*Mycopath* (2018) 16(2): 73-80 **Research Article**

# Antibacterial activity of *Citrullus colocynthis* extracts

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## Abstract

The use of aromatic and medicinal plants for therapeutic aims has developed since ancient times because of their richness in bioactive substances. Research was carried out to explore the most suitable and standardized method to extract bioactive molecules from plant. The aim was to test the effect of the change of extraction time (24, 48, 72 h) and the use of different solvents (water, ethanol) on both phytochemicals levels and antibacterial activity of *Citrullus colocynthis* (L.) Schrad. extracts. The results revealed that the time of extraction and the nature of the solvent used had a considerable influence on the level of the molecules compounds. The variation of the time correlates with the extraction yield because changing extraction time from 24 h until 72 h the extraction yield with ethanol rose. However, extraction yield decreases inversely when the time of extraction increases by water as solvent. The ethanol extract after 72 h extraction was characterized by the highest phenolic content 61.58 mg gallic acid equivalent per g dry weight. Ethanolic extracts 24 h and 72 h and aqueous extract 24 h were the most active against *S. aureus* with inhibition zone 20, 18 and 16, respectively. The results, also, showed that the aqueous extract after 72 h had no action on all bacteria strains.

***Keywords:*** Antibacterial activity, Bioactive compounds, *Citrullus colocynthis,* Ethanol, Medicinal plants.

## Introduction

Almost all cultures and civilizations, from antiquity to the present day, depended wholly or partly on herbal medicine because of its efficacy, accessibility, availability, low toxicity, and acceptability. Currently, more than 100 countries have regulations for herbal medicines (Rezaei-Golestani *et al.,* 2015; Khoudali *et al*., 2014). Plants which possess unique chemical profiles, therapeutic properties and which offered flavors would be more explored by scientists. Now, these plants are widely used as a source for many derivative compounds such as seasonings and colorings, and as preservatives and medicines (Kumar and Jnanesha, 2016). Due to the inappropriate consumption and abuse of antibiotics that lead to the emergence of multi-drug resistant microorganisms in addition to ever-increasing cost of drug production, a renewed interest in the pharmacopeia is, thus, noted in order to search for new effective molecules having antimicrobial properties and having a broad spectrum of action. One of the most recommended strategies, and an interesting track, is to explore plants used in traditional medicine (Traoré *et al.* 2012; Chebaibi, 2011).

The species *Citrullus colocynthis* belongs to the family Cucurbitaceae that contains nearly 120 genera and 825 species. *C. colocynthis* is a xerophilic, perennial and herbaceous plant, naturally growing on sandy soils of Arabia, West Asia, tropical Africa, and the Mediterranean regions. This plant is endemic to southern Algeria and is widely used in Algerian traditional medicines. This plant is one of the few indigenous species that grows under arid conditions and improving soil fertility. In addition, its seeds oil has the potential to be used as a raw material for biodiesel (Menon *et al.,* 2014; Milovanović & Pićurić-Jovanović, 2005). This species is used to treat fever, visceral and cerebral congestions, liver diseases (Mehni *et al.,* 2014), rheumatism, cancer and bacterial infections (Dehghani *et al.,* 2008; Mukherjee and Patil, 2012).

To identify and quantify the plants' substances and as each plant sample has its unique characteristics during extraction, there is a need to develop an optimal extraction method. This study deals with the valorization of medicinal plants of the Algerian flora, in order to find new bioactive natural products. The interest is also to select the most suitable experimental conditions for the extraction of those molecules by studying the effect of variation of extraction times and the nature of the solvent used. The aim was to evaluate the antibacterial ability of ethanolic and aqueous extracts of the Algerian ethnomedicinal plant *C. colocynthis*.

## Materials and Methods

### Plant material

The plant material was collected from Bechar (South-west of Algeria), between May and July (arid and semi-arid zones). Aerial parts of *C. colocynthis* were air-dried at room temperature and crushed.

