_f, which was also observed with EA extract, whilst products with the R_f for catechin spot (0.92) were observed with the E1 and HM extract. Another eluant, *i.e.* ethyl acetate/chloroform/methanol (90/5/5), yielded five spots with the E1 extract and six spots with the *n*-B extract, none of these spots corresponds to a given standard. Seven spots were observed with the HM extract, one of which yielded the same R_f as gallic acid (0.55), whilst another spot yielded the same R_f as myricetin and quercetin (0.87). Likewise, eight spots were obtained with EA extract, of which one spot yielded the same R_f as gallic acid (0.55) and another spot the R_f of myricetin (0.87) and quercetin (0.88). These preliminary findings indicate the presence of gallic acid, the major component of polyphenols, and myricetin and quercetin belonging the flavonols subclass of flavonoids, in the HM and EA extracts, whilst catechin which also belong to the flavonol substrate, may be present in the E1 and HM extract.

Table 1
Characteristics of *C. colocynthis* (L.) seeds extracts.

Extracts	Physical aspect	Color	Yield (%)	Solubility
Aqueous (E1)	Powder	Brown	0.9	Water
Aqueous (E2)	Powder	Yellow	0.2	Water
Water-methanol (HM)	Paste	Red-orange	4.2	Water:methanol (70:30)
Ethyl acetate (EA)	Powder	Orange	1.1	Water:methanol (70:30)
n-Butanol (*n*-B)	Powder	Brown	1.2	Water
Fraction A	Paste	Brown	-	Water:methanol (70:30)

Table 2
Phytochemical screening of *C. colocynthis* (L.) seeds extracts.

	Aqueous (E1)	Aqueous (E2)	Water-methanol (HM)	Ethyl acetate (EA)	*n*-Butanol (*n*-B)	Fraction A
Tannins	+++	−	+++	++	+	+++
Flavonoids	+	−	+++	+++	−	+++
Terpenoids (Lieberman-Burschart)	++	−	+	−	++	−
Alkaloids	−	−	−	−	−	−
Redusing sugars	−	−	−	−	−	−
Anthraquinones	−	−	−	−	−	−
Quinones	−	−	−	−	−	−
Coumarins	−	+	−	++	++	−

+++: Strong positive test; ++: Low positive test; +: Weak positive test; −: Negative test.

Table 3
Total polyphenols and flavonoids content of *C. colocynthis* (L.) seeds extracts.

	Polyphenols[a]	Polyphenols[b]	Flavonoids[a]	Flavonoids[b]
Aquous (EI)	166.39	150.00	91.02	82.00
Aquous (EII)	61.62	12.32	24.49	5.00
Water-methanol (HM)	238.80	1 002.00	147.76	620.00
Ethyl acetate (EA)	298.88	329.00	219.18	241.00
n-Butanol (*n*-B)	154.06	185.00	78.37	94.00

a: mg gallic acid equivalent per g extract; b: mg per 100 g plant matter.

Table 4
DPPH inhibition percentage of ascorbic acid and *C. colocynthis* (L.) seeds extracts.

	Concentrations μg/mL (% DPPH inhibition)					IC_{50} (μg/mL)
Ascorbic acid	1 (49.0%)	2 (82.0%)	4 (89.5%)	6 (91.5%)	8 (92.6%)	1.1
EI	–	500 (50.0%)	1 000 (62.0%)	1 500 (64.5%)	2 000 (66.2%)	500
HM	–	500 (46.5%)	1 000 (60.5%)	1 500 (67.6%)	2 000 (74.5%)	580
EA	300 (39.4%)	500 (67.4%)	1 000 (82.0%)	1 500 (86.2%)	2 000 (88.8%)	350

3.4. Free radical scavenging activity

At a concentration of 2 000 μg/mL, the EA, HM and E1 decreased under the present experimental condition, by 88.8%, 74.5% and 66.2% the DPPH signal, as compared to a 92.6% decrease with 8 μg/mL ascorbic acid. The IC_{50} found in this study for the EA, HM and E1 extracts were great high, being estimated, respectively, as 350, 580 and 500 μg/mL, as compared to 1 μg/mL for ascorbic acid (Table 4).

4. Discussion

The defatting of the *C. colocynthis* seeds powder with hexane revealed a lipid content of 18%-21%, in fair agreement with published values of 18% and 17%-19%[11,12]. The oil extract from *C. colocynthis* seeds is rich in unsaturated fatty acids (80%-85%), especially oleic acid (13.1%) and linoleic acid (70.1%)[11]. In streptozotocin-induced diabetic rats, the inclusion of *C. colocynthis* oil in the diet opposes insulin resistance and increases the mass of pancreatic insulin producing β-cells[13].

