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# Antiproliferative, Proapoptotic, Antioxidant and Antimicrobial Effects of *Sinapis nigra* L. and *Sinapis alba* L. Extracts

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**Abstract:** High *Brassicaceae* consumption reduces the risk of developing several cancer types, probably due to their glucosinolate amount. Extracts from *Sinapis nigra* L. and *Sinapis alba* L. have been obtained from leaves and seeds under different conditions using ethanol/water mixtures because well accepted by food industry. The EtOH/$H_2O$ 8:2 mixture gives better yields in glucosinolate amounts from grinded seeds, mainly sinalbin in *S. alba* and sinigrin in *S. nigra*. The highest antiproliferative activity in both non-tumour and tumour cell lines was induced by *S. alba* seeds extract. To evaluate whether *Sinapis spp* effect was only due to glucosinolate content or it was influenced by the extracts' complexity, cells were treated with extracts or glucosinolates, in the presence of myrosinase. Pure sinigrin did not modify cell proliferation, while pure sinalbin was less effective than the extract. The addition of myrosinase increased the antiproliferative effects of the *S. nigra* extract and sinigrin. Antiproliferative activity was correlated to MAPKs modulation, which was cell and extract-dependent. Cell-cycle analysis evidenced a proapoptotic effect of *S. alba* on both tumour cell lines and of *S. nigra* only on HCT 116. Both extracts showed good antimicrobial activity in disc diffusion tests and on ready-to-eat fresh salad. These results underline the potential effects of *Sinapis spp* in chemoprevention and food preservation.

**Keywords:** *Sinapis alba* L.; *Sinapis nigra* L.; ultrasound-assisted extraction; antiproliferative; proapoptotic; antimicrobial.

## 1. Introduction

The *Brassicaceae* family is composed of around 375 genera and about 3,200 species [1], including broccoli, cabbage, cauliflower and mustard that originate from a common ancestral cabbage (*Brassica oleracea*), which still grows in Atlantic and Mediterranean areas [2]. Recent years have seen this family become the focus of growing amounts of interest as several epidemiological studies have demonstrated that high consumption levels of these vegetables reduce the risk of a great number of cancers, particularly cancers of the gastrointestinal tract, lung, bladder and prostate [3-5]. The ability of cruciferous vegetables to reduce cancer risk is not only due to the presence of fibre, carotenoids, lutein, flavonoids, phytosterols, folic acid and vitamin C, but also due to sulphur-containing secondary metabolites, namely glucosinolates that are responsible for their bitter or spicy taste. Glucosinolates are composed of three moieties; a β-thioglucose moiety, a sulfonated oxime moiety and a variable side chain aglycone derived from a α-amino acid [6]. The biological activity of glucosinolates can be attributed to their hydrolytic products [2,4], which are derived from the action of myrosinase (β-thioglucosidase glucohydrolase, EC 3.2.3.1), an enzyme localized in districts where

glucosinolates are not usually found. Glucosinolates and myrosinase come into contact when plant tissue is damaged, releasing unstable aglicones which spontaneously rearrange into a variety of reactive compounds, mainly isothiocyanates (ITCs) [4,6]. This process can be mediated, in humans and animals, by the bacterial microflora of the gastrointestinal tract. However, the yield is lower in this process than when the process is mediated by plant enzymes [7]. The activity of myrosinase, whose stability differs by origin, can be preserved during the preparation of cruciferous vegetables, although it is often inactivated by a number of cooking methods, including boiling, steaming and microwaving at high power, thus decreasing the bioavailability of the bioactive compounds. *Sinapis alba* (white or yellow mustard) and *Sinapis nigra* (black mustard) are plants belonging to the *Brassicaceae* family that are extensively consumed by humans. They contain glucosinolate sinalbin (SNB) and sinigrin (SNG), respectively, which can be converted, by endogenous myrosinase, into benzyl isothiocyanate (BIT) first, and then, allyl isothiocyanate (AIT), which are responsible for the mustard flavour. It should be noted that black mustard myrosinase is more resistant to pressure and thermal treatment than the yellow mustard form. Moreover, AIT from SNG is also considered a food additive because of its flavouring and antimicrobial properties [8-11].

The aim of this study is to test the antiproliferative, proapoptotic, antioxidant and antimicrobial activities of a variety of extracts obtained from *S. alba* and *S. nigra* leaves and seeds, that had been cultivated in Piedmont, Italy. A variety of extraction conditions were used, while glucosinolate amounts (HPLC analyses) and antioxidant activity (DPPH$^\bullet$ radical inhibition) were determined for all extracts in order to facilitate the selection of suitable samples on which to perform the antiproliferative and antimicrobial tests. Potential chemopreventive activities were evaluated on both non-tumor (hTERT-HME1 and podocytes) and colon cancer cell lines (HCT 116 and HT-29). The antiproliferative activities of hydroalcoholic seed extracts were compared to those of the main glucosinolates, SNB (syn. sinapine glucosinalbate, SNP-GSB) in *S. alba* and SNG in *S. nigra*, to evaluate whether any activity present is only due to glucosinolate or whether it is influenced by the extracts' complexity. We also investigated whether the antiproliferative activity of these extracts is correlated to the modulation of the MAPK pathway, cell cycle progression and antioxidant activity. Finally, the antimicrobial activity of the hydroalcoholic seed extracts has been evaluated using both a disc diffusion test and a growth inhibition test on fresh salads, in order to combine their food preservation with chemoprevention potential.

## 2. Results

### 2.1. Extract Preparation

Fresh leaves and seeds of *S. alba* and *S. nigra* were extracted under different conditions (Table 1), in order to obtain the highest amounts of glucosinolates (SNG and SNB), using solvents that are suitable for the food industry.

It is well known that the mechanical effect of ultrasound (US), promotes the release of soluble compounds from plant bodies, during extraction processes, by disrupting cell walls, enhancing mass transfer and facilitating solvent access to cell content [12]. For extraction methods A and B, ground vegetable matrices were therefore immersed in solvent (EtOH 96%; EtOH/$H_2O$ 8:2; $H_2O$/β-cyclodextrin), and irradiated with US, for 20 min (20 kHz, 150 W), before maceration at room temperature (r.t.), for 24 h. The HPLC analyses of *S. alba* and *S. nigra* leaf extracts show amounts of SNB sulfonate or glucosinalbate (GSB) and SNG that were lower than 1 mg/g (Table 1, method A), at w/w% extract yields of 2.54% and 1.86%, respectively. Extraction yields were 3.68% for white mustard and 3.43% for black mustard leaves. Ground seeds (different batches and different aliquots of the same batch), extracted according to method B, gave a mean yield value of 12.5% for *S. alba* and 11.1% for *S. nigra*., while method C, following the procedure described by Toribio *et al.* [13], granted 15.5% and 17.4% raw extract yields for white and black mustard seeds, respectively. Finally, an extraction protocol that involves the same conditions as method B was tested using 1.5% β-cyclodextrin (β-CD) in water, with the aim of achieving the selective complexation of glucosinolates [14].

