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Cytotoxic and antioxidant effect of the ethanolic extract of *Citrullus colocynthis L.* plant fruits

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Abstract

Citrullus colocynthis L. of the Cucurbitaecea botanical family is a plant widely used in traditional medicine in Algeria. It seems empirically effective in the treatment of various diseases. The aim of the present research is to evaluate the antioxidant and cytotoxic effects, in vitro, of the ethanolic extract of *C. colocynthis* fruits. The quantitative analysis has shown that the ethanolic extract of *C. colocynthis* is rich in total polyphenols with a content of 443.62 ± 2.13 µg EAG/mg of extract. The results obtained showed a strong anti-free radical activity of the ethanolic extract of *C.colocynthis* exerted against the DPPH free radical scavenging effect (CI_{50} = 6.31 µg/ml) and highlighted a powerful ferric reducing antioxidant power (CI_{50} = 27.94 µg/ml). We should also note a good antioxidant activity against the OH radical, obtained with the concentration (IC_{50} = 67.13 µg/ml). Furthermore, the obtained results indicate that the treatment of the three cancer cell lines (HepG2, SH-SY5Y and Raw 264.7) with the different concentrations of the used extract reduced the number of cells in a dose-dependent way. Based on our results, we can consider that *Citrullus colocynthis* is a plant with a strong pharmacological power and can therefore be used in phytotherapy.

Keywords: *Citrullus colocynthis L.*; ethanolic extract; polyphenols; antioxidant activity; cytotoxicity.

Introduction

Free radicals are constantly produced physiologically by aerobic organisms, notably through mitochondrial respiration and phagocytosis (Ateba and *al*., 2019). They can also be the result of external factors such as UV radiation, pollution, tobacco and alcohol as well as some herbicides and pesticides. However, this production is controlled by enzymes with antioxidant activity that are naturally present in our bodies: superoxide dismutase, catalase and glutathione peroxidase (Fu and *al*., 2018). The balance between ROS and these antioxidants is fragile and an imbalance can lead to oxidative stress (Akter and *al*., 2016 ; Ateba and *al*., 2019).

Oxidative stress is linked to many diseases, for example atherosclerosis, cancers, type 2 diabetes, neurodegenerative and rheumatic diseases. This is why research on antioxidants in plants has developed a lot in recent years, in order to find the best possible antioxidants in the hope of protecting our health and even curing these different diseases (Fu and *al*., 2018 ; Santos and Silva, 2020).

Indeed, natural antioxidants are the subject of much research and a new impetus towards the exploitation of secondary metabolites generally and polyphenols particularly in health and pernicious diseases (cancer) as well as in the food industry (Akter and *al*., 2016). Noting that the powerful efficiency of these substances to stop radical reactions by neutralizing free radicals is mainly due to their phenolic structures with the presence of hydroxyl groupings (Santos and Silva, 2020). It is in this sense that the study of the antioxidant activity of plants has become important

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today, as powerful antioxidants can be found in plants. Building on this vision, a revival of phytotherapy towards this green wave that produces a host of antioxidants to counter and trap these oxidants (Teng and *al*., 2017).

For this purpose, and in the framework of the valorization of the Algerian flora, we were interested in a spontaneous species of medicinal nature from the Algerian Sahara. It is *Citrullus colocynthis* or coloquinte, a perennial herbaceous plant of the cucurbitaceous family, known since antiquity (Davatgaran-Taghipour and *al*., 2017). This plant, which is very abundant in Algeria and has the local common name Handhal, Hdaj or Delaa El-Wad, resembles a small watermelon with a bitter taste. The various components of the plant are widely used in traditional medicine as a treatment for various ailments such as diabetes, hypertension, rheumatism, certain urinary, gynaecological, gastrointestinal and pulmonary infections (Chawech and *al*., 2015 ; Ogbuji and *al*., 2012 ; Shafaei and *al*., 2012). In Algeria, as in the rest of the Mediterranean, the parts of the plant most used for therapeutic purposes are the fruits and/or seeds. The fruits of the coloquinte grow naturally in the desert, sandy regions and in many tropical countries (Ogbuji and *al*., 2012 ; Hussain and *al*., 2013).

In our study, we were interested in assessing the antioxidant and cytotoxic effects of the ethanolic extract of the fruits of *Citrullus colocynthis L*. with a view to its valorization. This work is part of a contribution to a better knowledge and exploitation of medicinal plants in Algeria, to the valorization and possible discovery of extracts with pharmacological activities.