According to Koko *et al.*[14], an ethanolic extract (80%) of *C. colocynthis* fruits is deprived of alkaloids, anthraquinones, coumarines and tannins, but contains flavonoids and terpenoids.

Ethanolic (80%) and aqueous extracts from *C. colocynthis* leaves and fruits were also reported by Najafi *et al.* to contain alkaloids, flavonoids, glycosides and saponosides[15]. Likewise, Sultan *et al.* found 1.39 mg flavonoids, 0.52 mg saponosides, 1.64 mg alkaloids, 1.64 mg phenolic compounds and 30.12 mg ascorbic acid per 100 g of entire *C. colocynthis* plants[16].

Another study by Gill *et al.* documented the presence of alkaloids, steroids, terpenoïdes, flavonoids, as well as coumarins, glycosides in methanolic and hydromethanolic extracts of *C. colocynthis* seeds[17]. Even the roots of this plant were found to contain alkaloids, flavonoids, terpenoids and glycosides, but no saponosides and anthraquinones[18]. Incidentally, minor differences between the result of distinct studies could be related to differences in local climate and soil composition. The distribution of phytoconstituants such as saponins, tannins, flavonoids and alkaloids, may also vary in distinct parts of *C. colocynthis*, *e.g.* in leaves, fruits, roots and seeds.

Phenolic compounds or polyphenols, represented in majority by tannins and flavonoids, are presently a major axis of research, because they are considered as potent antioxidants, anti-inflammatory, anti-bacterial, antiviral and anti-cancer agents[19]. The results either as mg of gallic acid (polyphenols) or catechin (flavonoids) equivalent per g extract or as mg/100 g dry matter. The highest values were recorded in the HM an EA extracts, the water-methanol and ethyl acetate solvents being indeed currently used for the extraction of polyphenols and flavonoids[20]. The hierarchy for such content in these two fractions differed, however, depending on the mode of expression of results. This difference is accounted for by the yield of dry matter for each fraction under consideration. The recovery of distinct compounds (*e.g.* probably polar flavonoids versus polar aglycones and flavonoids glycosides) is also dependent on the solvent used for their recuperation[21]. For purpose of comparison, Kumar *et al.* reported that a methanolic extract of *C. colocynthis* fruits collected in the area Haryana in India contained[10], as polyphenols, 740 mg of gallic acid equivalent per 100 g plant matter and, as flavonoids, 130 mg of catechin equivalent par 100 g plant matter.

A number of chemical compounds were already identified in different extracts obtained from different parts (seeds, pulps, leaves and roots) of *C. colocynthis*. For instance, Afifi *et al.* reported the presence in the whole plant of three alkaloids ($C_{10}H_{15}NO_3$, $C_{20}H_{32}NO$ and $C_{16}H_{24}NO_7$[22]. Hatam *et al.*[23] documented the presence of two sterols ($C_{29}H_{48}O$ and $C_{29}H_{50}O$) in a petroleum extract of *C. colocynthis* fruits collected in Basra area in Iraq. In our extracts, the screening by the Liberman-Burchard

reaction did not reveal the presence of sterols. Sonja *et al.* identified cucurbitacins I, E, L, J, and T in a methanol extract of *C. colocynthis* fruits[24], whilst the same extract was also found to contain cucurbitacin glycosides by Nayab *et al.*[25] and Seger *et al*[26]. Such cucurbitacins are relevant to the bitterness and toxicity of the plants, as well as their anti-inflammatory, purgative and anti-cancer activities, such as the inhibition of cell adhesion resulting from the cytoskeleton destabilizing in cancer cells exposed to cucurbitacin E[27]. As far as flavonoids are concerned, isovitexin, *iso*-orientin and *iso*-orientin-3′-methylether were isolated from the hydromethanolic extract of *C. colocynthis* fruits, whilst the leaves and stems contains other flavonoids such as C-p-hydroxybenzyl derivatives, 8-C-p-hydroxybenzoylisovitexin, 6-C-phydroxybenzoylvitexin, 8-C-p-hydroxybenzoyl isovitexin-4′-O-glucoside[28,29]. Another flavonoid, quercetin, was identified in different parts of the plant (fruit, stem, leave and root) in ethyl acetate and diethyl ether fractions recovered from water-methanol (80%) extract[30]. Thus, *C. colocynthis* contain flavonoids such as quercetin, myricetin and kaemoferol[19].