**Table 1.** *S. alba* and *S. nigra* extraction conditions, extraction yields and sinalbin sulfonate (GSB), sinigrin (SNG) and sinapine (SNP) percentages in the obtained extracts and in the vegetal matrices.

| *S. alba* sample | Extr. solvent (plant/solvent) | Extr. method | Yield w/w % | GSB/EXT[1] w/w % | GSB/SAM[1] mg/g | SNP/EXT[1] w/w % | SNP/SAM[1] mg/g |
|---|---|---|---|---|---|---|---|
| Fresh leaves | EtOH 96% (1:7) | A | 3.68 | 2.54 | 0.93 | - | - |
| Seeds | EtOH/$H_2O$ 8:2 (1:10) | B | 12.5 | 11.5 | 14.4 | 6.96 | 8.75 |
| Seeds | $H_2O$/β-CD 1.5% (1:10) | B | 11.9 | 0.30 | 0.35 | 0.20 | 0.24 |
| Seeds | $H_2O$ (ppt EtOH) (1:10) | C | 15.5 | 2.77 | 4.43 | 3.41 | 5.29 |
| *S. nigra* sample | Extr. solvent (plant/solvent) | Extr. method | Yield w/w % | SNG/EXT[2] w/w % | SNG/SAM[2] mg/g | SNP/EXT[2] w/w % | SNP/SAM[2] mg/g |
| Fresh leaves | EtOH 96% (1:7) | A | 3.43 | 1.86 | 0.64 | - | - |
| Seeds | EtOH/$H_2O$ 8:2 (1:10) | B | 11.1 | 17.7 | 19.7 | 4.5 | 5.1 |
| Seeds | $H_2O$/β-CD 1.5% (1:10) | B | 10.1 | - | - | 0.32 | 0.32 |
| Seeds | $H_2O$ (ppt EtOH) (1:10) | C | 17.4 | 2.15 | 3.75 | 1.42 | 2.47 |

[1] The % w/w GSB/EXT and SNP/EXT correspond to the percentage of GSB and SNP in the obtained extract, while the % w/w GSB/SAM and SNP/SAM correspond to the percentage of GSB and SNP in the original sample (leaves or seeds). [2] The % w/w SNG/EXT and SNP/EXT correspond to the percentage of SNG and SNP in the obtained extract, while the % w/w SNG/SAM and SNP/SAM correspond to the percentage of SNG and SNP in the original sample (leaves or seeds).

Unfortunately, obtained yields were lower than those derived from hot water extraction (10-12%). The EtOH/$H_2O$ 8:2 mixture gave the highest amount of GSB and SNG w/w % in the extracts (11.5±1.4% and 17.7±1.2%), with 14.4±1.3 and 19.7±1.8 mg per g being obtained from white and black mustard seeds, respectively. No SNG was found in the 1.5% β-CD-aqueous-solution black mustard seed extract, while a very low amount of GSB was obtained from white mustard. Despite literature data indicating the contrary, the aqueous extracts of mustard seeds were less rich in GSB and SNG w/w % than the EtOH/$H_2O$ 8:2 ones.

### 2.2. Antioxidant activity

A DPPH• assay was used to characterize the antioxidant capacity of all the extracts obtained as it is one of the most accurate and responsive methods for analysing vegetal extracts. The scavenging effect of the various extracts was screened to evaluate their DPPH• inhibition percentage (I%), at a variety of concentrations. DPPH• scavenging activity is expressed as the mean of the $EC_{50}$ values ± standard deviation and is compared with the Trolox® standard (data not shown). Trolox® equivalents (TE), expressed as µmol/g of extract, were calculated based on $EC_{50}$. Leaf and aqueous seed extracts

showed the lowest antioxidant activity of all the samples screened, with $EC_{50}$ values that were around a hundred times higher than Trolox® (around 400 *vs* 3.94±0.50 $\mu$g/mL), and a TE of nearly 40 $\mu$mol/g (Table 2). The EtOH/$H_2O$ 8:2 mixture furnished seed extracts with higher antioxidant capacity than the $H_2O$ extract. In particular, the highest antioxidant activity was found for white mustard seed extract, which exhibited an $EC_{50}$ of 56.6±4.6 $\mu$g/mL and a TE of 278.1±22.6 $\mu$mol/g.

**Table 2.** Antioxidant activity of the various extracts compared with the Trolox® standard expressed as $EC_{50}$ values (mean ± standard deviation) and as $\mu$mol Trolox® equivalents (TE) per g of extract.

| Sample | Extraction solvent | $EC_{50}$±SD $\mu$g/mL | TE/EXT $\mu$mol/g |
|---|---|---|---|
| Trolox® | - | 3.94±0.50 | - |
| *S. alba* L. leaves | EtOH 96% | 437.6±37.2 | 36.0±3.1 |
| *S. alba* L. seeds | EtOH/$H_2O$ 8:2 | 56.6±4.6 | 278.1±22.6 |
| | $H_2O$/$\beta$-CD 1,5% | 146.4±13.0 | 107.5±9.5 |
| | $H_2O$ (ppt EtOH) | 343.3±23.7 | 45.9±3.2 |
| *S. nigra* L. leaves | EtOH 96% | 372.9±29.7 | 42.2±3.4 |
| *S. nigra* L. seeds | EtOH/$H_2O$ 8:2 | 194.7±21.8 | 80.9±9.1 |
| | $H_2O$/$\beta$-CD 1.5% | 230.9±15.1 | 68.2±4.5 |
| | $H_2O$ (ppt EtOH) | 447.2±48.7 | 35.2±3.8 |

*2.3. Antiproliferative activity*

2.3.1. Effect on the cell viability of non-tumour and tumour cell lines

We compared the antiproliferative activity of extracts from the fresh leaves and seeds of *S. alba* and *S. nigra* (extracted in EtOH/$H_2O$ 8:2 mixture), on two non-tumour cell lines, hTERT-HME1 and podocytes (Fig. 1A). Cells were treated with six different extract concentrations, in the 15.6-500 $\mu$M range, for 6 days and then cell viability was determined using the luminescent detection of cellular ATP content; each point was performed in triplicate.