**Preparation of extracts**

One gram of powdered leaves and stems of *C. colocynthis* was extracted for different periods (24, 48 and 72 h) via cold maceration technique using two different solvents, ethanol or water at volumes 50 mL under agitation at room temperature. The extracts were, then, filtered and concentrated under reduced pressure at 40 °C via a rotary evaporator the extracts were stored at 4 °C (Bandar *et al.,* 2013).

**Bacterial strains**

For the antibacterial assays, three bacterial strains (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*) were used, which were provided by the Clinical Laboratory of Bechar Public Hospital, Algeria.

**Determination of total polyphenols**

For the determination of total phenolic content (TPC), Folin-Ciocalteu reagent test was used. A volume of 0.05 mL diluted extract was mixed with2.5mL of diluted Folin-Ciocalteu reagent (1:10) and 0.45 mL of distilled water. Then, 2mL of sodium carbonate (7.5%, w/v) was added. After incubation at 50 °C for 5 min, the absorbance of samples was measured at 760 nm. To estimate TPC, gallic acid was used as a standard curve. All tests were done on triplicate and results were expressed as mg gallic acid equivalent (GAE) per g dry weight (DW) (Koczka *et al*., 2018).

**Determination of total flavonoids**

Total flavonoid content of plant extracts was evaluated using aluminum chloride assay. Three milliliters of methanol was added to 1 mL of plant extracts or standard. In addition, 200 µL of potassium acetate (1M), 200 µL of aluminum chloride solution (10%) and 5.6 mL of distilled water were added to the reaction mixture that was then incubated at room temperature for 30 min. The absorbance was measured at 415 nm against methanol served as blank. The TFC was mentioned as quercetin equivalent mg $g^{-1}$ dry weight (Islam *et al*., 2016). All phytochemical tests were repeated three times.

**Antibacterial properties assays**

Antibacterial activities of plant extracts were assessed using the disc diffusion method (Murray *et al.,* 2016). The aromatogram represents an essential point of reference since this technique is identical to the antibiogram, a routine test for bacteriological laboratories that used to measure the bactericidal activity of antibiotics (El kalamouni, 2010; El Amri *et al.,* 2014). By this technique, the discs loaded by plant extracts were seton the solidified medium (Mueller Hinton Agar) already inoculated with the bacterium tested. The next step was the incubation at 27 °C 48 h for fungi and for 24 h at 37 °C for bacteria. A negative control was prepared using the respective solvent. Tetracycline antibiotic (TR) at 30 µg $mL^{-1}$ was served as positive control. Then, minimum inhibitory concentrations (MICs) were deduced via the dilution method consists of putting a standardized bacterial inoculum in direct contact with a range of plant extract increasing concentrations. A volume of 100, 200 and 300 µL of extract were tested and were added to 20mL of Mueller Hinton medium and then were poured into dishes. MIC is the lowest concentration at which the extract still showing a visible antibacterial activity. Experiments were repeated three times.

**Statistical analysis**

Data were expressed as means ±standard deviation (S.D) and were deemed to be significant at $p < 0.05$. The two-way analysis of variance (ANOVA) was used as a statistical analysis for registered Data.

# Results and Discussion

Medicinal plants are an important source of natural products to treat infectious bacteria. Pharmaceutical industries search for new therapeutic compounds from alternative sources such as medicinal plants because of the widespread of multidrug-resistant bacteria, increasing the cost of synthetic medicines and the negative side effects of drugs (Niranjan *et al.,* 2017).

Table 1 shows extraction weights obtained via maceration of aerial parts of *C. colocynthis* for various times of extraction using different solvents. The total phenolic content (TPC) and the total flavonoid content (TFC) were determined from *C. colocynthis* ethanolic and aqueous extracts. Results are summarized in Tables 2 and 3. As shown in Table 1, extraction yield obtained by ethanol was significantly more important in the samples where extraction time was 72 h (122.8 mg) than in those of 24 and 48 h. Conversely, the highest extraction yield (155.1 mg) is obtained at 24 h by water. The variation of the time correlates with the extraction yield because as seen in the table, changing extraction time from 24 h until 72 h the extraction yield with ethanol rose. However, extraction yield decreases inversely when the time of extraction increases by water as solvent. All the plant extracts had higher phenolic content, but the *C. colocynthis* aqueous extract after 48 h (aqueous extract 48) had significantly ($P \leq 0.05$) the highest TPC than other extracts.TPC values for analyzed *C. colocynthis* ethanol extracts ranged from 36.28 mg to 61.58 mg GAE $g^{-1}$ DW. The ethanol extract 72 h of *C. colocynthis* was characterized by the highest phenolic content and has manifested remarkable TFC about 31.72 quercetin equivalent mg $g^{-1}$ dry weight. Significantly lower values of TPC were found for ethanol extract of 24 h. Appreciable levels of TPC were deduced for aqueous extract 48 with 102 mg GAE $g^{-1}$. However, TFC resulted in no remarkable