Materials and Methods

Vegetal material

The fruits of the coloquinte (*C. colocynthis*) are harvested after ripening in August/September in the region of Bir El Ater, in the province of Tebessa, in the east of Algeria. The fruits are cleaned of impurities, dried in the laboratory at room temperature, away from sun and light. The dried fruit, free of seeds, is passed through an electric grinder and the resulting shred is stored at -20°C.

Extraction of a crude extract rich in polyphenols

The extraction is carried out by macerating 50 g of vegetable powder in 500 ml of ethanol/water mixture (v/v: 60/40). The mixture is filtered after 24 hours, the ethanol and water are eliminated respectively by the rotavapor (T°=40°C) and the freeze-dryer. The dry extract obtained is kept at -20°C for the study of biological activities (Addab and *al*., 2020).

Dosage of total polyphenols

The determination of the total polyphenols of the ethanolic extract of *C. colocynthis* fruit is carried out according to the Folin-Ciocalteu method (Adam and *al*., 2001). To 200µl of the ethanolic extract dissolved in distilled water is added 1 ml of Folin-Ciocalteu reagent (diluted 10 times in distilled water). The solution, after standing for 4 minutes, 800µl of Na_2Co_3 (7.5%) also diluted in distilled water, is added to the preparation. The previously stirred mixture is kept away from light for 2 hours. The absorbance is then read at 765 mm by a UV-visible spectrophotometer. The concentration of total polyphenols is calculated from the regression equation of a calibration range in aqueous medium (0.025-0.6 mg/ml), established with gallic acid under the same operating conditions as the extract. The results are expressed in microgram equivalents of gallic acid per milligram of extract (µg EAG/mg extract). The equation of the calibration curve of gallic acid was: $Y= 1.286x+ 0.022$ ($R^2 = 0.997$).

Antioxidant activity in vitro

DPPH free radical scavenging effect

According to the protocol described by Addab and *al*. (2020). DPPH (1,1-diphenyl-2-picrylhydrazyl) solution is prepared by solubilising 4 mg of this product in 100 ml of methanol. 50 µl different concentrations of sample and control solutions are added to 1.95 ml of DPPH solution. The mixture is left in the dark for 30 min and the absorbance reading is taken against a blank at 517 nm. The positive

control is represented by a standard antioxidant: ascorbic acid whose absorbance is measured under the same conditions at concentrations of (0-1.9 mg/ml). The results of the anti-radical activity or the inhibition of free radicals are expressed as a percentage of inhibition (I%) estimated according to the equation below : *I% inhibition = [(Abs control- Abs test)/ Abs control] x 100*

I %: Percentage of anti-radical activity.

Abs Control: Absorbance of the DPPH solution at time 0.

Abs test: Absorbance of the extract.

The IC_{50} being the concentration of the extract or standard that allows the 50% reduction of DPPH. A low IC_{50} value indicates a high antioxidant activity. A difference is statistically significant at $P<0.05$.

Reducing power (FRAP, Ferric Reducing Antioxidant Power)

This method is based on the ability of extracts to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The mechanism is known to be an indicator of electron-donating activity, characteristic of the antioxidant action of polyphenols (Yen and Duh, 1993). It consists of mixing 1 ml of the extract at different concentrations with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of a potassium ferricyanide solution ($K_3Fe(CN)_6$ at 1% (w/v)). The resulting mixture was incubated at 50°C for 20 min, then 2.5 ml of 10% trichloroacetic acid (CCl_3COOH) was added to stop the reaction. The mixture is centrifuged at 3000 g for 10 min at room temperature. To 2.5 ml of the supernatant are added 2.5 ml of distilled water and 0.5 ml of 0.1% iron chloride ($FeCl_3$). The absorbance of the reaction medium is determined at 700 nm. The increase in absorbance in the reaction medium indicates an increase in the reducing power of the tested extracts. The EC_{50} concentration, which is defined as the effective concentration at which the absorbance is equal to 0.5 for reducing power and was obtained from linear regression analysis, is an index used to compare and express the strength of the reducing capacities of bioactive substances. The increase in absorbance in the reaction medium indicates the increase in iron reduction. Ascorbic acid is used as a positive control.