The extracts obtained from the fresh leaves did not modify hTERT-HME1 viability, while those from seeds were able to inhibit cell proliferation. The *S. alba* extract was more active than *S. nigra* and the $IC_{50}$ values were 156.7±1.08 $\mu$g/mL and 249.6±2.12 $\mu$g/mL, respectively. Podocytes were sensitive to both seed extracts, but not to those obtained from leaves, which induced cell proliferation in a dose dependent manner. However, the seed extracts of both species were less effective on podocytes than on hTERT-HME1 and cell viability was reduced to 55% by *S. alba* and to 64% by *S. nigra*, at their maximum concentrations. Since seed extracts obtained with the EtOH/$H_2O$ 8:2 mixture showed the highest antioxidant activity and inhibited cell proliferation in non-tumour cell lines, their antiproliferative activity was also evaluated in two colorectal cancer cell lines, HCT 116 and HT-29. Cell lines were also treated with the principal glucosinolates present in these extracts, SNB in *S. alba* and SNG in *S. nigra.* The glucosinolates were used at the concentrations measured in each extract. Cells were treated with six different concentrations of glucosinolates, which were either pure or contained within the extracts, in the range of 2.77-88.5 $\mu$g/mL for SNG and 2.95-94.5 $\mu$g/mL for SNG, for 6 days.
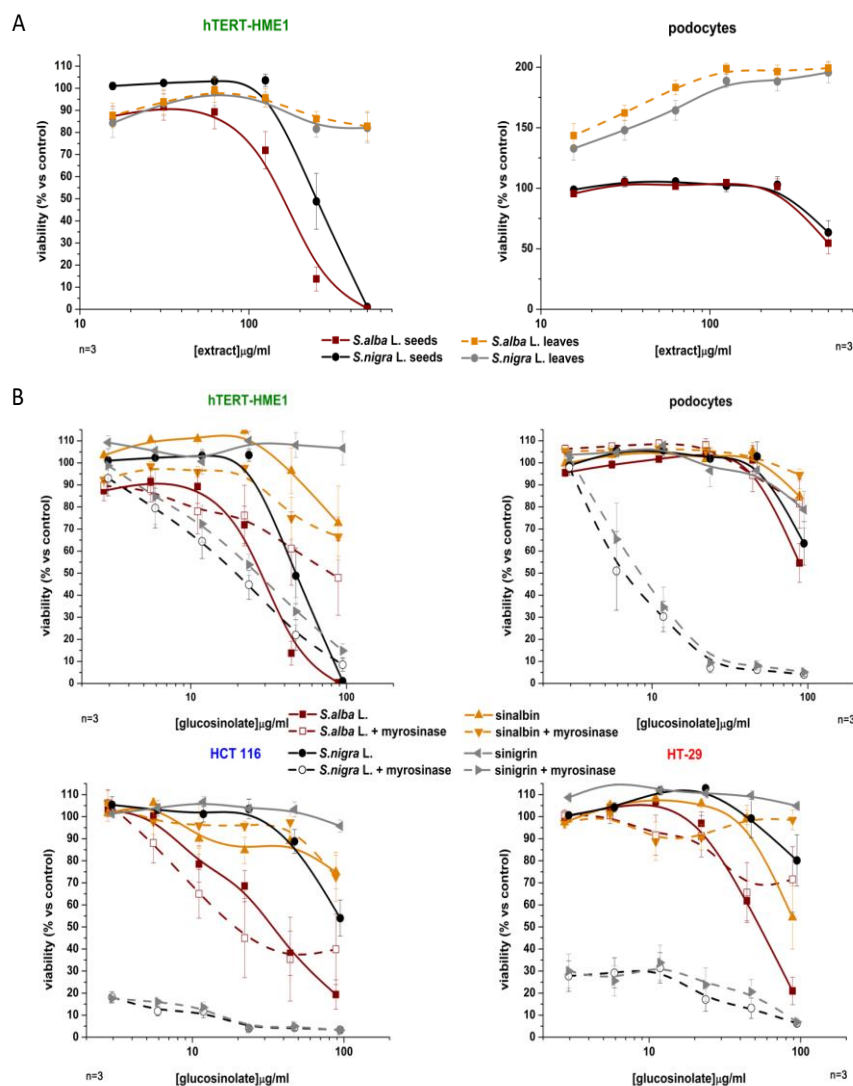
**Figure 1. (A)** Antiproliferative activity of a number of extracts of *S. alba* and *S. nigra* in hTERT-HME1 and podocytes. **(B)** Antiproliferative activity of sinalbin (SNB) and sinigrin (SNG), pure and obtained from the ethanolic seed extract of *S. alba* and *S. nigra*, in the presence and absence of myrosinase.

Seed extracts from *S. alba* were more active than those from *S. nigra* in both cell lines. *S. alba* $IC_{50}$ values were 33.69±1.11 $\mu$g/mL in HCT 116 and 54.10±1.06 $\mu$g/mL in HT-29 (corresponding to 189.83 $\mu$g/mL and 305.65 $\mu$g/mL of *S. alba* extract, respectively). HCT 116 cell viability was reduced to 55% and HT-29 viability to 81% at the maximum tested concentration of *S. nigra*. Extracts could not be tested at concentrations greater than 500 $\mu$g/mL because those extracts were not soluble in the medium used for cell cultures (Fig. 1B). Cell viability was not modified by pure SNG, while SNB inhibited cell proliferation despite being less active than the extract obtained from *S. alba* seeds. When cells were treated at the maximum extract concentration, HCT 116 and HT-29 viabilities were 55% and 33.5% lower, respectively, than when treated with pure SNB (Fig. 1B).

SNG did not influence cell viability in hTERT-HME1, while SNB reduced it in a less significant manner than the *S. alba* extract did (Fig. 1B). Podocytes are insensitive to both pure glucosinolates. This result may be partly explained by the fact that glucosinolates are not active as of themselves but must be converted into ITCs by myrosinase. In order to evaluate whether SNB and SNG need enzymatic activation to exert their antiproliferative activity, one group of cells was treated with extracts and another with glucosinolates in the presence of myrosinase 0.1 U/mL (Fig. 1B). The

antiproliferative effects of SNG, whether pure or present in the *S. nigra* seed extract, increased in the presence of myrosinase. It inhibited hTERT-HME1 viability in a dose-dependent manner, with an IC$_{50}$ value of 18.62±1.20 $\mu$g/mL for SNG present in the extract (corresponding to 98.52 $\mu$g/mL of *S. nigra* extract, significantly lower than what was obtained without myrosinase, 249.6 $\mu$g/mL), and 26.00±0.13 $\mu$g/mL for pure SNG. When myrosinase was added, podocyte viability was reduced by both pure SNG and the extract-borne analogue in a dose-dependent manner, with IC$_{50}$ values of 8.73±1.12 $\mu$g/mL and 7.17±1.42 $\mu$g/mL (corresponding to 37.94 $\mu$g/mL of *S. nigra* extract), respectively. HCT 116 and HT-29 viability was reduced to 20% and 30%, respectively, at the lowest SNG concentrations in the presence of myrosinase. As regards *S. alba*, the antiproliferative effect of the extract was greater than that of pure SNB. The addition of myrosinase to the medium did not modify the antiproliferative activity of SNB, whether pure or extracted from seeds.

2.3.2. Effects on MAPK

In order to evaluate whether the antiproliferative activity of the seed extracts was correlated to the modulation of the MAPK pathway, cells were treated with the extracts at a variety of times (5-60 min), either at the IC$_{50}$ values or at the maximum concentrations used in the proliferation assay if 50% proliferation inhibition was not reached (data not shown). Cells were processed for western blot analysis of the phosphorylated (ph-) and total forms of p38, p42/44 and JNK, as described in the methods section. The results reported are those obtained after treating cells for 30 min, as they were the most significant (Fig. 2).
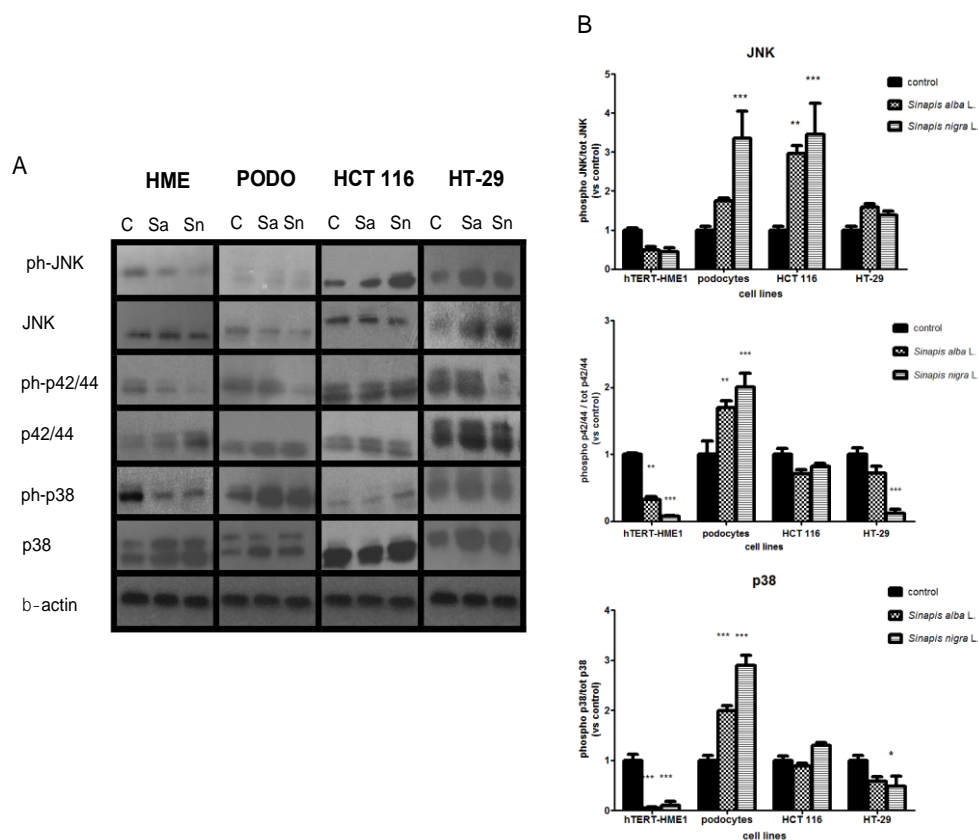