differences between the three samples using water for the extraction times 24, 48 and 72 h, for which the best TFC was 13.41 (aqueous extract 72 h). Since antiquity, natural and herbal products had been used by folk medicine all over the world. An increasing interest in the therapeutic benefits related to these products is observed (Bramorski *et al.,* 2010). The detection of the polyphenols in an extract is done by measuring the intensity of blue coloring which results from their reduction in an alkaline medium by the Folin-Ciocalteu reagent at $\lambda = 760$ nm (Georgé *et al.,* 2005). This process, which is today the most widespread, its simplicity and its reproducibility have made it one of the oldest methods used to determine the TPC of plant extracts. (El-Haci *et al.*, 2012). The flavonoids content of each extract is estimated by the determination of yellow coloration produced during the formation of the aluminum-flavonoids complexes measured by direct colorimetric test using aluminum chloride. In fact, the flavonoids possess a free hydroxyl group in the 5-position capable of giving, in the presence of aluminum chloride, a yellow-aluminum-flavonoids complex by chelating of $Al^{+3}$. This resulted in yellow coloration is proportional to the amount of flavonoids present in the extract, maximum absorbance is detectable around 510 nm (Basli *et al.,* 2012).

With more than 5000 flavonoids compounds already identified, this is the most important class of polyphenols (Gómez-Caravaca *et al.,* 2006). Flavonoids protect cellular structures by their function as UV filters, besides they prevent its harmful effects (Oliveira *et al.,* 2009). The extraction of phenolic components from the plant materials depends on the nature of the solvent chosen to recover the soluble fraction of the solid. To extract polyphenols, which are strong antioxidants, the most effective solvents are polar, such as methanol and ethanol. Polar solvents, such as methanol and ethanol, are better to exhibit rich polar constituents and thus extracting polyphenols from plant material (Nizam and Mushfiq, 2012). To extract high polar compounds, highly polar solvents, like water, are not suitable. In addition to this, the use of them as the only extraction solvents generate a high level of impurities such as soluble proteins, sugars and organic acids by the side of polar compounds which could interfere during quantification and identification. Extraction using absolute alcoholic solvents also reduces the extraction yield (Bandar *et al.,* 2013). Genetic characteristics besides environmental factors like light intensity, temperature, and water availability strongly influence both the quantity and quality of secondary metabolites such as phenolic molecules (Król and kiełtyka-Dadasiewicz, 2015; Kleinwächter *et al.*, 2015). According to (Chan *et al.,* 2009), extraction prolonged time contributes to oxidation of phenolics because of exposure to oxygen and light. Hence, the law of Fick about diffusion predicts a final equilibrium that exists between the concentrations of solute in the plant material and among a solvent after a certain extraction period. Therefore, a prolonged time of extraction process is not effective to extract more phenolics (Silva *et al.,* 2007). In addition, these variations in phenolic profiles can be explained by the growth conditions such as geographical location, soil type, climate, precipitation, altitude, harvest season, the duration of conversation, diseases that can affect the plant and the treatments carried out, that directly interfere with the content of chemical constituents and consequently their therapeutic effects (Özgüven and Tansi, 1998; Park and Cha, 2003; Naghdi-Badi *et al.,* 2004; Ebrahimzadeh *et al.,* 2008). Plant oil and phenol content vary from year to year, for example, maximal yields of *Thymus vulgaris* in oil and in phenol content were obtained with the closest culture spacings and after completion of flowering (Shalby and Razin, 1992; Piccaglia and Maroti, 1991; Mcgimpsey *et al.,* 1994). The largest extractive value of *C. colocynthis* leaves was found for the aqueous extract (28.31%), followed by ethanol extract (25.22%), and then chloroform extract (3.53%) (Brown and Pice-Evans, 1998). The dry matter yields of *T. vulgaris* increases gradually by 15% under natural light to 29% under an additional light (Letchamo *et al.,* 1995a). Some studies have reported that agricultural factors have a critical effects on the quantitative and qualitative characteristics of thyme, such as places where plants are grown, physiochemical characteristics of soil, seed source, crop spacing, harvest period, plant age, chemotype taken from the same botanical species, the part used for extraction and also the oil extraction method (Naghdi-Badi *et al.,* 2004; Sharafzadeh *et al.,* 2010; Rolli *et al.,* 2014). The extraction yields of *C. colocynthis* seeds using methanol and water were 4.89% and 2.72%, respectively. Phytochemical analysis of aqueous extracts revealed the richness of *C. colocynthis* in tannins, glycosides, terpenoids, saponosides as well as coumarins (Gacem *et al.,* 2013). Phytochemical analysis of aqueous extracts revealed the richness of *C. colocynthis* in tannins, glycosides, terpenoids, saponosides as well as coumarins (Gacem *et al.,* 2013). The extraction yield could be improved using a mixture of alcohol and water to increase the contact surface between the solvent used and the plant material (Bandar *et al.,* 2013). Due to cytotoxic behavior, cucurbitacins appear to play an important role in discovering new drugs especially in the development of anticancer drugs. (Hussain *et al.,* 2014). The phytochemical analysis of *C. Colocynthis* revealed the presence of a wide range of active compounds: terpenoids, carotenoids, alkaloids, steroids, saponins, tannins, flavonoids, phytosterols glycosides, citrullol, citrulline, colocynthine, colocynthetin, cucurbitacin E (α-elaterin), cucurbitacin L. (dihydroelatericin B), cucurbitacins A, B, C, D, E, F, I, J, citbittol (a bitter