Hydroxyl radical scavenging effect

The Hydroxyl radical scavenging method adopted in this study is that of Fetni et *al*. (2020), with some modifications. The reaction mixture consists of the following reagents: 1 ml of (9mM $FeSO_4$) and 1 ml of 0.3% H_2O_2, 0.5ml of (9mM) of the ethanol-salicylic acid solution, 1 ml of the extract at different concentrations. After incubation at 37°C for 60 minutes, the reading is taken at a wavelength of 510 nm. The scavenger effect of the hydroxyl radical is calculated according to the following equation: *Inhibition OH· (%) = [(A control-A extract)/A control X 100]*.

The inhibitor concentration of ·OH for each extract is then calculated from the equation which determines the percentage inhibition as a function of the inhibitor concentration. This concentration is expressed in µg/ml and compared with that of ascorbic acid. The IC_{50} value is defined as the concentration of antioxidants corresponding to 50% inhibition. It is calculated by plotting the curve of the inhibition percentages as a function of the extract concentrations.

Cytotoxic effect of the extract on some cancer cell lines

Cell culture conditions

In order to assess the cytotoxic effect of the ethanolic extract, three *tumor* cell lines are used: HepG2, SH-SY5Y and Raw 264.7. These cells usually grow in a moisture-saturated atmosphere containing 5% CO_2 at 37°C and in a DMEM culture medium with a high glucose content of 4.5 mg/l. The culture medium used for the development of cancer cells contains 10% of the fetal bovine serum (FBS) inactivated for 1 hour in a water bath at 56°C. All media also contain 584 mg/l L-glutamine and 5ml streptomycin/penicillin (5000 U/ml).

Viability cell assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) test was used to measure toxicity as described above (Fetni and *al*., 2020). Briefly, the cells were seeded in 96-well plates overnight. After 72 h incubation with a range of different crude extract concentrations, the cells were rinsed with phosphate saline buffer (PBS) and incubated in 100 µl at 0.5 mg/ml MTT at 37°C. After 30 min incubation, the dark blue crystals of formazan (metabolites of MTT) are dissolved in 100 µl of DMSO and incubated at 37 °C for 30 min. The level of reduced MTT was determined by measuring the difference in absorbance at 570 nm using a BioTek Synergy HT multi-mode reader (BioTek Instruments Inc., Vermont, USA) and the acquired data is processed by BioTek's Gen5TM software (BioTek Instruments Inc.). The crude extract is generally considered to have cytotoxic activity in vitro at an IC_{50} value ≤ 20 µg/ml.

Statistical analyses

The results of in vitro tests are expressed as the mean ± standard deviation (SD). The IC_{50} values (50% effective concentration) are calculated by the linear regression method from the curve [% inhibition = f (concentrations)]. Multiple comparisons and determination of significance levels are made by the univariate ANOVA test followed by the Dunnett test. Differences are considered statistically significant at the 0.05 threshold. The comparison of means and variances is determined using the "Graphpad Prism" software version 5.0.

Results

Dosage of total polyphenols

The yield of crude ethanolic extract is around 42.15%. In order to determine the characteristics of the prepared extract, a polyphenol assay is carried out. The method used to determine total polyphenols is that of Folin-Ciocalteau (Fetni and *al*., 2020). Gallic acid is used as the standard and the total polyphenol content is determined from the linear regression equation of the calibration curve expressed in micrograms of gallic acid equivalents per milligram of extract (µg GGE/mg extract). The ethanolic extract of the fruits of *C. colocynthis* shows a total phenol content of the order of 443.62±2.13 µg GGE/mg extract.

Antioxidant activity

The evaluation of the antioxidant activity of the extract of the *C.colocynthis* fruit is carried out by three methods. These methods are based on two different antioxidant mechanisms: the free radical scavenging effect, evaluated using the two free radical scavenging tests DPPH and ·OH, and the reducing power using the iron reducing power test (Table 1).

Table 1. Antioxidant effect (IC_{50} and EC_{50}) on DPPH radical, hydroxyl radical and iron reduction of the *C. colocynthis* fruit ethanol extract.

Antioxydant activity	**Iron reduction**	**Free radical scavenging (DPPH)**	**Hydroxyl radical scavenging**
Ethanolic extract (µg/ml)	27.94	6.31	67.13
Ascorbic acid (µg/ml)	31.62	5.59	52.20

Table 2. Correlation coefficients (R) for relationships between assays.

