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Antiproliferative effect on human cancer cells and antioxidant activity of Tunisian *Citrullus colocynthis* Schrad. immature fruit and seed organic extracts.

Belsem Marzouk¹, Francesca Mussi^{2,3}, Serena Galati², Nadia Fenina⁴, Annamaria Buschini², Zohra Marzouk⁴

1. Laboratory of Transmissible Diseases and Bio-active Substances, Faculty of Pharmacy of Monastir; University of Monastir; Tunisia.

2. Department of Biosciences; University of Parma, Italy.

3. Departement of Life Sciences; University of Modena and Reggio Emilia; Italy.

4. Laboratoire of Genome, Diagnostic and Valorisation; Faculty of Pharmacy of Monastir; Tunisia.

ABSTRACT

Citrullus colocynthis Schrad., traditional Tunisian medicinal plant, showed beneficial effects against oxidative stress mediated diseases, namely its fruits and seeds which contain several compounds with biological activity. The present study reports the antioxidant and the antiproliferative properties of different seed and fruit organic extracts. Antioxidant activity was assessed by the ability to quench the free DPPH and the superoxide anion radicals and inhibit the ABTS cation. Methanol extracts presented the highest DPPH scavenging (seeds IC₅₀ = 0.178 mg/ml; fruit IC₅₀ = 0.223 mg/ml) and superoxide scavenging (seeds IC₅₀ = 28.102 µg/ml; fruits IC₅₀ = 30.793 µg/ml) activities. All extracts inhibited the ABTS radical formation. The most interesting TEAC values were registered with methanol extracts (seeds = 1.225 and fruits = 1.120). Petroleum ether seed extract showed the lower antioxidant action. Seed and fruit organic extracts were also tested for their antiproliferative activity on HT-29 human cell line. All extracts induced a concentration dependent growth inhibition. Petroleum ether seed extract exhibited the higher growth inhibition activity (IC₅₀ = 321 µg/ml), while methanol fruit extract showed the less antiproliferative efficiency (IC₅₀ >500 µg/ml). Data obtained indicate that seeds and fruits constitute an excellent source of effective natural antioxidants and chemopreventive agents.

Keywords: *Citrullus colocynthis* ; phenolic contents; antioxidant; antiproliferative.

*Corresponding Author Email: belsemmarzouk@yahoo.fr

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INTRODUCTION

Nowadays, the interest in exploring the antimutagenic potential of medicinal plant constituents is increasing. In particular, antioxidant and chemoprotective agents are sought as they offer some degree of protection against environmental mutagens and carcinogens, that represents one of the most important potential causes of increased incidence of cancer across the globe (Kaefer and Milner, 2008)¹.

One of the plants that was believed to possess potential anticancer activity and is currently under investigation in our laboratory is *Citrullus colocynthis* Schrad. (family: Cucurbitaceae). This species is widely distributed throughout the world and known to the Tunisian as “Handhal”, “Hdaj”, or “Dellaa el wed” (Marzouk *et al.*, 2009)². The plant has been used by practitioners of traditional medicine in Arab countries to treat various types of ailments such as inflammations and infections (Marzouk *et al.*, 2010)³. At the scientific level, many *Citrullus colocynthis* extracts have been shown to possess anti-tumour (Tannin-Spitz *et al.*, 2007)⁴, immunostimulant (Bendjeddou *et al.*, 2003), anti-microbial (Marzouk *et al.*, 2009)², antioxidant (Marzouk *et al.*, 2010)³ properties and they are efficient against hepatic diseases (Gebhart, 2003)⁵, hyperglycaemia (Al-Gaithi *et al.*, 2004)⁶ and hair loss (Roy *et al.*, 2007)⁷. These biological and pharmacological activities of *Citrullus colocynthis* could be related to different bio-active substances such as cucurbitacins: A, B, C, D and I (Adam *et al.*, 2001)⁸ which are known to exhibit potent biological properties (Tannin-spitz *et al.*, 2007)⁴. The aim of the present study was to determine the *in vitro* antioxidant activity of the petroleum ether, chloroform, ethyl acetate, acetone and methanol extracts of *Citrullus colocynthis* seeds and fruits using the DPPH radical scavenging, the superoxide scavenging and the ABTS cation assays; furthermore their antiproliferative effectiveness is evaluated on HT-29 human cell line by MTS assay.