**Figure 2.** Effect of *S. alba* (Sa) and *S. nigra* (Sn) seed ethanolic extracts on the phosphorylation of p38, p42/44 and JNK MAPKs at 30 min treatment (**A**) Immunoblotting of one representative experiment of the two, at least, performed. (**B**) Densitometric results, normalized to total protein, expressed in densitometric units (O.D.) with control=1 (means ± SE of at least two separate experiments).

In hTERT-HME1, we observed a decrease in the phosphorylation of all the MAPKs analysed, which was significant for p42/44 and p38, when cells were stimulated with *S. nigra* (p<0,001), and *S. alba* (p<0,01) (Fig. 2B).

On the other hand, both the extracts had the effect of increasing MAPK phosphorylation in podocytes. *S. nigra* activated all the MAPKs analysed (p<0.001), while *S. alba* was more active with p38 (p<0.001), and p42/44 (p<0.01) (Fig. 2B). The only MAPK activated in HCT 116 by both extracts was JNK (p<0,01 for *S. alba* and p<0,001 for *S. nigra*). p42/44 was significantly dephosphorylated (p<0,001), in HT-29 stimulated with *S. nigra*, while p38 was dephosphorylated, but to a lesser extent (p<0,05).

### 2.3.3. Effects on cell cycle

The inhibition of cell proliferation by *Sinapis* spp. seed ethanolic extracts was only partly associated with cell cycle modulation (Fig. 3). The treatment of hTERT-HME1 and podocytes, for 24 h with *S. alba* and *S. nigra* extracts, did not modulate the cell cycles. *S. alba* doubled the amount of cells in apoptosis in both tumour cell lines (p<0,05 *versus* the control), while *S. nigra* was only effective in inducing apoptosis in HT-29 (p<0,05 *versus* the control).

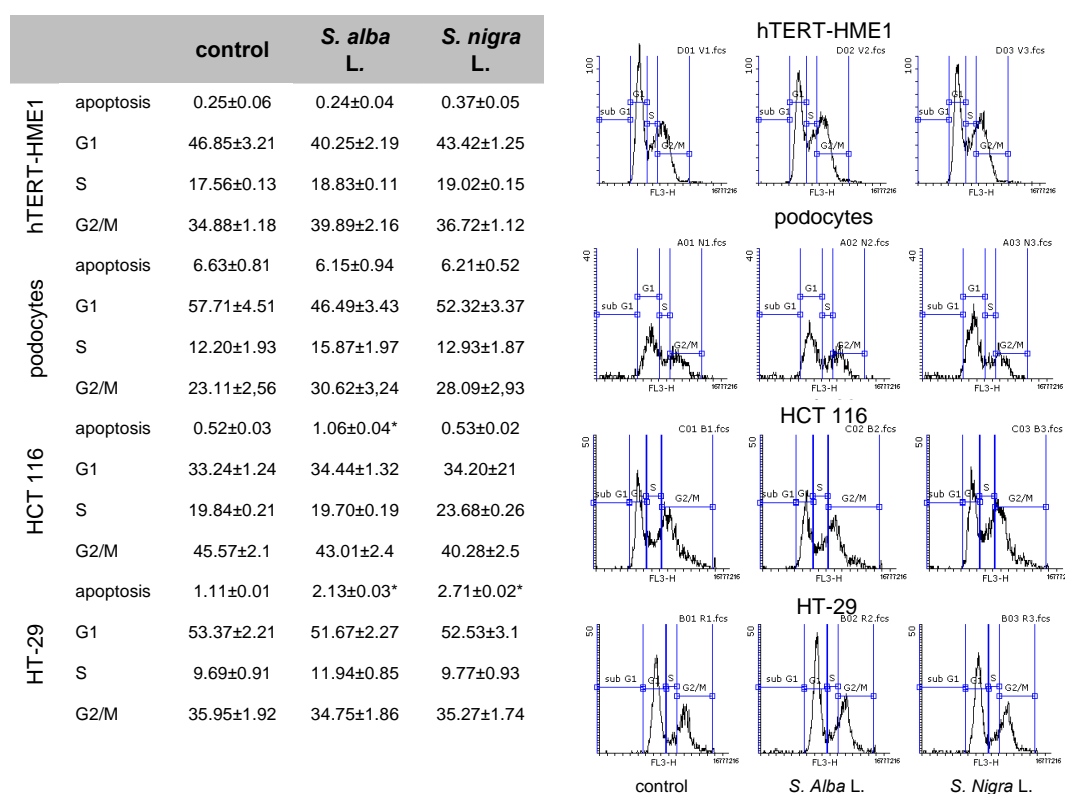| | | control | S. alba L. | S. nigra L. |
|---|---|---|---|---|
| hTERT-HME1 | apoptosis | 0.25±0.06 | 0.24±0.04 | 0.37±0.05 |
| | G1 | 46.85±3.21 | 40.25±2.19 | 43.42±1.25 |
| | S | 17.56±0.13 | 18.83±0.11 | 19.02±0.15 |
| | G2/M | 34.88±1.18 | 39.89±2.16 | 36.72±1.12 |
| podocytes | apoptosis | 6.63±0.81 | 6.15±0.94 | 6.21±0.52 |
| | G1 | 57.71±4.51 | 46.49±3.43 | 52.32±3.37 |
| | S | 12.20±1.93 | 15.87±1.97 | 12.93±1.87 |
| | G2/M | 23.11±2,56 | 30.62±3,24 | 28.09±2,93 |
| HCT 116 | apoptosis | 0.52±0.03 | 1.06±0.04* | 0.53±0.02 |
| | G1 | 33.24±1.24 | 34.44±1.32 | 34.20±21 |
| | S | 19.84±0.21 | 19.70±0.19 | 23.68±0.26 |
| | G2/M | 45.57±2.1 | 43.01±2.4 | 40.28±2.5 |
| HT-29 | apoptosis | 1.11±0.01 | 2.13±0.03* | 2.71±0.02* |
| | G1 | 53.37±2.21 | 51.67±2.27 | 52.53±3.1 |
| | S | 9.69±0.91 | 11.94±0.85 | 9.77±0.93 |
| | G2/M | 35.95±1.92 | 34.75±1.86 | 35.27±1.74 |



**Figure 3.** Effect of *Sinapis alba* and *Sinapis nigra* seed ethanolic extracts on cell cycle. Values are percentage of cells in each phase of the cell cycle, as a mean ± SEM (n=3) (p<0.05 compared with the control).

