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ORIGINAL PAPER

Phytochemical Composition and Antitumor Activities of New Salad Greens: Rucola (*Diplotaxis tenuifolia*) and Corn Salad (*Valerianella locusta*)

R. P. Ramos-Bueno[1] · **M. A. Rincón-Cervera**[2] · **M. J. González-Fernández**[1] · **J. L. Guil-Guerrero**[1]

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Abstract *D. tenuifolia* and *V. locusta*, two greens, were analyzed for active compounds and antitumor actions on colorectal cancer cells. Phenolics were determined by UHPLC-Orbitrap-MS; carotenoids and glucosinolates by HPLC-MS; and sterols and fatty acids by gas–liquid chromatography (GLC). For antitumor effects, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) tests were run on HT-29 colorectal cancer cells, and in CCD-18 untransformed enterocyte cells. Six main carotenoids were identified in both vegetables, while total carotenoids accounted for 3520 and 2970 $\mu g \cdot g^{-1}$ dry weight in *D. tenuifolia* and *V. locusta*, respectively. Six phenolics were detected in *D. tenuifolia* (68,600 $\mu g \cdot g^{-1}$ dry weight) and five in *V. locusta* (139,000 $\mu g \cdot g^{-1}$ dry weight). Three glucosinolates (GSL) were found in *D. tenuifolia* (1960 $\mu g \cdot g^{-1}$ dry wt. total). Low-polarity extracts from *V. locusta* and *D. tenuifolia* showed $IC_{50} \sim 150$ and ~200 $\mu g \cdot mL^{-1}$ on HT-29 cells, while both plants lacked actions on CCD-18 cells. *V. locusta* inhibited HT-29 cancer cells viability more efficiently than *D. tenuiofolia*, but induced less cytotoxicity. This work highlights the importance of functional foods for colorectal cancer prevention.

Keywords Bioactive compounds · Cytotoxicity · *Diplotaxis tenuifolia* · HT-29 colorectal cancer cells · *Valerianela locusta*

Abbreviations

4-MSB	4-methylsulphinylbutyl (glucoraphanin)
AIF	All-ion fragment
ALA	α-linolenic acid
APCI	Atmospheric pressure chemical ionization
BSTFA	Tris (2-carboxyethyl) phosphine hydrochloride bis-(trimethylsilyl) trifluoroacetamide
EFA	Essential FA
ESI	Heated electrospray interface
FA	Fatty acid
FBS	Fetal bovine serum
FID	Flame ionization detector
GSL	Glucosinolate
LA	Linoleic acid
LDH	Lactate dehydrogenase
MTBE	Methyl tert-butyl ether
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PUFA	Polyunsaturated fatty acid

Introduction

Food preferences in Western societies have recently shifted towards healthier options in contrast to previous high caloric diets inherent to processed foods. This way, new leafy vegetables are being consumed worldwide in salads and cut-fresh products [1]. Leaves of *Valerianella locusta* L. (Valerianaceae) and *Diplotaxis tenuifolia* L. (Cruciferae), more commonly known in Europe as corn salad and rocket or rucola respectively, are among the most representative

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✉ J. L. Guil-Guerrero
jlguil@ual.es

[1] Food Technology Division, University of Almería, Almería, Spain

[2] Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile

vegetables of this trend [2]. Both species have been reported to have a positive influence on certain diseases such as diabetes, cardiovascular disorders and cancer [1]. Moreover, many studies show that cruciferous vegetables usually display strong inhibitory effects on several cancer types. Specifically, a diet rich in Brassica vegetables such as *Eruca sativa* has been related to prevent specifically colon carcinogenic processes [3]. *E. sativa* is a green which is commonly marketed and consumed together with *D. tenuifolia*, and both are known under the common name of rucola. The relationship between vegetables consumption and lower risk of suffering some diseases seems to be related to certain bioactive compounds they contain, such as carotenoids, phenols, glucosinolates (GSL) and fatty acids (FAs) [4].