MATERIAL AND METHOD

Plant materials

Citrullus colocynthis Schrad. plants were collected in August 2007 near Medenine, Tunisia in the municipality of Sidi Makhoulouf (33°33N, 10°27W). The identification was performed according to the flora of Tunisia (Pottier-Alapetite, 1981) and a voucher specimen (C.C-01.01) deposited in the biological laboratory of the Faculty of Pharmacy of Monastir.

Chemical and drugs

The organic solvents used were 99 % pure. All the chemicals were obtained from Sigma (St. Louis, MO, USA).

Extraction protocol

Fresh materials (seeds and fruits) were dried and powdered using a tissue blender. Different solvents, in ascending polarity (petroleum ether, chloroform, ethyl acetate, acetone and methanol) were used for Soxhlet extraction to fractionate the soluble compounds from the plant material. The extraction was performed with dried powder (100 g) placed inside a thimble made by thick filter paper, loaded into the main chamber of the soxhlet extractor, which consisted of an extracting tube, a glass balloon and a condenser. The total extracting time was 6 h for each solvent continuously refluxing over the sample at a temperature not exceeding the boiling point. The resulting extracts were evaporated at reduced pressure to obtain the crude extracts. Yields are calculated according to the formula: Yield Extract = ((Weight Plant material – Weight Extract) / Weight Plant material) x 100.

Determination of the total phenolic contents

Phenolic compounds concentration in the different extracts was determined by using the Folin–Ciocalteu’s phenol reagent, according to a previously described procedure (Singleton and Rossi, 1965)⁹, with some modifications. Briefly, 100 µl of the extract solution was mixed with 100 µl of Folin–Ciocalteu’s phenol reagent. After 3 min, 100 µl of saturated sodium carbonate solution was added to the mixture and adjusted to 1 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 720 nm. Gallic acid was used for constructing the standard curve. The contents of total phenolics are expressed as mg of gallic acid equivalents (GAE)/g of extract. Data were reported as means ± SD for at least three replications.

Determination of DPPH radical scavenging activity

The ability to scavenge the DPPH free radical was monitored according to a method first introduced by Blois (1958)¹⁰ and developed by Brand-Williams *et al.*, (1995)¹¹. Various concentrations of sample extracts (0.5 ml) were mixed with 0.5 ml of methanolic solution containing DPPH radicals (6 x 10⁻⁵ M). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discolouration using the equation: % scavenging effect = [(ADPPH x AS)/ADPPH] x 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. Three experiments were performed in triplicate. The antiradical activity was expressed in terms of the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50%

(IC50). The IC50 value for each extract was determined graphically by plotting the percentage of DPPH scavenging as a function of extract concentration.

Superoxide anion scavenging activity

The superoxide anion radical scavenging activity was performed using the method adopted by Jung *et al.*, (2006)¹². The superoxide radicals were generated *in vitro* by the hypoxanthine/xanthine oxidase system. The scavenging activity of the extract is determined by the nitro blue tetrazolium (NBT) reduction method. The sample solution (0.1 mg.ml⁻¹) in 5% DMSO was added to 1 ml of a mixture of 0.1 mM xanthine and 0.2 mM NBT in a 50-mM potassium phosphate buffer (pH 7.5) containing 0.05 mM ethylenediamine tetra-acetic acid. Xanthine oxidase (0.1 ml) (0.8 unit.ml⁻¹) diluted in 50 mM phosphate buffer (pH 7.5) was added, and the resulting mixture was incubated at 37 °C for 20 minutes. The addition of 2 ml of 2.5 N HCl to the mixtures terminated the reaction, followed by increase of coloration of NBT, which was measured at 540 nm. The percent of removal rate by sample was calculated relative to the control and results are expressed as IC50.

ABTS+ assay

This assay was carried out according to the procedure described by Re *et al.*, (1999)¹³. Tested materials were dissolved and diluted with ethanol. After adding the test solution to ABTS+ solution having absorbance of 0.70 ± 0.02 at 734 nm, absorbance was recorded up to 20 min in 5 min intervals. Results were expressed as Trolox equivalent antioxidant capacity (TEAC) only at 20 min and for a concentration of 1 mg/ml. TEAC is defined as the mM concentration of a Trolox solution whose antioxidant activity is equivalent to the activity of 1.0 mM test solution. In order to find TEAC values, a separate concentration response curve for standard Trolox solutions was prepared.