oil), spinasterol (Chauhan *et al*., 2011; Abou Zaid *et al*., 2013; Elgerwi et *al*., 2013; Dehghani *et al.*, 2008; Prasad, 2014). The efficiency of the extraction process depends on numerous parameters: temperature, pH, nature of the solvent used, the ratio of solvent/solid, number of extraction steps, and component size of the solid matrix (Chirinos *et al.*, 2007). The disc-diffusion and the checkerboard methods were used to test antibacterial properties and to deduce minimum inhibitory concentrations (MICs) of plant extracts. The data pertaining to the antibacterial potential of the *C. colocynthis* ethanol and aqueous extracts were presented in Tables 4 and 5. The tested and antibacterial assays were performed by the checkerboard and disc diffusion methods. Growth in each dish was quantified using a visual observation and then interpreted as visible or non-visible growth of bacteria. The recorded results shown in Table 4.

The results reveal variable responses according to the strains examined, the nature of the solvent and the time that allowed to extract plant material. Ethanolic extracts 24 and 72 h and aqueous extract 24 h were the most active against *S. aureus* with inhibition zone 20, 18 and 16, respectively. The MICs for these extracts were 45.5 and 60.9 and 63.1 µg $mL^{-1}$, respectively ($P \leq 0.05$). The results also showed that the aqueous extract 72 extract had no action on all bacteria strains. In addition, no inhibition of *P. aeruginosa* was observed by each of aqueous extract 24, 72 h and ethanol extract 24 h. Tables 4 and 5 show significant differences in the antibacterial activities of the plant extracts tested. These results are impressing as some of the extracts tested allowed the inhibition of certain highly pathogenic strains: *S. aureus, P. aeruginosa*, *E. coli*, such as aqueous extract 48 h which prevented their growth. The antibacterial effect noticed for ethanol extract 48his difficult to overlook. Generally, Gram-negative bacteria are more resistant to the antimicrobial substances of medicinal plants than Gram-positive strains which could be related to the presence of an outer-membrane permeability barrier outside the cytoplasmic membrane of Gram-negative bacteria which limiting the access of such effective agents to their targets within the bacterial cells (El-Aasr *et al.*, 2016). The antimicrobial power of molecules, *in vitro*, can be demonstrated by conventional techniques, both in liquid and in solid medium. When it fulfilled correctly, disk diffusion and agar dilution, which performed in solid medium, showed repeatable and reproducible results (Threlfall *et al.*, 1999; Walker, 2000). Plants synthesize an array of molecules with very diverse structures such as polyphenols, flavonoids, and terpenoids with low antibiotic activity compared to those produced by microorganisms (Sarker *et al.*, 2005). Both flavonoid and phenolic molecules produced by medicinal plants have a powerful antibacterial ability. Probably, they induce antibiotics synergistically, directly inhibit bacterial species or reduce and weaken its pathogenicity (Messaoudi *et al.*, 2019). Since antiquity, plants aqueous extracts such as decoctions or infusions, were used as cures for various human and animal illnesses (Kaufman *et al*., 2006). Aqueous extracts such as infusions have biological effects (Martins *et al.