### 2.4. Antimicrobial activity

Our results led us to choose *S. nigra* and *S. alba* seed EtOH/$H_2O$ 8:2 extracts for antimicrobial activity verification. The results of antibacterial tests on *E. coli*, *S. aureus*, *B. cereus*, *P. aeruginosa*, *S. pyogenes* and *C. albicans* are shown in Fig. 4A. Both black and white mustard seed ethanolic extracts were effective against all tested strains, showing inhibition zones of between 8 and 22.7 mm (20 mg/mL in DMSO), in disc diffusion tests, confirming the antimicrobial properties of these spices and their potential benefits in food preservation. DMSO was used as the solvent and did not show any

activity. The greatest inhibition was exerted towards *S. aureus* and *E. coli* by *S. nigra* and *S. alba* (around 22 and 20 mm inhibition zones, respectively, 20 mg/mL conc.). *S. nigra* seed extracts showed stronger antimicrobial activity than *S. alba*, except against *E.coli*.
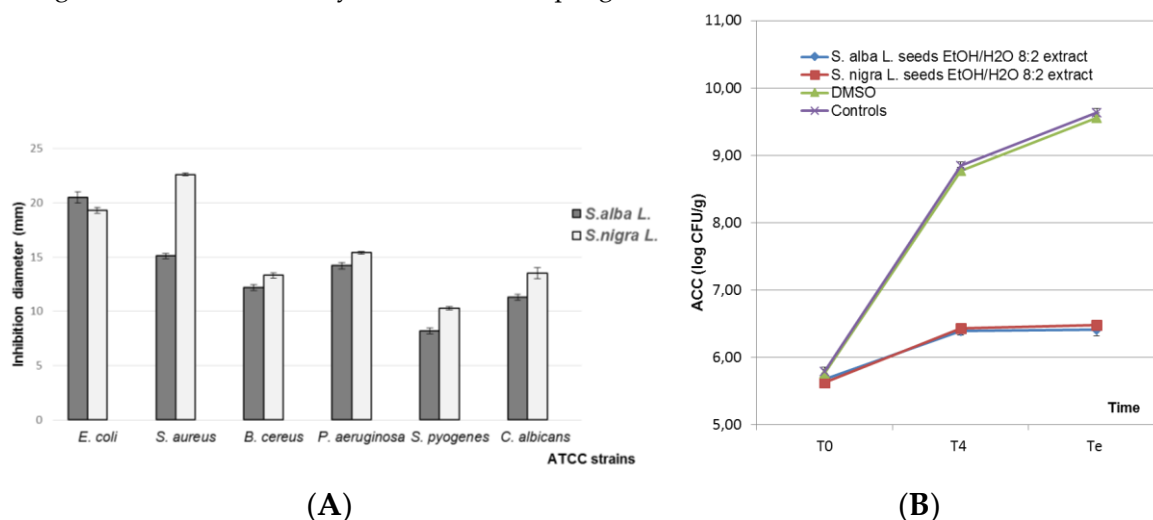


**Figure 4.** Antimicrobial activity of *S. alba* and *S. nigra* seed ethanolic extracts: (**A**) disc diffusion tests on several ATCC strains (inhibition zones in mm); (**B**) bacterial growth trends in fresh-to-eat salad (Aerobic Colony Count: ACC at different times).

The antimicrobial activity of *S. nigra* and *S. alba* seed EtOH/$H_2O$ 8:2 extracts was also evaluated on ready-to-eat salad, in order to explore the possibility of extending shelf life. Untreated salad and salad treated with DMSO alone were examined as controls. Fig. 4B shows bacterial growth trends of the treated and untreated samples which had been analysed on the packaging date ($T_0$), after 3 days ($T_3$), and on the expiry date ($T_{exp}$, day 6) [15]. At $T_0$, all samples showed similar aerobic colony count (ACC), ranging from 5.6 to 5.8 log CFU/g with an average of $5.7\pm0.02$ log CFU/g. On the expiry date, salads treated with black and white mustard seed EtOH/$H_2O$ 8:2 extracts had ACC values of $6.36\pm0.30$ and $6.45\pm0.26$ log CFU/g, respectively, while the DMSO-sprayed and untreated samples showed ACCs of $9.29\pm0.29$ and $9.31\pm0.42$ log CFU/g, respectively. Analyses revealed 2.95 and 2.86 log decreases in bacterial growth in the salad packages treated with *S. nigra* and *S. alba* seed EtOH/$H_2O$ 8:2 extracts, respectively, as compared to control and DMSO treated samples. The distribution curves of 45 samples, analysed as controls (same production batches as the sprayed samples), are compared with samples treated with the extracts and DMSO (data not shown). Results show that the values of total bacterial load in 45 packages of ready-to-eat salad, used as controls and examined at expiry date, approximate to a normal distribution with a mean of 9.31 log CFU/g and a standard deviation of 0.42. A comparison of the controls and treated samples shows that the probability of obtaining values that are lower than or equal to the treated cases without treatment is very low, thus confirming the observed difference in bacterial growth decrease is statistically significant and not random, despite the small sample size considered.

### 3. Discussion

Several epidemiological and case-control studies have lent their weight to the idea that the intake of cruciferous vegetables can reduce the risk of developing various types of cancer. This property is thought to be related to the presence of ITCs [5,7,16,17]. There are many examples of SNB and SNG extraction from varying species of mustard seed in the literature. Importantly, a number of solvents have been used, including aqueous acetonitrile [18], aqueous methanol [19], pure water and aqueous buffers [20,21]. In a number of protocols, a myrosinase inactivation step is carried out at high temperature (100 °C), before extraction [13,22]. A comparison of a variety of extraction temperatures and solvents for *S. nigra* seed extraction has given SNG recovery values of between 1.6 and 2.35% (16-23.5 mg/g seeds) [20], while *S. alba* gave SNB yields of between 0.3 and 2.5% (3-25 mg/g seeds) [13,22].

In the present work, different parts (leaves and seeds) of *S. alba* and *S. nigra* have been extracted, using several methods and solvents, in order to find the best conditions under which to give samples with the highest amounts of glucosinolates. The US-assisted extraction of ground seeds using an EtOH/H2O 8:2 mixture provided extracts with the highest GSB and SNG, content (11.5 and 17.7% respectively), while the highest antioxidant activity was detected in *S. alba* seed extracts (278 TE μmol/g). Recent studies have described the DPPH• radical scavenging activity of white mustard seed water extracts as giving inhibition values of 5.6%, 30.9% and 36.4 at concentrations of 1, 2 and 4 mg/mL, respectively [23], while black mustard seeds have shown a TE value of 4.80±0.1 μmol/g [24]. The antioxidant activity of these water extracts was much lower than those found in our extraction protocols (even for water extracts), while comparable results have been obtained for *S. nigra* seed essential oil (EC50 value of 155 μg/mL) [25]. Selective β-CD complexation did not increase the antioxidant properties of seed extracts. This trend is more closely related to the presence of polyphenols, such as SNP, than to glucosinolate amount. In fact, the SNP content is higher in white mustard seeds. Moreover, GSB hydrolysis generates *p*-hydroxybenzyl isothiocyanate (HBIT), a phenolic structure. Results reported in the literature assert that glucosinolate content appears to play a minor role in the antioxidant properties generally exhibited by cruciferous vegetables, since purified glucosinolates have exhibited only weak antioxidant properties [26].