Antiproliferative assay

The antiproliferative activities of fruit extracts was measured by MTS assay (Promega, Madison, WI) (Cory *et al.*, 1991)¹⁴. Human colon adenocarcinoma cell line HT29, obtained from the American Type Culture Collection. Cells (ATCC, Rockville, MD), was maintained in 25 cm³ flasks with 10 ml of Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 0.5 % penicillin/streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were seeded (5x10⁴ cell/ml) in 96-well flat-bottom plates. After 24 h of incubation, HT-29 cells were treated, in quadruplicate, with increasing concentrations of seed and fruit extracts or negative control and incubated for 24h; 20 µl of MTS solution were added directly to culture wells and after 4h of incubation the absorbance at 450 nm

with a 96-well plate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland) was recorded.

Statistical analysis

The data were analyzed using the statistical and graphical functions of SPSS18 (SPSS Inc., Chicago, IL, USA). Differences were assessed using analysis of variance (ANOVA), followed by Bonferroni's post-hoc test as appropriate. Significance was accepted at $p < 0.05$ level.

RESULTS AND DISCUSSION

Extraction yields

Yields of prepared extracts are given in Table 1. Petroleum ether extraction gave the worse yield for fruits and the better one for seeds. For this last organ, the lowest yield is obtained for chloroform extraction. The highest yield is noted for fruit methanol extract.

Table 1. Yields (%) of *Citrullus colocynthis* seed and fruit extracts.

	P.E.		Chl.		E.A.		A.		M.	
	Seeds	Fruits								
Yields	8.96	0.54	0.71	8.35	0.08	0.93	4.74	6.55	6.83	18.02

P.E.: petroleum ether extract; Chl.: chloroform extract; E.A.: ethyl acetate extract; A: Acetone extract; M.: Methanol extract.

Total phenolic contents

Citrullus colocynthis Schrad. seeds and fruits contains several active constituents, especially polyphenols (Marzouk *et al.*, 2009). Total phenolic contents of the tested extracts are shown in Figure 1. As expected, methanolic extracts obtained from seeds and fruits presented a higher phenolic content than the other ones. Total phenolic contents of the tested extracts are shown in Figure 1. As expected, methanolic extracts obtained from seeds and fruits presented a higher phenolic content than the other ones. This fact correlates with the remarkable differences in the polarity of the extraction solvents used and the solubility of phenolic compounds in them. Methanol is considered as one of the best solvents for phenolic extraction (Bruneton, 1999)¹⁵. Among methanolic extracts, seeds presented the highest total phenolic content (mean value of 558 mg of GAE/g of extract), followed by fruits (292.166 mg of GAE/g of extract). While the non-polar extracts namely petroleum ether extracts are very poor in phenolic compounds (49.666 mg of GAE/g of seed extract and 18.666 mg of GAE/g of fruit extract) mainly enriched in no-polar constituents such as tocopherols and tocotrienols, sterols and fatty acids.

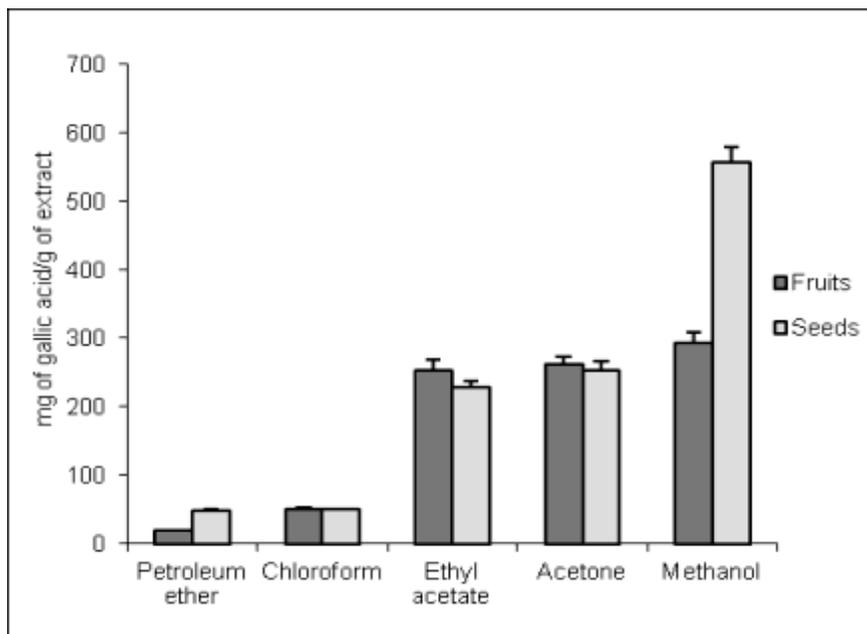


Figure 1. Total phenolic contents of organic extracts from *Citrullus colocynthis* seeds and fruits.