*, 2015). As they can interact with the bacterial cell wall and could disrupt it, It has been reported that flavonoids have the greatest antimicrobial role. Glycosylated flavonoids are hydrosoluble and may be responsible for the observed antibacterial effect (Marston and Hostettmann, 2006). In the study made by (Messaoudi, 2016), aqueous extracts resulted from *C. colocynthis* by decoction were found to be the most active against all the strains examined (*Staphylococcus aureus, Enterobacter cloacae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Citrobacter freundii*, *Salmonella typhimurium*). Inhibition zone diameter ranged between 7–11 mm and the best MICs result was 162.8 µg $mL^{-1}$. In the same study, infusion extracts to be ineffective against *E. coli, S. Typhimurium* and *K. pneumoniae.* Inhibition zone noticed for the other bacteria situated from 9 to 14 mm. Essential oils are soluble in the water of an infusion when the temperature is that of boiling water (Messaoudi, 2016). During a decoction, they disappear by slow evaporation. At the time of the cooling of the infusion, they precipitate on the surface. Of all the compounds, the polyphenols are among the most found in the infused extracts. (Gonzàlez and Marioli, 2010).

Plants active agents against microorganisms are generally saturated organic or aromatic compounds that's why they are resulted after initial extraction using methanol or ethanol (Cowan, 1999). Due to the simple nature of this assay and the reduced amount of extract required, the use of agar diffusion method is generally recommended for the evaluation of many extracts on a single microorganism, before highlighting and characterized deeper those with the highest capacity (Chorianopoulos *et al.*, 2007). The practical difficulties that can be encountered in conventional antimicrobial assessment techniques result from the insolubility of constituents in water, their volatility and the need to test them at low concentrations (El Kalamouni, 2010). Some researchers have explained the absence of antibacterial activity in aqueous extracts by the fact that the compounds constituting the apolar fractions of the plant extracts are at the origin of the antibacterial action and probably be the phenolic diterpenoids, because of their highly lipophilic character, which allows them to be extracted with low polarity solvents such as chloroform. **(**Fernandez-Lopez *et al.*, 2005; Albano and Miguel, 2011). The variations in the antibacterial ability of plant extracts tested are due to both taxonomic properties of the plant and biological

properties of the tested bacteria (Abdel Rahman *et al.*, 2011). The study of Eloff (1998) was about the extraction of solvent that is more appropriate to the quantification, isolation, and identification of plants' antimicrobial agents. Several parameters like the amount of extraction, the quantity, and variety of components resulted, toxicity registered during biological tests, biological risks, number of inhibitors obtained and the facility of solvent elimination were tested for each extraction solvent. The best results obtained using acetone as solvent followed by those of methanol: chloroform: water mixture, then by methanol, ethanol or water.