The preliminary findings of thin layer and column chromatography indicate the presence of gallic acid, the major component of polyphenols, myricetin and quercetin belonging the flavonols subclass of flavonoids, in the HM and EA extracts, whilst catechin which also belong to the flavonol substrate, may be present in the E1 and HM extract. Prior work also revealed the presence of quercetin in an ethyl acetate extract of *C. colocynthis* fruit, steam, leave and root[19].

The EA, HM and E1 decreased DPPH signal by 88.8%, 74.5% and 66.2% respectively as. These values are in fair agreement with those reported by Kumar *et al*[10]. According to Delazar *et al.*[29], the IC_{50} (yielding a 50% decrease of DPPH) for selected flavonoids isolated from a methanol extract of *C. colocynthis* fruit yielded respective values of 71.3 μg/mL for isosaponarin, 0.56 μg/mL for isovitexin and 3.47 for isoorientin 3′-O-methyl ether compared to 27.8 μg/mL for a quercetin control. The IC_{50} found in this study for the EA, HM and E1 extracts were great high as compared to ascorbic acid. Thus, at variance with the antioxidant effect of pure ascorbic acid, the high values found with our crude extract probably reflect their variable content in several chemical compounds, with a low IC_{50} of the EA extract, when compared to the mean and other extracts, deprive the higher content of polyphenols and flavonoids in the low HM extract then in the former EA extract.

According to the literature, polyphenols and flavonoids, are known for their antioxidant power. Such as antioxidant activity of flavonoids depends on their chemical structure, especially of 3′,4′-orthodihydroxy group on the B cycle and the 4-carbonyle group on the C cycle. The 3-OH and 5-OH groups on the C cycle are also relevant to the antioxidant effect. The absence of an O-dihydroxy structure on the B cycle provides a catechol structure which may compensate the antioxidant activity of flavonoids. In the case of flavonols (quercetin, myricetin, kaemoferol) the hydroxyl group located on the C_3 of the C cycle is recommended as a critical site for the scavenging free radicals[30,31].

In conclusion, *C. colocynthis* represent a plant rich on such phytocompounds as polyphenols and flavonoids, which confer to the plant an interesting antioxidant activity. It thus seems pertinent to identify the majority chemical components of *C. colocynthis* extracts and to document both their free radical scavenging activity and other therapeutic effects.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Medicinal plants represent a source of chemical compounds such as tannins, flavonoids, saponins, resins and alkaloids with curative properties. In traditional medicine, several medicinal plants are used for the treatment of diabetes mellitus. among which *C. colocynthis* represent one of the most commonly used species in ethopharmacology. According to lietrature, Infusion prepared from the fruit and seeds of this plant are indeed recommended to diabetic patients. The chemical compounds identified in antidiabetic plants are often alkaloids, polyphenols and polysaccharides, gums and glycans. Objective of examined work, consists in a phytochemical screening of different extracts from *C. colocynthis* seeds, to determine the total content of polyphenols and flavonoids in such extracts, and to assess their antioxidant activity on the DPPH free radical scavenging.

Research frontiers

The studie include Phytochemical screening, total content of polyphenols and flavonoids and antioxidant activities of *C. colocynthis* seeds extracts, Differents extracts are tested (aqueous (E1), a defatted aqueous(E2), Hydromethanolic, Ethyl acetate and n-Butanolic extracts).

Related reports

Data about the distribution of phytoconstituants such as saponins, tannins, flavonoids and alkaloids, in distinct parts of *C. colocynthis*, *e.g.* in leaves, fruits, roots and seeds. Thus, study conducted by Gill *et al.* (2011) documented the presence of alkaloids, steroids, terpenoïdes, flavonoids, as well as coumarins, glycosides in methanolic and hydromethanolic extracts of *C. colocynthis* seeds. Even according to Suman (2010) the roots of this plant were found to contain alkaloids, flavonoids, terpenoids and glycosides, but no saponosides and anthraquinones.

Author of this paper explain that the minor differences between the results of distinct studies could be related to differences in local climate and soil composition.

Innovations and breakthroughs

This study has showed that *C. colocynthis* represent a plant rich on as polyphenols and flavonoids, which confer to the

plant an interesting antioxidant activity.

Applications

It is interesting to extract and identify the pur polyphenol or flavonoids of the bioactive extract . So , it is important to continuing the works of the phytochemical part, and is interesting to study the relation between antioxidant activity and content of polyphenols

Peer review

The work and results obtained are interesting. Its will certainly enrich research into valorisation of medicinal plants as antioxydants source.

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