Antioxidant and anti-proliferative activities

Figures 2, 3 and 4 show the antioxidant activities of the petroleum ether, chloroform, ethyl acetate, acetone and methanol extracts of *Citrullus colocynthis* seeds and fruits assessed using the DPPH radical and superoxide scavenging tests and also the ABTS radical assay.

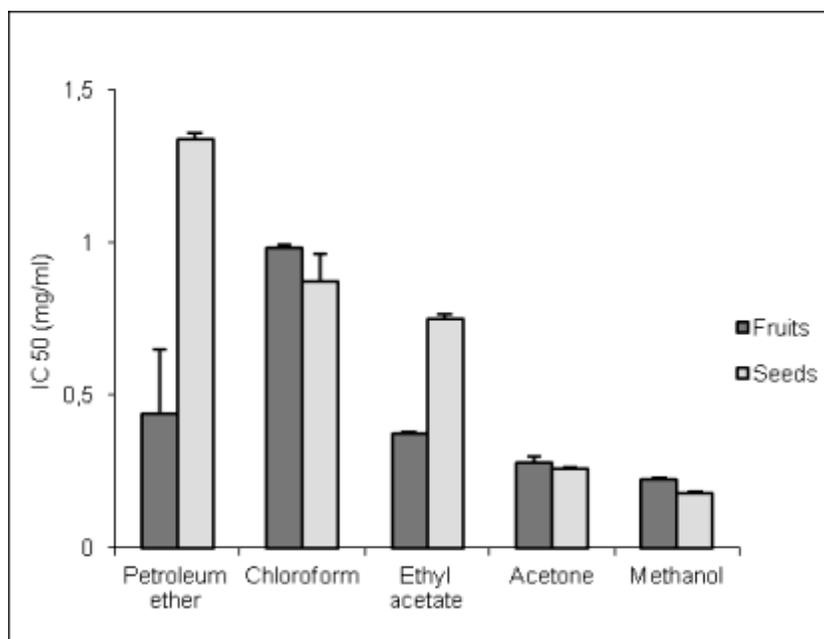


Figure 2. DPPH scavenging activity of organic extracts from *Citrullus colocynthis* seeds and fruits expressed as IC50.

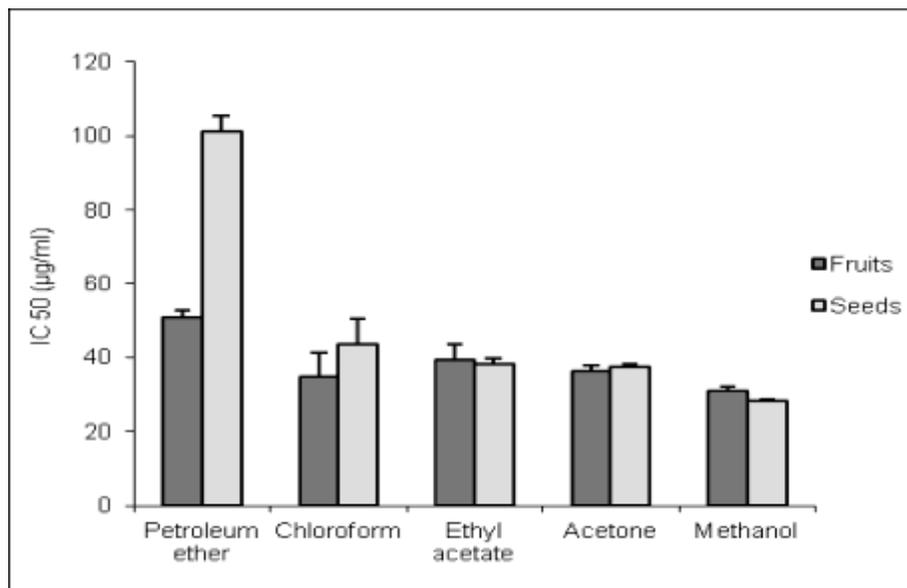


Figure 3. Superoxide scavenging activity of organic extracts from *Citrullus colocynthis* seeds and fruits expressed as IC₅₀.