Unlike other related species, a high lipid content has been reported in *D. tenuifolia* [5], and studies indicate suitable percentages of n-3 polyunsaturated FAs (PUFAs) in leaves of green vegetables [6], whose effects comprise the prevention of a large number of diseases such as cancer, inflammatory disorders and coronary heart disease [7]. Carotenoids are also powerful antioxidant molecules inherent to vegetables, whose consumption is related to the prevention of several cancer types, such as colorectal ones [3]. Lutein, which has been related to the prevention of several eye disorders, is the most abundant carotenoid in leafy vegetables, being β-carotene and β-cryptoxanthin also relevant due to their important role as protective agents and that they are metabolized to vitamin A [8]. Among phytochemicals, sterols have been shown to exert a beneficial influence on several health disorders such as heart diseases and colon cancer cell proliferation [9]. They are obtained mainly from fruits and vegetables, being β-sitosterol, stigmasterol and campesterol found in higher amounts than others [9].

Phenolic compounds possess important antioxidant and other potential health-promoting effects, including anti-inflammatory, antimicrobial and antitumor activities [10]. There are strong evidences pointing out that some flavonoids such as quercetin, kaempferol and isorhamnetin are responsible of the protective effects associated to these compounds [11]. Furthermore, glucosinolates (GSLs) have shown beneficial effects on human health. They are secondary metabolites with protective effects against several cancer types including colorectal cancer through the regulation of carcinogen-metabolizing enzymes [3, 12]. For instance, glucoraphanin (4-MSB) is hydrolysed after tissue disruption by the enzyme myrosinase to yield sulforaphane as its breakdown product, which is believed to exhibit beneficial effects on human health through a variety of mechanisms, being considered as a promising natural anticancer compound [12]. GSLs have been cited in *D. tenuifolia* [13] but not in *V. locusta* [14].

This work was designed to characterize the bioactive composition of two recently introduced greens in our diets, *D. tenuiofolia* and *V. locusta*. This paper constitutes the first report about the FAs and sterols composition of the leaves from both species. In addition, the antitumor effects of both plants on colorectal cancer cells, which is unknown for *V. locusta* and only partially established for *D. tenuifolia*, have been assessed.

Material and Methods

Samples *V. locusta* and *D. tenuifolia* were purchased in local markets in Almería (Spain). Leaves of the two vegetables were washed with cold water and freeze-dried (LyoQuest, Telstar) for 48 h. Then, dried leaves were ground and kept at −70 °C until they were used for further processing.

Moisture The moisture content was determined by drying 5 g of sample in an air circulation oven at 105 °C until constant weight (24–48 h).

Fatty Acids Analyses Prior to derivation, 500 mg of freeze-dried sample of each leafy vegetable was treated with 7 mL of ethanolic sodium hydroxide (10 %, *w*/*v*) and this solution was maintained at 82 °C for 2 h, with constant stirring. Thereafter, the saponified solution was cooled at 4 °C and washed with water, removing by decantation the ether upper layer. Then, the soaps were acidified to pH 1 with HCl/H_2O 1:1 (*v*/*v*) and FAs were extracted with n-hexane (3 × 10 mL), and the hexane was evaporated. Derivation to FA methyl esters (FAMEs) and gas liquid chromatography (GLC) analyses were carried out as previously reported [15].

Carotenoids Extraction and analyses were accomplished by means of HPLC-MS [15].

Phenolics Phenolic compounds were extracted following previous methodology [16]. Analyses were carried out by using a UHPLC-Orbitrap-MS as previously described [10].

Sterols Samples were processed according to previous reports [17], and analyzed using a Focus GLC (Thermo Electron, Cambridge, UK) equipped with a flame ionization detector (FID) and an Omegawax 250 capillary column (30 m X 0.25 mm i.d. X 0.25 μm film thickness; Supelco, Bellefonte, USA). The column temperature was programmed from 150 to 260 °C at 6 °C min^{-1}, then to 300 °C at 2.5 °C min^{-1} and constant temperature at 300 °C for 7 min. Nitrogen was used as carrier gas (1 $mL \cdot min^{-1}$). A 50:1 split ratio was programmed, and injection volume was 4 μL. Temperatures in the injection port, transfer line and detector were set at 260, 280 and 220 °C, respectively. Sterols were identified using commercial standards, and 5α-cholestane was used as internal standard for quantification purposes.