Today, secondary plant metabolites, such as polyphenols, have taken a place in food, cosmetics, perfumes, the detergent industry and in the pharmaceutical industry because of their antibacterial, antifungal, antioxidants, anti-carcinogens, disinfectants of the respiratory system, antivenom and healing properties (Benzeggouta, 2005; Berdowska *et al.*, 2013; Rezaei-Golestani *et al.*, 2015). It was, also, found that the richness of plants polar extracts with active components such as phenols and terpenes is responsible for their main capacity (Messaoudi *et al.*, 2019).

# Conclusion

The plant species *C. colocynthis* examined in the case of aqueous and ethanolic extracts exhibited extremely high levels of phenolic compounds, which partly explained the antibacterial properties registered. The existence of other compounds with beneficial, biological and antimicrobial effects that can be elucidated by future studies remains a non-neglected proposition. The results confirmed the existence of important antibacterial ability probably linked to the richness of as phenolic molecules.

Further studies should be conducted to better clarify both the action mechanisms and the nature of bioactive molecules. The best extraction time depended on the chosen solvent, in addition, for optimal conditions of extraction, water needs to be moderately polar medium by combinated it with other organic solvents.

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**Table 1:** Extraction yields (mg) produced by maceration technique.

| Extraction Time (h) | Ethanol | Water |
|---|---|---|
| 24 | 27.4 ± 1.665 | 155.1 ± 0.325 |
| 48 | 59 ± 0.551 | 137 ± 1.114 |
| 72 | 122.8 ± 2.335 | 99.7 ± 0.882 |

**Table 2:** Phytochemical amounts of ethanol extracts of *C. colocynthis*.

| Extraction Time (h) | TPC (mg EAG $g^{-1}$) | TFC (mg EQ $g^{-1}$) |
|---|---|---|
| 24 | 36,28 ± 0,51 | 24,77 ± 1,14 |
| 48 | 57 ± 0,435 | 19,49 ± 0,22 |
| 72 | 61,58 ± 2,23 | 31,72 ± 0,82 |

**TPC:** Total phenolic content (mg GAE $g^{-1}$), **TFC:** Total flavonoids content (mg QE $g^{-1}$).

**Table 3:** Phytochemicals amounts of aqueous extracts of *C. colocynthis*.

| Extraction Time (h) | TPC (mg EAG/g) | TFC (mg EQ/g) |
|---|---|---|
| 24 | 91.44 ± 3.10 | 9.15 ± 0.38 |
| 48 | 102 ± 0.69 | 11.32 ± 2.15 |
| 72 | 76.58 ± 1.19 | 13.41 ± 0.19 |

**TPC:** Total phenolic content (mg GAE $g^{-1}$), **TFC:** Total flavonoids content (mg QE $g^{-1}$).

**Table 4:** Antibacterial ability of *C. colocynthis* extracts via disc diffusion method.

| Bacterial Strains | Inhibition zone diameter (mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ethanol extracts | | | Aqueous extracts | | | Positive control (30 µg mL$^{-1}$) |
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | Tetracycline |
| *Escherichia coli* | $9^{A}$ | $13^{a}$ | - | - | 16 | - | 15 |
| *Pseudomonas aeruginosa* | - | 8 | - | 14 | 11 | - | - |
| *Staphylococcus aureus* | $16^{B}$ | $13,5^{b}$ | - | 20 | - | 18 | 11 |

Capital letters (A–B) and lowercase letters (a–b) indicate significant differences at $P \leq 0.05$.

**Table 5:** Minimum inhibitory concentrations (MICs) of *C. colocynthis* extracts by checkerboard technique.

| Bacterial Strains | MIC (µg mL$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ethanol extract | | | Aqueous extract | | | Positive control |
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | Tetracycline |
| *Escherichia coli* | $187^{A}$ | $91,8^{a}$ | - | - | 77 | - | 10 |
| *Pseudomonas aeruginosa* | - | 208 | - | 56 | 73 | - | - |
| *Staphylococcus aureus* | $63,1^{B}$ | $98,4^{b}$ | - | 45,5 | - | 60,9 | 30 |

(-) : Absence of activity; Negative controls did not show any activity.
Capital letters (A–B) and lowercase letters (a–b) indicate significant differences at $p < 0.05$.

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