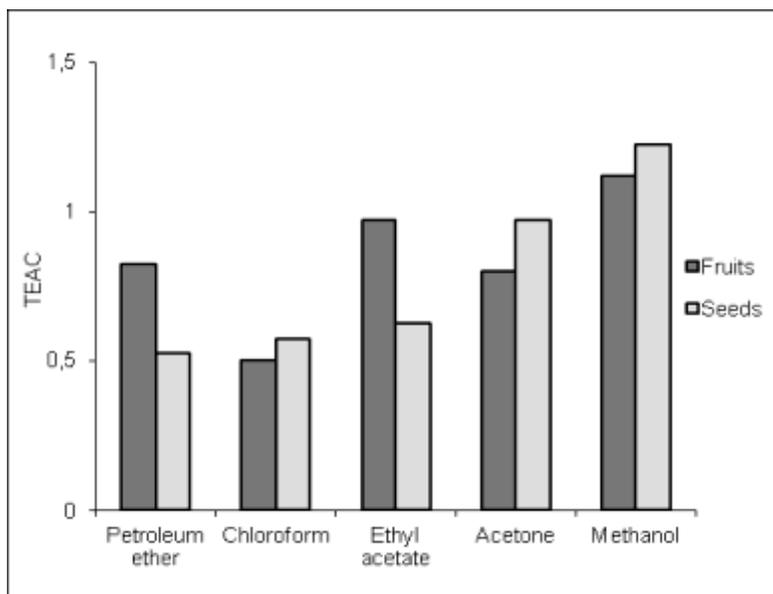


Figure 4. TEAC values of *Citrullus colocynthis* seed and fruit extracts.

All extracts produced a DPPH radical scavenging activity. Polar extracts showed the high antioxidant effects. Ethyl acetate extracts produced a DPPH radical scavenging activity with an IC₅₀ of 0.747 mg/ml and 0.372 mg/ml, a superoxide scavenging effects with an IC₅₀ of 38.022 µg/ml and 39.503 µg/ml and TEAC values of 0.625 and 0.975, respectively for seeds and fruits. While acetone extracts produced a DPPH radical scavenging activity with an IC₅₀ of 0.261 mg/ml and 0.281 mg/ml, a superoxide scavenging effects with an IC₅₀ of 37.293 µg/ml and 36.344 µg/ml and TEAC values of 0.975 and 0.800, respectively for seeds and fruits. Methanol extracts

exhibited the high antioxidant activity of all producing a DPPH radical scavenging activity with an IC₅₀ of 0.178 mg/ml and 0.223 mg/ml, a superoxide scavenging effects with an IC₅₀ of 28.102 µg/ml and 30.793 µg/ml and TEAC values of 1.225 and 1.120, respectively for seeds and fruits. Petroleum ether seed extract showed the less activity on scavenging the DPPH (IC₅₀ = 1.339 mg/ml), the superoxide anion (IC₅₀ = 101.187 µg/ml) and the ABTS radicals (TEAC = 0.525).

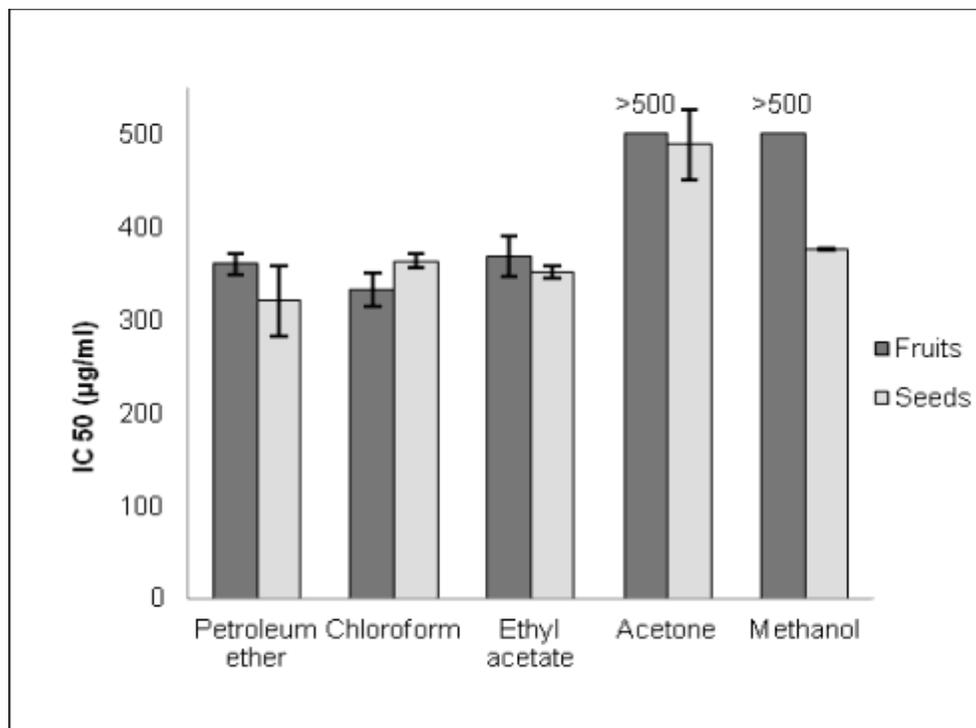


Figure 5. Antiproliferative effect of *Citrullus colocynthis* seed and fruit extracts on HT-29 cancer cells.