Glucosinolates (GLS) Sample extraction was carried out according to previous work [18]. Analyses were performed by HPLC-MS; a Hewlett-Packard HP11100 with a C18 Phenomenex Luna column (250 × 4.6 mm, 5 μm particle size) was used, as well as a mobile phase based on acetonitrile (eluent A) and a water solution of ammonium formate 50 mM with formic acid pH 3.5 (eluent B) at a flow rate of 1 mL · min^{-1}. Injection volume was set at 20 μL. The gradient method for GSLs separation was carried out as follows: t = 0 min 90 % B; t = 30 min 0 % B; t = 35 min 0 % B; t = 40 min 90 % B; t = 45 min 90 % B.

The drying gas flow was 6 L · min^{-1}, the nebulizer pressure was 40 psig, the drying gas temperature was 325 °C, the vaporizer temperature was 450 °C, the capillary voltage was 2500 V and the corona current was 3 mA. The interface between the LC and MS was APCI (atmospheric pressure chemical ionization) positive (fragmentor 100 V). Peak identification was based on comparison of HPLC retention times and mass spectra with chemical standards of glucosativin, glucoraphanin and glucoerucin. These compounds were identified by their UV spectra, molecular weight and their characteristic m/z fragments. Standard solutions were used for calibration with quantification purposes.

Cell Assays Freeze-dried samples were extracted by using with two different solvent systems, a mixture of distilled water-absolute ethanol (1:1, *v*/*v*) and a mixture of chloroform-methanol (1:1, *v*/*v*) using a sample:solvent ratio of 1:10 (*w*/*v*) [19].

Cell viability and selectivity were assessed using two cell cultures, the HT-29 colon cancer cell line and the CCD-18 untransformed colon fibroblast cell line, which were supplied by the Technical Instrumentation Service from the University of Granada (Spain). Cell cultures and MTT assay were accomplished as detailed in previous works [19].

Lactate dehydrogenase (LDH) assay (Cytotoxicity Detection Kit PLUS, Roche, Mannheim, Germany) was carried out using similar culture conditions than in the MTT assay, to allow a comparative study between both tests. A lower cell density (5×10^3 cell/well) was determined in preliminary experiments to improve the procedure on cells HT-29. Cell treatment in the LDH assay was the same as in the MTT assay until the steps of the addition of extracts and the incubation (48 h). Data were acquired according to manufacturer's instructions. Results of cytotoxicity were quantified by measuring the absorbance at 450 nm with a reference filter at 690 nm. A "high control" was used to estimate the total LDH content, treating cells with lysis solution to release all LDH. The percentage of cell death was calculated using the following equation:

$$\%\,\text{of LDH activity} = \frac{\text{exp. value-low control}}{\text{high control-low control}} \times 100$$

All parameters are based on absorbance data from samples and controls by triplicate. Low control determined the LDH activity released from the untreated cells; high control determined the maximum releasable LDH activity in the cells and experimental value (exp. value) determined the LDH activity released from treated cells with the plant extracts. Assayed extract concentrations ranged from 12.5 to 1000 μg · mL^{-1}.

Different tested extracts as well as negative controls for MTT and LDH assays were evaluated in independent tests, being the results reported as mean ± S.D.

Statistical Analysis Data in tables and figures are expressed as the average ± SD of the analysis of five different sampled leaves of each variety analyzed in triplicate. All values are presented as mean ± SD. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) followed by assessment of differences using STATGRAPHICS Plus version 5 (Statistical Graphic Corp., Warrenton VA, USA.

Results and Discussion

Moisture Content

Moisture contents of *D. tenuifolia* and *V. locusta* were 941 and 934 g · kg^{-1}, respectively, values in agreement with previous ones [20].