Our data confirm the antiproliferative effects that *Sinapis* spp have on tumour and non-tumour cell lines and the possible chemopreventive activity that these products present. We have observed that the extracts obtained from the leaves of *Sinapis* spp. do not influence the viability of hTERT-HME1, but that they increase that of podocytes. On the other hand, both *Sinapis* seed extracts inhibit the cell proliferation of both non-tumour and tumour cell lines. This is not surprising since the greatest glucosinolate concentrations are found in seeds (by HPLC analyses). In fact, the amount of SNG in the seed extracts is about 17.7%, whereas it is 1.86% in the leaf extracts for *S. nigra*. The average values for GSB are about 11.5% and 2.54% in the seeds and leaves of *S. alba*, respectively. These results are confirmed by literature data which state that the glucosinolate concentration in seeds is ten times higher than that found in the leaves [27]. Moreover, myrosinase content is also higher in seeds than in leaves [28]. We cannot exclude the possibility that extraction methods completely inactivate myrosinase, meaning that the glucosinolates present in these extracts cannot be converted into the ITCs that are responsible for cell proliferation inhibition. Extracts obtained from *S. alba* seeds inhibit cell proliferation in a more significant manner than those obtained from *S. nigra*. Since the amount of glucosinolates in these two extracts is similar (17.7% for *S. nigra* and 18.9% for *S. alba* for the extracts used herein), the difference in the antiproliferative effects may be correlated to the different structure and physicochemical properties of the ITCs that originate from glucosinolate hydrolysis, namely HBIT from SNB, and AIT, a small volatile compound, from SNG. As regards *S. alba*, the antiproliferative effect of the extract was greater than that of pure SNB. The activity of myrosinase in the extract may be conserved, at least in part, so that SNB can be hydrolysed to HBIT. Nevertheless, pure SNB exerted a weak antiproliferative effect, while pure SNG did not modify cell viability. When myrosinase was added to the medium, the inhibitory effects of the *S. nigra* extract and pure SNG were comparable, meaning that the antiproliferative activity of the black mustard extract can be mainly attributed to SNG hydrolysis and AIT formation.

The difference between the antiproliferative activities of the black and white mustard extracts and their glucosinolates may also be ascribed to the presence of sinapine (SNP). SNP is a small molecular alkaloid extracted from the seeds of plants belonging to the cruciferous species [29]. Several studies have shown that SNP has various pharmacological activities, including antioxidant [30], and anti-inflammatory effects [31]. For example, SNP has inhibited the proliferation of Caco-2 cells in a dose-dependent manner and increased the cytotoxic effect of doxorubicin suppressing P-glycoprotein expression [29]. In our work, both pure SNB and the SNB present in the extract were in the form of the salt of sinalbin glucosinolate (GSB) with SNP, while pure tested SNG was the potassium salt (SNP present in black mustard extract was not stoichiometric). The amount of SNP was greater in *S. alba* than it was in the *S. nigra* (6.96% vs 4.5%, Table 1A and B) seed extract.

In order to understand the mechanism behind the antiproliferative activity of these extracts, their effects on the activation of the MAPK pathway were investigated. Several studies in the literature have reported that both BITC and AITC treatment have increased the phosphorylation of MAP kinases in a dose dependent manner and suppressed cancer cell growth [17,32]. We have observed that the modulation of MAPK activity is cell-dependent. In hTERT-HME1 and in HT-29, the antiproliferative activity of the extracts can be correlated to the considerable inhibition of p42/44 and p38 phosphorylation, even though a significant decrease in phosphorylation is only observed in HT-29 when cells are treated with *S. nigra*. Both *Sinapis* extracts activated all the MAPKs evaluated in podocytes, with *S. nigra* inducing a greater increase in phosphorylation. In HCT 116, the antiproliferative effects of *Sinapis* extracts can be explained by the activation of JNK, which directly regulates mitochondria-dependent apoptosis in a proapoptotic direction [33]. These results can be explained by MAPKs' different modes of involvement in regulating proliferation and apoptosis in a cell specific manner. For example, even though p42/44 plays a crucial role as an antiapoptotic factor [33], a few recent studies have suggested that the activation of p42/44 is correlated to apoptosis, such as in human pancreatic cancer cells [32] and in ovarian cancer cells [34]. The p38 signalling pathway is also involved in a variety of cellular responses; it has been demonstrated that p38 promotes the death of melanoma cells [35], cardiomyocytes and fibroblasts [36], but also that this signalling pathway enhances survival and cell growth, such as in human cervical cancer cells [37] and in osteoblasts. Our cell cycle analysis showed that *Sinapis* spp was only able to induce apoptosis in the two tumour cell lines. Once more, there is a cell dependent effect, as these two cell lines also have different reactions to *Sinapis* spp: *S. alba* was able to drive both cell lines to apoptosis, while *S. nigra* was only proapototic for HT29.

We also tested the antimicrobial activity of both *Sinapis* spp. seed extracts on several bacterial strains and on ready-to-eat salad. Of the bacterial strains tested, we observed that *S. nigra* and *S. alba* exerted the greatest inhibition towards *S. aureus* and *E. coli*, while the *S. nigra* seed extract showed stronger antimicrobial activity than *S. alba*, except against *E.coli*. However, data reported in 2014 by Aliakbarlu *et al.* on *S. alba*, gave quite different antimicrobial activity [23]; white mustard seed water extracts inhibited *Listeria monocytogenes* (15.3 mm inhibition zone at 150 mg/mL), while it was not active against *E. coli* and *B. cereus*. On the other hand, a *S. nigra* water extract showed good inhibition against several bacterial strains (for example, *L. monocytogenes*, *Salmonella typhimurium*, *E. coli*). In particular, the MIC found for *E. coli* was 10 mg/mL [24]. Disc diffusion tests on black mustard essential oil has shown similar antimicrobial properties against *S. aureus* (~19 mm inhibition zone at 10 mg/mL), while they were lower against *E. coli* (12 *vs* 20 mm). *B. cereus* was found to be the most sensitive to this oil (20-23 mm) [25]. Several papers have recently described the antibacterial activity of yellow and oriental mustard powder and their pure glucosinolates (SNB and SNG) in foods [38-41]. Tests were carried out against inoculated *E.coli* and *L. Monocytogenes* in a variety of meats using non-deodorized and deodorized seed flour, seed extracts and pure glucosinolates applied directly to the meat surface or incorporated in the packaging. Both active and inactivated myrosinase powder samples were found to be active against inoculated pathogens, proving the key role that myrosinase-like activity has to play. The best results were obtained when the extended shelf life of foods allowed the bacterial conversion of glucosinolates to corresponding ITCs to occur. Oriental mustard showed greater inhibition than yellow mustard due to the greater volatility and moisture stability of that AIT shows over *p*-HBIT. Our tests on ready-to-eat salad have revealed that *S. nigra* and *S. alba* seed EtOH/H$_2$O 8:2 extracts reduced bacterial growth in salad packages, as compared to control and DMSO treated samples, paving the way for a possible extension in their shelf life.