	IC_{50} DPPH	**IC_{50} Hydroxyl Radical**	**EC_{50} Iron reduction**
Polyphenols	0.827	0.938	0.991

Free radical scavenging DPPH

The IC_{50} results presented in table 1 show that the extract has a high anti-free radical activity, close to that of ascorbic acid (6.31 µg/ml). Ascorbic acid is the most active with an IC_{50} of 5.59 µg/ml. The results of the correlation between DPPH IC_{50} and total phenols showed that they were significantly

correlated (R= 0.80 ; Table 2). This allows us to infer that the capacity of the trapped radical DPPH is mainly due to the 83 % of polyphénols.

Reducing power of iron

From the results shown in table 1, we can see that the reducing activity of iron is proportional to the concentration of the reference molecule and the extract. The values of the effective concentrations EC_{50} indicate that the extract has an EC_{50} (27.94 µg/ml) higher than that of ascorbic acid, which has an EC_{50} of 31.62 µg/ml. The results have shown a correlation between the level of total polyphenols with value of R= 0.991 (Table 2). This enables us to deduce that this capacity is due to the 99% of polyphenols.

Hydroxyl radical scavenging

The hydroxyl radical, the most well-known reagent, can attack and damage some bio-macromolecules in living cells (Yoshikawa and *al*., 2007). We have determined the values of IC_{50} concentrations to compare the potency of the hydroxyl radical scavenging activity, where the ethanolic extract has a scavenging activity of the radical ˙OH equal to 67.13 µg/ml compared to ascorbic acid which is 52.20 µg/ml (Table 1). A correlation is established between hydroxyl radical IC_{50} and the content of polyphenols (R= 0.938 ; Table 2). This allows us to infer that the capacity of the trapped radical hydroxyl is due to the 94% of polyphenols.

Cytotoxic effect on cancer cell lines

The MTT is used to evaluate the cytotoxic effect of the ethanolic extract on the three studied cancer cell lines. After 24 hours of treatment, the results obtained showed that the extract reduced the number of cancer cells with effective "IC_{50}" doses of 15.69; 4.82 and 10.72 µg/ml for HepG2, SH-SY5Y and Raw 264.7 respectively (Table 3) and (Fig. 1). The 100 µg/ml concentration reduced the number of all cancer cells very significantly ($P<0.001$). However, it is interesting to note that the concentrations of the ethanolic extract from 1 to 200 µg/ml did not have the same effect on the three tumor cell lines, whose reduction in cell number differed from one line to another. The SH-SY5Y cell line is the most sensitive to the treatment. In fact, with 3.14 µg/ml of the extract a significant reduction in the number of these cells was obtained ($P<0.001$). The results show that all the other cancerous cell lines are sensitive to the effect of 12.5 µg/ml of the extract and their numbers decreased significantly ($P<0.001$).

Table 3. IC_{50} value (µg/ml) of the *C. colocynthis* ethanolic extract.

Cancer cell lines	**IC_{50} (µg/ml)**
HepG2	15.69
SH-SY5Y	4.82
Raw 264.7	10.72

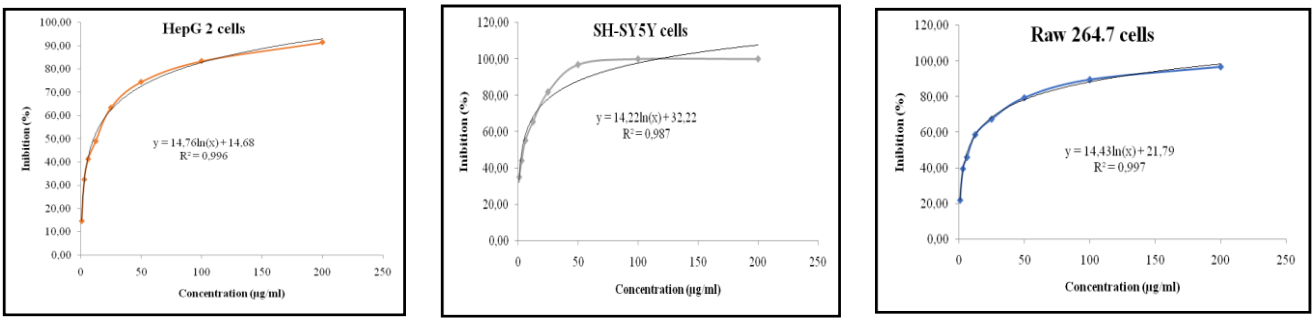

Figure. 1. Inhibition (%) of cell growth by the ethanolic extract of the fruits of *C.colocynthis* on cancer cells of the cell lines HepG2, SH-SY5Y and Raw 264.