Carotenoids Content

The following carotenoids were identified in both vegetables: neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene (Table 1, Online Resource 1), which eluted in order of decreasing polarity from polar oxycarotenoids to lipophilic hydrocarbons. Neoxanthin was the first detected carotenoid followed by violaxanthin, lutein and zeaxanthin. Then, β-cryptoxanthin eluted at 20.35 min and finally β-carotene at 30.25 min. Total carotenoid was 3520 and 2970 μg · g^{-1} dry weight (dw) in *D. tenuifolia* and *V. locusta*, respectively. References reporting the carotenoid profile of *D. tenuifolia* are very scarce; Žnidarčič et al. [21] indicated a value of 1030 μg · g^{-1} dw for *D. tenuifolia* leaves, which is lower than that found in this work. However, large differences are found for carotenoid content even in works reporting values for *E. sativa* (Table 1, Online Resource 1) due to different factors such as climate conditions, nutrient intake and growth practices among others, making difficult the comparison among studies. *V. locusta* showed also intermediate figures in relation to other ones previously reported (Table 1, Online Resource 1). The main contributor to both carotenoid profiles was neoxanthin, while β-carotene was at the bottom of the range. However, other works reported lutein as the most

prominent carotenoid in both vegetables (Table 1, Online Resource 1). Again, differences in carotenoid profiles can be related to the multiple conditions that affect the phytochemical content of vegetables, such as plant variety, soil type, agronomic treatments, storage variables, post-harvest treatments, and the employed methodology for the quantification of the target analytes [8]. Given that lettuce, which is the most common vegetable consumed as salad, provides 1230 $\mu g \cdot g^{-1}$ dw (Table 1, Online Resource 1), it is shown that in comparison, both greens assessed in this work become more suitable options as salad ingredients regarding carotenoids content.

Phenolic Compounds

Phenolic profiles for *V. locusta* and *D. tenuifolia* are reported in Table 2 (Online Resource 2). Total phenolics accounted for 68,600 and 139,000 $\mu g \cdot g^{-1}$ dw in *D. tenuifolia* and *V. locusta*, respectively. Total amounts were in good agreement with previous works in the case of *D. tenuifolia*, and higher for *V. locusta* (Table 2, Online Resource 2). Six phenolic compounds were detected in *D. tenuifolia* leaves: chlorogenic acid, rutin, luteolin, tamarixetin, isorhamnetin-3-O-glucoside, and quercetin-3-0-galactoside/quercetin-3-0-glucoside, being rutin in the upper range (41,100 $\mu g \cdot g^{-1}$ dw). Kaempferol-3-O-rutinoside, isorhamnetin-3-O-glucoside and quercetin-3-0- glucoside have been previously identified in this species (Table 2, Online Resource 2).

Five phenolic compounds were detected in *V. locusta* leaves: chlorogenic acid, rutin, luteolin, kaempferol-3-O-rutinoside and genistein, with chlorogenic acid in the top with 116,100 $\mu g \cdot g^{-1}$ dw. The remaining quantified compounds represent only 16.5 % of the total amount. Using again lettuce as a reference green due to its large use in salads, it can be noted that phenolics of both analyzed species were much higher than those found in lettuce (Table 2), both in quantity and diversity, therefore an increased consumption of these crops may be linked with long-term, decreased incidences of several chronic diseases.

Sterol Composition

Sterol composition for both greens is shown in Table 3 (Online Resource 3). Four compounds were detected and quantified. Total sterols accounted for 5140 and 10,480 $\mu g \cdot kg^{-1}$ dw in *D. tenuifolia* and *V. locusta*, respectively. Three of them -campesterol, stigmasterol, and β-sitosterol- are usually found in commonly consumed vegetables [22]. β-sitosterol was the predominant sterol in both vegetables (3730 and 9550 $\mu g \cdot kg^{-1}$ dw in *D. tenuifolia* and *V. locusta*, respectively), while stigmastanol was quantified at the bottom of the range (50 $\mu g \cdot kg^{-1}$ dw). Sterols amounts found in both vegetables were somewhat lower than those reported for other vegetables such as celery [22]. However, *V. locusta* contains high amounts of β-sitosterol, which possess the highest anticancer activity among all sterols [24].