HT-29 cell proliferation was significantly inhibited by *Citrullus colocynthis* seeds and fruit extracts in a dose-dependent manner. Antiproliferative activity was expressed as concentration able to inhibit 50% cells growth (IC₅₀). Based on the IC₅₀ values (Figure 5), the inhibitory effects of the seed extracts on HT-29 cell proliferation are in the decreasing order: acetone extract (489 µg/ml) > methanol extract (374 µg/ml) > chloroform extract (363 µg/ml) > ethyl acetate extract (351 µg/ml) > petroleum ether extract (321 µg/ml).

Regarding the inhibitory effects of the fruit extracts on HT29 cell growth, the chloroform extract and the petroleum ether one had the highest inhibitory activity with an IC₅₀ value of 332 µg/ml and 360 µg/ml respectively. On the contrary, the methanol and the acetone extracts had a less antiproliferative activity with an IC₅₀ >500 µg/ml.

Some cancer cells live under oxidative stress because this condition increases their potential to survive by activating redox signaling that may lead to the activation of prosurvival factors such as

NFkB and AP-1 (Ripple *et al.*, 1999; Chen *et al.*, 2008; Yang *et al.*, 2012)^{25,26,27} and the inactivation of tumor suppressor genes such as p53 (Sun and Oberley, 1996; Striteskà, 2005)^{16,17} or mutations (Shinkai and *al.*, 1986; Lozano, 2007; Szabova *et al.*, 2012)^{18,19} In addition, mild levels of reactive oxygen species have been shown to induce the proliferation of cancer cells (Emin *et al.*, 2009)²⁰. Many *in vitro* and *in vivo* studies demonstrated that some molecules (*i.e.* polyphenols, catechins, *etc.*) present in food are anti-carcinogenic by inducing apoptosis and inhibiting cell-growth. Probable mechanisms of action include antioxidant and free-radical scavenging activity. Epidemiologic studies suggest that the consumption of a phytochemical-rich diet could be associated with a reduced risk of cancer. Plants contain high levels of several phytochemicals, which include polyphenols like flavonoids, hydrolysable, and condensed tannins and phenolic acids (Lu *et al.*, 2011)²¹. In particular, polyphenols have been shown to exert anticancer effects via mechanisms that include antioxidant and antiproliferative activities (Kaefer and Milner, 2008)¹.

CONCLUSION

The present study has revealed the potential antiproliferative and antioxidant properties of the various extracts of *Citrullus colocynthis* seeds and fruits. Methanol extracts showed the highest antioxidant activity, while non-polar extracts (petroleum ether from seeds and chloroform from fruits) presented the most important antiproliferative action. High levels of phenolic content are present in the methanol extract and in trace in the non polar extracts. Extracts with high total phenolic content, including the acetone and methanol ones, were found to exhibit high antioxidant capacity. It is plausible to suggest that the phenolic contents of those extracts contribute directly or indirectly to the obtained antioxidant activity. We have also studied the antiproliferative activity and it is believed that the inhibition of cancer cell proliferation by *Citrullus colocynthis* seed and fruit extracts was not correlated with their total phenolic content. In particular, the polar extracts seem to present the most effective antiproliferative activity. This activity could be related to different molecules from cucurbitacins that usually enrich the chloroform extracts (Tannin-Spitz *et al.*, 2007). There is an increasing consent that the health benefits of vegetable product mixtures could not be related to single components, since they involve synergistic, additive and antagonistic effects (Liu, 2003; Mertens-Talcott *et al.*, 2005; Kurin *et al.*, 2012)^{22,23,24}. Further studies are ongoing to understand if the antiproliferative detected is due mainly to a specific constituent of the extract or to a mixture of biologically active molecules. *Citrullus colocynthis* Schrad. should be an inexpensive and easily accessible source of effective natural antioxidants and chemopreventive agents, and future clinical investigations on this medicinal plant should be encouraged.

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