Discussion

We contribute with our results, which have never been published, to the valorization of the *C. colocynthis* plant from eastern Algeria. This investigation is considered as the first report on its antioxidant properties related to polyphenol content. The presence of a positive correlation between the antioxidant potential and the phenolic content evaluated by the tests should be highlighted. A concept that has been proven in several works is that the high content of total phenols in the extracts and the synergistic interactions explain the strong antioxidant properties of this plant (Fetni and *al*., 2020 ; Jian and *al*., 2015 ; Marzouk and *al*., 2010 ; Negri and *al*., 2014; Shawkey and *al*., 2014 ; Yoshikawa and *al*., 2007).

The crude ethanolic extract of the *Citrullus colocynthis* fruit has a pasty greenish-brown appearance. The selection of appropriate methods and conditions for the extraction of phytoconstituents is a crucial step in the analytical process. It is well known that the yield is only relative and depends on the method and conditions for carrying out this extraction. According to our results, maceration with agitation seems to be the ideal extraction method because it allows the extraction of a relatively large quantity of phenolic compounds from the fruits of *C.colocynthis*. Also, the impact of the type of solvent on the extraction of phenolic compounds is widely discussed and the use of polar organic solvent mixtures (ethanol, methanol) with water is often recommended (Marzouk and *al*., 2010 ; Negri and *al*., 2014). The extraction efficiency is 42.15±0.13% (W/W). A yield of only 3% ethanolic extract is recovered after Soxhlet extraction (Shawkey and *al*., 2014). On the other hand, a yield of 18.02% in ethanolic extract is obtained by Soxhlet extraction for 6 hours by (Jian and *al*., 2015) (explain the difference between 3 and 6%, 6 hours). This yield is higher compared to that obtained by extraction of *C. colocynthis* fruits with chloroform (8.35%), acetone (6.55%), water (2.94%), ethyl acetate (0.93%) and petroleum ether (0.54%).

The antioxidant effects of the ethanolic extract are evaluated by three complementary methods: scavenger of DPPH and hydroxyl radicals, and FRAP. According to Koleva et *al*. (2002), polyphenols appear to be effective hydrogen donors to the DPPH radical, due to their ideal structural chemistry. The mechanism of the reaction between the antioxidant and DPPH depends on the structural conformation of the antioxidant. The name of the author(s) suggests further in vivo experiments to confirm the scavenger effect of these extracts (Roy and *al*., 2007). Studies on the relationship between the chemical structure of phenolic compounds and their free-radical scavenging power have shown that the anti-radical activity is dependent on the number, position and nature of the substituents on the B and C rings (hydroxyl, metaxyl, glycosyl groups) and the degree of polymerisation (Ali and *al*., 2013 ; Chawech and *al*., 2015 ; Seger and *al*., 2005 ; Najafi and *al*., 2010). The crud extract has shown a high antiradical efficacy with respect to the DPPH radical compared to BHT (Mahsa and *al*., 2014), which confirms the results of the present study, with an antiradical power almost 3 times greater. Results of research undertaken by Marzouk et *al*. (2011) show that the antiradical activity is not only dependent on the concentration of phenolic compounds, but also on the degree of hydroxylation and polymerization of the compounds contained in the extracts. Another study has shown that the most effective flavonoids are those containing 3', 4'-dihydroxy groups on the B ring and/or a 3-OH group on the C ring. The C2-C3 double bond in conjugation with the 4-oxo function on the C ring increases the radical scavenger capacity of the flavonoids (Abdalla and *al*., 2019 ; Archana and *al*., 2013). The presence of the 3-OH group in combination with the C2-C3 double bond also increases the scavenger effect of flavonoids. The results of the antiradical activity obtained are in agreement with those of Uma et *al*. (2014) and Marzouk et *al*. (2010).The latter found that extracts of *C. colocynthis* fruit showed very strong antioxidant and antiradical activities.