Fatty Acids

FA compositions are shown in Table 4 (Online Resource 4). α-linolenic acid (ALA, 18:3n-3) was the most abundant FA, accounting for 48.5 and 57.4 % of total FAs in *D. tenuifolia* and *V. locusta*, respectively. This PUFA belongs to the n-3 family and is considered an essential FA (EFA), being reported its protective effect against certain types of human cancer [25]. n-3 PUFAs are also involved in the biosynthesis of eicosanoids with anti-inflammatory activity [12]. Other main FAs found in *D. tenuifolia* and *V. locusta* were linoleic acid (LA, 18:2n-6), with 10.6 and 12.0 %, and palmitic acid (PA, 16:0), with 16.4 and 11.7 %, respectively. A peak compatible with heptadecenoic acid (17:1) was found at 17.69 min retention time (Rt) in the chromatogram of *D. tenuifolia* and *V. locusta* leaves. However, although this signal came from a compound contained in a saponified extract and thus it has a FA structure, such amount for this FA in greens is unusual, and it could be due to any unidentified branched or hydroxilated FAs [26] thus we classified this signal as "unidentified".

Both analyzed greens showed good percentages of n-3 PUFA, comparable to other figures found in lettuce (Table 4, Online Resource 4), and an n-6/n-3 ratio lower than that of the latter; therefore, the intake of the species studied in this work might provide derived n-3 health benefits to a greater extent than lettuce [7]. It highlights that both greens contain almost three times the amounts of FAs found in lettuce (Table 4, Online Resource 4).

Glucosinolates (GSLs)

GSLs found in *D. tenuifolia* are shown in Table 5 (Online Resource 5). Three GSLs were detected and quantified by HPLC-MS: glucoraphanin, glucosativin and glucoerucin. These compounds have been previously identified as the most prominent GSLs in *D. tenuifolia* and *E. sativa* leaves (Table 5, Online Resource 5). The most abundant GSL found in this work was glucosativin (1030 $\mu g \cdot g^{-1}$ dw), accounting for 52.4 % of total GSLs. Total GSLs detected in this work (1960 $\mu g \cdot g^{-1}$ dw) was in good agreement with results revealed by Pasini et al. [23] (Table 5, Online resource 5).

Antitumor Assays

To evaluate the effects of extracts on HT-29 and CCD-18 cells, both *D. tenuifolia* and *V. locusta* were extracted by using ethanol:water (1:1, *v*/*v*) and chloroform:methanol (1:1, *v*/*v*). These solvent mixtures have different polarities, thus they extract hydrophilic or lipophilic molecules and showing up activities of compounds having similar polarity. Lipophilic tomato extracts were composed by a mixture of glyceryl esters of FAs, mainly ALA, LA, PA and oleic (OA, 18:1n-9) acids, together with small amounts of carotenoids and other non-

polar bioactive compounds; conversely, ethanol:water extracts are composed by several carbohydrates types, mainly fructose and glucose [19]. Although both extracts from each vegetable showed inhibitory effects on HT-29 cells growth, the chloroform-methanol extracts were the most effective (Fig. 1). IC_{50} values obtained for this extract were ~150 and ~200 $\mu g \cdot mL^{-1}$ in *V. locusta* and *D. tenuifolia*, respectively. Nonetheless, in both vegetables, ethanol-water extracts did not reach IC_{50} values at the assayed concentrations, reaching the lowest values in *V. locusta*. Besides other active compounds such as phenolics, differences might be related to FAs content, that is, *V. locusta* was an n-3 rich plant, and these PUFAs have been associated to a lower risk of suffering cancer and metastasis [3]. Altogether, active compounds such as phenols and carotenoids are only partially soluble in water, thus fat can improve their dispersion, enabling contact with cell membranes, and then promoting cytotoxic effects on targeted cells. Focusing on the results of this work, *V. locusta* showed the highest phenolic and FA amounts, which was surely responsible for the noted effects on HT-29 cell growth inhibition.

In order to discern selectivity of both plant extracts in cell viability of normal colon cells, chloroform-methanol extracts were also assayed on the CCD-18 untransformed colon cell line at different concentrations, obtaining a lack of effects on cell viability in all cases (Fig. 1). Therefore, these extracts act selectively on tumour cancer cells. Although an absence of immediate effects on normal colon cells was obtained by inducing proliferation or decreasing viability; identifying the effects of both plant extracts on normal cells during a higher number of cell cycles might be helpful in order to discern the scope of such absence.