## 4. Materials and Methods

### 4.1. General Experimental Procedures

Analytical-grade ethanol (Carlo-Erba Reagenti, Milan, Italy), was used for extractions. The SNG ((−)-sinigrin hydrate, ≥99.0%, CAS 3952-98-5, MW 397.46 for anhydrous base), reference sample was purchased from Sigma-Aldrich (Milan, Italy), while the SNB (HPLC grade >99.0%, sinapine

glucosinalbate, CAS 20196-67-2, MW 734.79), reference sample was obtained from Appen Lab S.r.l. (Turin, Italy). β-CD was kindly provided by Wacher Chemie (Munich, Germany). HPLC-grade acetonitrile (Carlo-Erba Reagenti), Milli-Q water and ammonium acetate (Sigma-Aldrich), were used for the HPLC mobile phases. The *S. alba* and *S. nigra* leaf samples were cultivated and characterized by Agroselviter (Prof. S. Nicola, Dipartimento di Agronomia, Selvicoltura e Gestione del Territorio, University of Turin, Turin, Italy), while the seeds were obtained from Martin Bauer S.p.A. (Turin, Italy; *S. alba* Cod. 1-879 Lotto A110014992/002; 25/10/12; *S. nigra* Cod. 1-880 Lotto A110014992/003; 25/10/12).

## 4.2. Extraction procedures

### 4.2.1. Method A

Leaves were immersed in EtOH 96% and ground in an immersion blender. The suspension was sonicated using a titanium immersion horn at 20 kHz (20 min, 150 W), and then kept at room temperature for 24 h for maceration. The suspension was filtered on paper in a Buchner funnel and the filtrate was dried under vacuum. The optimized plant/solvent ratio was 1:7.

### 4.2.2. Method B

Liquid-nitrogen frozen seeds were subjected to fast cryogenic blender grinding and kept at -20°C until extraction. Varying aliquots of seeds were poured into an appropriate solvent (Table 1), sonicated (20 kHz immersion horn at 150 W for 20 min), and kept at room temperature for 24 h for maceration. The suspension was filtered on paper in a Buchner funnel and the filtrate was dried under vacuum. The optimized plant/solvent ratio was 1:10.

### 4.2.3. Method C

Liquid-nitrogen frozen seeds were subjected to fast cryogenic blender grinding and kept at -20°C until extraction. Seeds were poured into water and the suspension was heated to 100°C for 10 min, in order to inactivate myrosinase, and then kept at 70°C for 4 h under silent conditions, according to the procedure described by Toribio *et al.* [13]. The mucilage was precipitated and centrifuged via the addition of ethanol ($H_2O$/EtOH 1:1 ratio), whilst the supernatant was dried under vacuum. The plant/solvent ratio was 1:10.

## 4.3. HPLC analyses

HPLC analyses were performed on a Waters 1525 Binary HPLC pump equipped with a 2998 PDA [18]. Standard solutions of 0.22, 0.44, 0.66, 0.88, 1.1 and 7.1 mg/mL concentrations were prepared in water for the SNG calibration curve and a 30 $\mu$L amount of each was injected for HPLC analysis. Standard solutions of 0.115, 0.345, 0.69 and 1.15 mg/mL concentrations were prepared in water for the SNB calibration curve and a 20 $\mu$L amount of each was injected for HPLC analysis, using a Synergi Hydro RP C18 column (4 μm, 250 mm x 4.6 mm; Phenomenex). 0.025 M ammonium acetate in Milli-Q water (A) and acetonitrile (B) were used as the mobile phases. The gradient program started at 0% B, was kept constant for 6.5 min, was taken up to 50% B at 22 min, kept constant at 32 min and finally taken to 100% B at 42 min, followed by a 100% B step from 42-48 min. The PDA 3D data were collected between 210 and 400 nm, while the wavelengths at 228 and 242 nm were used to monitor the chromatograms.

## 4.4. Determination of antioxidant activity using the DPPH• radical scavenging method

The radical scavenging ability of the extracts was evaluated using the stable, free-radical DPPH•, according to the method described by Brand-Williams, Cuvelier & Berset [42]. A DPPH• solution (0.1 mM), was prepared to give absorbance at 517 nm, in the 0.45-0.55 range. 700 $\mu$L aliquots of the DPPH• solution were placed in cuvettes for the UV-Vis colorimetric assay. The reaction started when 700 $\mu$L of the diluted sample solutions were added to the cuvette containing the DPPH• solution. Mixtures

were shaken vigorously and kept in the dark for 20 min at room temperature (time required to reach equilibrium). At this point, the UV absorbance of each sample was measured at 517 nm, measured against a pure methanol blank, on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Results are expressed as DPPH$^\bullet$ inhibition percentage (I %), which was calculated using the following equation:

$$I \% = (Abs_{DPPH\bullet} - Abs_{sample} / Abs_{DPPH\bullet}) \times 100.$$

The bleaching rate of DPPH$^\bullet$ was monitored in the presence of a number of solutions of Trolox® (antioxidant standard), as well as in that of black and white mustard extracts in order to calculate the $EC_{50}$ (amount of compound/extract necessary to decrease the initial concentration of DPPH$^\bullet$ to 50% at equilibrium). Radical scavenging activity in the Trolox® solutions was measured at 1, 2, 4, 5, 7, 10, 12, 18 $\mu$g/mL concentrations. Various concentrations of mustard extracts, of between 5 and 2000 $\mu$g/mL, were also tested. Data analysis, the calculation of $EC_{50}$ and Probit Regressions were performed using an algorithm in Microsoft Visual Basic 6.0 (Microsoft Corporation, Redmond, WA, USA) [43]. All samples were prepared in triplicate and DPPH$^\bullet$ radical scavenging activity was expressed as $\mu$g compound/dry extract per ml solution ± standard deviation. Trolox® equivalents (TE) $\mu$mol/g of extract were calculated according to the $EC_{50}$ values.

### 4.5. Cell culture

HCT-116, HT-29 and hTERT-HME1 cell lines were purchased from American Type Culture Collection. Immortalized human podocytes were kindly provided by Prof. Gianluca Miglio (University of Turin, Dept. Scienza e Tecnologia del Farmaco).   HCT-116, HT-29 and podocytes were cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). hTERT-HME1 cells were grown in DMEM-F12 medium (Aurogene, Rome, Italy), supplemented with 2% FBS, 20 ng/mL EGF, 10 $\mu$g/mL insulin and 100 $\mu$g/mL hydrocortisone (Sigma-Aldrich). All cell culture media were supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.25 $\mu$g/mL amphotericin B and 2 mM glutamine (Sigma-Aldrich).

Extracts obtained from the leaves (method A), and seeds (method B), of *S. alba* and *S. nigra*, containing SNB and SNG, respectively, were dissolved in medium. Cells were seeded in complete medium (100 $\mu$L), at an appropriate density (1.500-2.000 cells/well), in 96-well plastic culture plates in triplicate. The following day, after serial dilutions in medium, which either contained 0.1 U/mL myrosinase (Sigma-Aldrich), or was left without, 100 $\mu$L of each product, in serum-free medium, was added to cells using a multichannel pipette, while medium-only containing wells were used as controls. Plates were incubated at 37°C in 5% $CO_2$ for 6 days, after which cell viability was assessed using ATP content and the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Italia Srl, Milano, Italy). All luminescence measurements (indicated as relative light units), were recorded on a Victor X4 multimode plate reader (Perkin-Elmer, Waltham, MA, USA).