The antioxidant capacity of the extracts is also evaluated by FRAP dosing as it also shows a high reproducibility (Chen and *al*., 2005). The antioxidant reducing activity is evaluated by transforming Fe^{3+} into Fe^{2+} by giving an electron. According to the results obtained, the reducing capacity of ethanolic extract of the fruits of *C. colocynthis* compared to ascorbic acid, it can be seen that the reducing power of this extract increases with the increase in their concentrations. Furthermore, our results show that the ethanolic extract contains compounds that compete with ferrozine to chelate

ferrous iron (Fe^{+2}). The chelating capacity of a product is very important because it reduces the concentration of the transition metals that are catalysts of lipid peroxidation in vivo (Yoshikawa and *al*., 2007). Transition metals, such as ferrous iron (Fe^{+2}) can facilitate the production of reactive oxygen species in biological systems. Thus, a product's ability to chelate iron provides valuable antioxidant capacity by delaying metal-catalyzed oxidation (Adam and *al*., 2001). Iron is known as the most important pro-oxidant in lipids because of its high reactivity. Thus, chelating products offer protection against oxidative damage to cells by eliminating Fe^{+2} ions that participate in lipid peroxidation. Indeed, iron can stimulate the oxidation of lipids by the Fenton reaction, and also accelerates this oxidation by breaking down hydroperoxides into pyroxyl and alkoxyl radicals which can in turn sustain the lipid peroxidation reaction (Adam and *al*., 2001 ; Uma et *al*., 2014 ; Yoshikawa and *al*., 2007). Several studies have shown that flavonoids and phenolic acids have a remarkable chelating activity towards metal ions (Chen and *al*., 2005 ; Hussain and *al*., 2013).

This study showed that the ethanolic extract had a cytotoxic effect on all the cancer cell lines studied. However, it should be noted that the response of cancer cells differs according to the concentration of the extract and the cell line. Indeed, the SH-SY5Y cell line is the most sensitive to the treatment. At high concentration (200 µg/ml), the extract exerted a cytotoxic effect on all tumor lines. This concentration was therefore used to evaluate the anti-cancer effect. In addition, all cell lines are sensitive to the effect of 100 µg/ml of the extract. This effect is associated with the ability of flavonoids, on one hand to alter the expression of some specific genes, which can promote carcinogenesis by their demethylation at low concentrations, and on the other hand to potentially inhibit carcinogenic processes by activating the pro-apoptotic cascade at high concentrations (Abdalla and *al*., 2019 ; Archana and *al*., 2013 ; Fu and *al*., 2018 ; Santos and Silva, 2020). The decrease in cell numbers for the three studied cancer lines following their treatment with 100 µg/ml of extract could be due either to the toxic effect, where the cells burst and die by necrosis, or to the apoptotic effect where the ethanolic extract activates the cellular machinery responsible for triggering apoptosis.

According to Oszmianski et *al*, the antioxidant activities against DPPH, hydroxyl radical scavenging and iron reduction have been related to the concentration, chemical structures and degrees of polymerization of organic antioxidants (Oszmianski and *al*., 2007 ; Tannin-Spitz and *al*., 2007).

Indeed, studies have shown that plant extracts rich in phenolic compounds can stabilize transition metal ions by complexing with them. The result is that they can no longer participate in metal-catalyzed initiation. Further biological tests should be organized to determine the new and beneficial activities of these plants. For this purpose, phytochemical research should be planned to identify the active molecules and to estimate the toxicity by laboratory tests.

Conclusion

This study was devoted to evaluating the antioxidant and cytotoxic effects of a polyphenolic extract obtained from the *Citrullus colocynthis* fruit. The antioxidant effect of the extract estimated in vitro using the DPPH, hydroxyl radials scanning and reducing power tests was significantly interesting. The anti-cancer activity of the extract was explored using three tumor cell lines that represent the most responsive human cancers (HepG2, SH-SY5Y and Raw 264.7). The extract induced a significant and dose-dependent decrease in the number of cancer cells. Therefore, the extract can play the role of a preventive agent and also a curative agent after the cancer has settled. In spite of the results obtained in this study, other studies are still necessary, namely the study and identification of the different fractions of the crude extract of the *C. colocynthis* fruit in order to deepen our knowledge of the different flavonoids with these biological activities.

Author's Contributions

Fetni S. and Bertella N. conceived and designed the experiments; Fetni S. and Bertella N. performed the experiments; Fetni S. and Bertella N. analyzed the data; Fetni S. and Bertella N. wrote the paper. All authors read and approved the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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