LDH is one of the marker enzymes used for the detection of colorectal cancer [3]. The LDH cytotoxicity assay was carried out to compare the release of the lactate dehydrogenase enzyme into the culture medium after cell membrane damage. Both MTT and LDH assays were carried out at similar culture conditions in order to compare results (Fig. 2). LDH assay was carried out with the most active extracts (chloroform-methanol 1:1, *v/v*) from both vegetables. As shown in Fig. 2, for *V. locusta*, LDH values at all tested concentration were lower than MTT values, while for *D. tenuifolia* LDH values were lower than MTT ones only for concentrations higher than 400 $\mu g \cdot mL^{-1}$. On the other hand, whereas MTT assay revealed no increased inhibitory cell growth effects when using higher extract concentrations (600, 800 and 1000 $\mu g \cdot mL^{-1}$), LDH test showed increasing cytotoxic activity in parallel with extract concentrations.

Differences in effects measured by both tests can be related to their respective sensitivities; that is, the MTT assay showed actions at an earlier stage than LDH because it detects any change in cells viability. Conversely, LDH leakage occurred at later stages, when reactive oxygen species related to cell

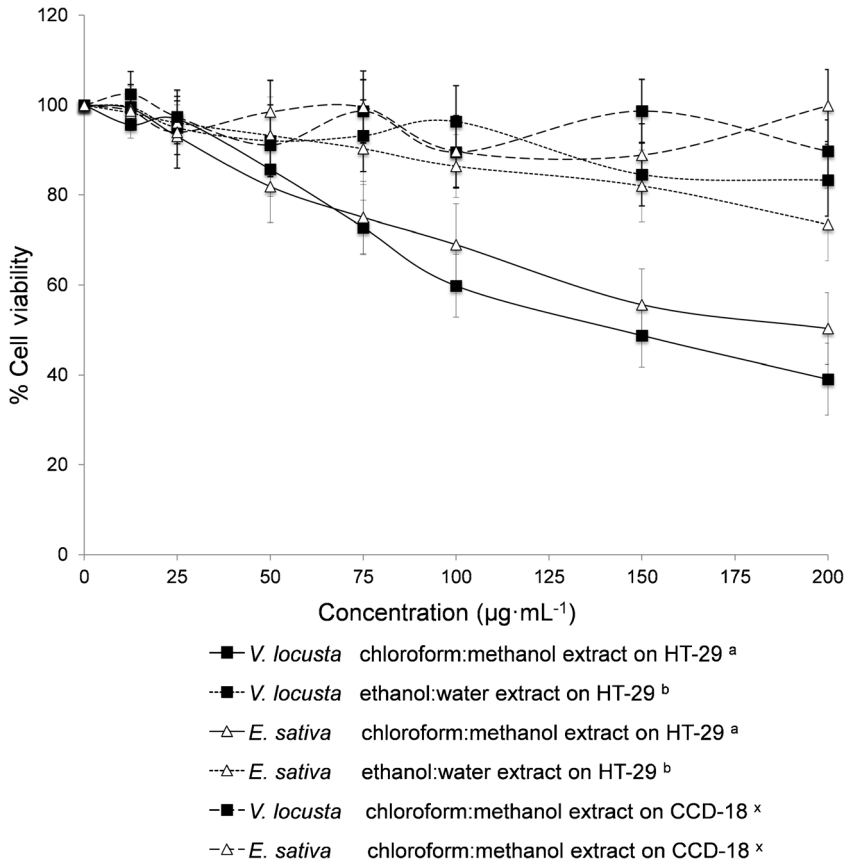

Fig. 1 MTT assays. Dose–response plot for HT-29 and CCD-18 cells following 48-h treatment with plant extracts. Data represent the mean of five complete independent experiments made in triplicate ± SD (error bars). Within each cell line, series not followed by the same letter are significantly different from one another by Duncan's multiple range test ($P < 0.05$)