### 4.6. Western blot analysis

Cells were grown in 6 well plates for 24 h and then starved and treated with extracts for different time periods (5, 15, 30, 60 min). All extracts were either used at the $IC_{50}$ calculated from the previously performed antiproliferative assays, or at maximum concentration if 50% proliferation inhibition had not been reached. All extracts were dissolved in medium. Cells were washed twice with ice-cold PBS and lysed with RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The protein concentrations of cell lysates were determined using the Pierce® BCA protein assay (ThermoScientific, Waltham, MA, USA), according to manufacturer's instructions. Cell lysates were then resolved using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Biorad, Hercules, CA, USA). The primary antibodies used for immunoblotting were; anti-p42/44 MAP Kinase, anti-phospho-p42/44 Map kinase (Thr202/Tyr204), anti-phospho-p38 (Tyr 1068), anti-p38, anti-phospho-SAPK/JNK (Thr183/Tyr185), and anti-SAPK/JNK, all from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies were diluted at 1:1000 in PBS containing 0.1% Tween-20 (PBS-T), and incubated overnight at 4°C, except

for anti-phospho-p42/44 which was diluted at 1:2000. The secondary antibody, used for all the primary antibodies reported above, was horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology), which was diluted at 1:2000 in PBS-T and incubated for 1 h at room temperature. In order to confirm the homogeneity of the protein loaded, the membranes were stripped and incubated with monoclonal anti-β-actin antibody (1:5000) (Sigma-Aldrich), for 30 min at room temperature, and subsequently with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000) (Cell Signaling Technology), for 1 h at room temperature. Protein bands were visualized using Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA, USA) [44]. Protein bands were quantified on the films by densitometry, using the ImageJ software (http://imagej.nih.gov/ij/, 1997-2015).

### 4.7. Cell cycle

Cell cycles were analysed using flow cytometry. $1.5 \times 10^6$ cells were grown on a 10 cm plate for 24 h and then incubated with *S. alba* and *S. nigra* extracts for 24 h, either at the $IC_{50}$ calculated from the previously performed antiproliferative assay, or at the maximum concentration if 50% proliferation inhibition was not reached. $1 \times 10^6$ cells were then washed twice with ice-PBS and treated overnight with ethanol 70%. All samples were incubated with 100 $\mu$g/mL RNase (Sigma-Aldrich), for 1 h at 37 °C and then with 100 $\mu$g/mL propidium iodide (Sigma-Aldrich), supplemented with 5 mM EDTA (Sigma-Aldrich), on ice. All fluorescence levels were detected using the flow cytometer BD AccuriTM C6 (Bd Biosciences, Erembodegem, Belgium). After doublet exclusion, extended analysis of DNA content and calculations of the percentage of cells in each phase of the cell cycle were performed using Flowing Software 2.5.1 (released 4.11.2013).

### 4.8. Antimicrobial diffusion test

Antimicrobial diffusion tests were carried out to determine the antimicrobial activity of *S. alba* and *S. nigra* seed extracts, which had been solubilized in dimethyl sulfoxide (DMSO) (20 mg/mL). DMSO was selected because it does not show antimicrobial activity against the selected microorganisms and easily dissolves all extract components. Six microorganisms were selected for the bioassays: gram-positive strains *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 12344 and *Bacillus cereus* ATCC 11778; gram-negative species *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 10145, and, finally, opportunistic fungus *Candida albicans* ATCC 10231. Each bacterial strain was inoculated in Tryptic Soy Broth (TSB; Biolife S.r.l., Milan, Italy), and incubated at 30 °C at a variety of different times (for *Bacillus cereus*), and at 37 °C (for other bacteria), to obtain a concentration, in the exponential growth phase, of $10^6$ CFU mL$^{-1}$. Fungal inoculum was prepared in Sabouraud Broth (Biolife S.r.l., Milan, Italy) and incubated for the time required at 25 °C to obtain a final concentration of $10^5$ CFU mL$^{-1}$. About 100 $\mu$L of tested microorganism solution, obtained as described above, was spread over the entire surface of Mueller-Hinton Agar plates (Biomerieux, Craponne, France), using a spatula. Sterile paper discs (7 mm), were soaked in 0.2 ml of extract solution and placed on the inoculated plates at a suitable distance. After Petri dish incubation, at the required temperature and time, the growth inhibition zone diameter was determined in mm via measurement with a calliper. A control test, using a paper disc soaked in DMSO, was also carried out. All tests were conducted in triplicate.

### 4.9. Antimicrobial activity in salad

The antimicrobial activity of extracts was tested to evaluate their influence on fresh-cut salad microorganism growth. A total of one hundred and eighty samples were analysed. Precisely four packs (200 g), for each of fifteen different production batches of the same salad brand were collected on packaging day ($T_0$). Tests were carried out in triplicate for each production batch. After opening and sampling at $T_0$, three packages in the same production batch were sprayed; one with 2 mL of *S. alba* seed extract solubilized in DMSO (20 mg/mL), another with *S. nigra* seed extract solubilized in DMSO (20 mg/mL), and the third with DMSO alone. The fourth was left untreated and was used as

a control test. Salads were sealed and kept refrigerated at 4°C for later analysis (after 3 days, $T_3$ and on the expiry date, day 6, $T_{exp}$). About 20 g of ready-to-eat lettuce was transferred to 180 mL of Tryptic Soy Broth (TSB, Biolife S.r.l.), in sterile stomacher bags and pummelled in a stomacher at 230 rpm for 2 min. Homogenized samples were serially diluted from $10^{-1}$ to $10^{-9}$ for subsequent plating on Plate Count Agar (PCA, Biolife S.r.l.) (ISO 4833). Plate count agar was incubated for 3 days at 30°C. Microbial counts were recorded as colony forming units per g (CFU/g), of sample. Data are shown as mean and standard deviation.

### 4.10. Statistics

The $IC_{50}$ for each compound was calculated using GraphPad Prism 4.0 software. Where indicated, the results are given as the mean ± SD. Statistical analyses were performed using the two-tailed t test with Bonferroni's multiple comparison correction and the Instat program (GraphPad). Differences in means were considered significant at a significance level of 0.05 (*, P< 0.05; **, P < 0.01; ***, P < 0.001).

## 5. Conclusions

It is well known the correlation between a high *Brassicaceae* consumption and a reduction in the risk of developing several cancer types. To obtain the highest concentration of glucosinolates from *S. alba* and *S. nigra,* considered the main responsible of their biological activity, we tested several methods of extraction, evidencing that the US-assisted extraction of grinded seeds using a EtOH/$H_2O$ 8:2 mixture afforded extracts with the highest glucosinolate (GSB and SNG) content.

Both extracts were able to inhibit cell proliferation, but with different potency, evidencing that their antiproliferative activity were strictly correlated to the chemical structure of ITCs, deriving from their hydrolysis, and counter anions (SNP).

Both black and white mustard seed hydroalcoholic extracts were effective against *E. coli*, *S. aureus*, *B. cereus*, *P. aeruginosa*, *S. pyogenes* and *C. albicans* in disc diffusion tests, but showed the greatest inhibition towards *S. aureus* and *E. coli*, respectively. Fresh-cut salads sprayed with these two mustard extracts revealed significantly lower bacterial growth, as compared to untreated salads, paving the way for a possible extension in their shelf life.

These results confirm the antiproliferative, proapoptotic, antioxidant and antimicrobial properties of these spices and their potential benefits in chemoprevention and food preservation.

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