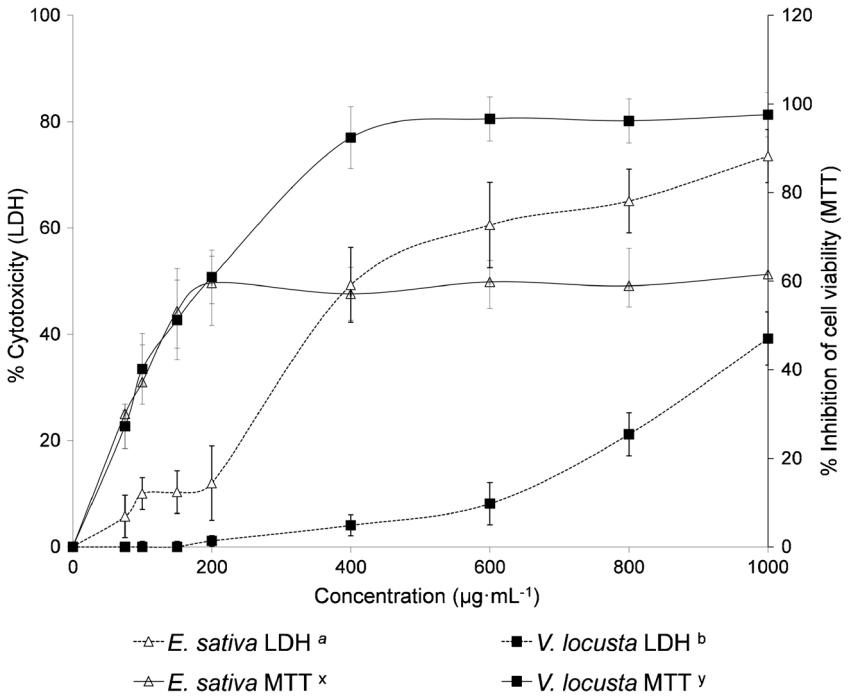

Fig. 2 MTT assays (% inhibition of cell viability) and LDH release assay (% cytotoxicity) of chloroform-methanol (1:1, *v/v*) extracts from *V. locusta* and *E. sativa* on HT-29 cell line. Data represent the mean of five complete independent experiments made in triplicate ± SD (error bars). Within each test, series not followed by the same letter are significantly different from one another by Duncan's multiple range test ($P < 0.05$)

death appear, being responsible of the mitochondria damages [3]. Cytotoxicity outcomes detected in extracts from both plants seem to be related to their different phytochemical profiles; that is, *D. tenuifolia* extracts induce cytotoxicity due to mitochondria damages that lead to cell death even at low concentrations, being such activity probably mediated by isothiocyanates. Conversely, *V. locusta* extracts induce a strong decrease of cell viability; however, these extracts induce a lower cell death than *D. tenuifolia*, being effects probably due to synergistic action of several of its phytochemicals.

In relation to the anticancer activity found in both plants, it must be pointed out that the NCI (National Cancer Institute, USA) indicates that a crude plant extract is promising for further purification when its IC_{50} values is lower than 30 $\mu g \cdot mL^{-1}$, looking for potential anticancer natural compounds [27]; therefore, the antitumor actions found in both species are not especially intense. However, considering that both plants are edible, it would not be logical to expect a very high anticancer activity, and the actions showed by such greens are in line with those effected by some other anticancer vegetables, such as that of tomatoes [19].

Conclusions

We have established that both, *D. tenuifolia* and *V. locusta*, constitute a healthy source of bioactive compounds such as carotenoids, phenols, sterols, fatty acids and glucosinolates. Both vegetables are able to produce antitumor actions on colorectal cancer cells without causing damage to untransformed cells. At the assayed extract concentrations, both plants exert differential antitumor actions; *V. locusta* induced a decreasing of colorectal cancer cell viability higher than *D. tenuifolia*, but it also induced less cytotoxic effects.

The results of this work highlight the importance of consumption of functional foods that are sources of physiologically-active components, such as those found in *D. tenuifolia* and *V. locusta*, which constitute an appropriate source of phytochemicals for colorectal cancer prevention.

Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects.

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