Antioxidant and in vitro Anti-cancer Activities of Brassica juncea (L.) Czern. seeds and sprouts

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ABSTRACT

Glucosinolates represent a large group of plant natural products long known for diverse and fascinating physiological functions and are widely consumed as part of a diet in the form of cruciferous vegetables. In the present study, two varieties of Brassica juncea (L.) Czern. (Indian mustard) (RSPR-01 and RSPR-03) seeds and different day sprout extract (3 days, 5 days and 7 days) were made in dichloromethane. These extracts were tested for the hydroxyl radical scavenging activity and in vitro cytotoxicity activity. The hydroxyl free radical scavenging of extracts was determined by using DNA nicking assay and in vitro cytotoxicity activity against the rat cancer cell line (C6) and three different human cell lines (PC3, HELA and A549) by using MTT dye assay. In addition to this, the morphological changes in the cells treated with extracts were observed under confocal microscope. A critical analysis of results showed that both the varieties were effective in scavenging the hydroxyl radicals as well as inducing the death of cancer cells by apoptosis but RSPR-01 was significantly effective than RSPR-03. The mode of action of extract was studied through confocal imaging. The presence of apoptotic bodies confirmed that the extracts triggered cell death by apoptosis. The seed extracts were more effective than sprout extracts and caused early cell death. This study, on the whole demonstrated that seed extracts of Indian mustard have more potential as chemo-preventive and chemotherapeutic agents than sprout extracts.

Keywords: Glucosinolates, Brassica juncea, confocal, chemopreventive

INTRODUCTION

Cancer is a group of diseases characterized by the over proliferation of the cells. Reports in literature indicate that about 30-40% types of cancers are directly or indirectly linked to improper diet and related factors [1]. Epidemiological studies indicated optimistic association between intake of fruits and vegetables and reduced mortality from cancers, heart and other degenerative diseases [2-5]. Plants, in addition to supplying essential nutrients for the mankind also possess a variety of bioactive substances like phenols, flavonoids, carotenoids and organo sulphur compounds having anti proliferative activities [6]. One of the major bioactive phytocompound class is glucosinolates. Among different natural plant products, glucosinolates and their hydrolytic products are considered to be very active. These are the substituted esters of thiophenolic acids and their synthesis is based on the corresponding two amino acids methionine and cysteine. Plants which accumulate glucosinolates possess a thioglucoside glucohydrolase activity known as myrosinase, which can hydrolyze the glucose moiety on the main skeleton and results in the formation of unstable aglycone and glucose. These two further rearrange to form isothiocyanate, nitriles and other products. Hydrolytic products in intact plants, appears to be hindered by the spatial separation of glucosinolates and myrosinase or it can be done by the inactivation of myrosinase [7]. These products mix with each other upon tissue damage, leading to the rapid formation of the hydrolytic products of glucosinolates. It has been reported that the factors such as the pH, availability of ferrous ions, presence of myrosinase-interacting proteins and structure of parent glucosinolates, determine the nature of the final hydrolytic product formed [8]. These hydrolytic products contribute towards the biological activities of the glucosinolates. The glucosinolates may act as a sink for the nutrients like nitrogen and sulphur while their hydrolysis products may have important roles in the defense of the plants against micro-organisms and insect [9]. Glucosinolates are present in rich amount in white mustard, brown mustard, radish, horse radish, cabbage, cauliflower, broccoli, turnip and rapeseed. The
Hydroxyl Free Radical Scavenging Activity

The seeds and sprouts extract of RSPR-01 and RSPR-03 varieties, were evaluated for their hydroxyl radical scavenging by using DNA nicking assay [16]. Supercoiled plasmid pBR322 DNA (5 μg) was incubated with Fenton’s reagent (30 mM H₂O₂, 50 mM ascorbic acid and 80 mM FeCl₃) containing different concentrations of extracts and standard i.e. rutin. Finally the volume of the mixture was raised up to 20 μl with DMSO. The reaction mixture was kept for 30 minutes at 37°C. 3μl of loading buffer (0.25% bromophenol blue and 50% glycerol) was added to each reaction mixture. Thereafter agarose gel electrophoresis, was done at 50V (1.5–2 V/cm) for 1.5 hours using 1% agarose gel in TBE buffer (40 mM Tris base, 16 mM acetic acid and 1mM EDTA, pH 8.0) and reagents used in Fenton’s reaction were prepared freshly, for each reaction. DNA bands were analyzed using Gel Doc system (Gel DOCXR, Bio-Rad, USA).

In vitro Cytotoxicity Studies

The seeds and sprout extract of RSPR-01 and RSPR-03, were evaluated for their cytotoxicity by using MTT colorimetric assay as per method described by Mosmann [17]. Four different cell lines, used in the study were obtained from National Centre for Cell Sciences (NCCS), Pune and from IIIM Jammu (Table1). The cell lines were grown in RPMI-1640 growth medium (Roswell Park Memorial Institute Medium) and in DMEM (Dulbeco’s Modification of Eagle’s Medium) growth medium in tissue culture plates. The cancer cells from the stock were revived and allowed to grow at 37°C temperature in an atmosphere of 5% carbon dioxide and relative humidity of 90% in a CO₂ incubator. The cells with subconfluent stage were harvested by trypsinization in Hanks Balanced Saline Solution (HBSS) and incubated for 5mins at 37°C. The cells were transferred to centrifuged tubes and cell pellet was separated from the medium by centrifugation at 1500 rpm for 5 minutes. Cell pellet was re-suspended in 5ml of fresh medium and their viability was checked by taking the cell count after staining with dye trypan blue. The cells were seeded in 96 well plate and kept for overnight incubation. Different concentrations of extract were made in DMSO and then diluting it with growth medium. 100 μl of extract concentration (6.25, 12.5, 25, 50 and100μl/ml) was added to first well and then i

Preparation of Extracts

The extraction of soluble bioactive phytocompounds was done by using the method proposed by Vaughan and Berhow [15] with slight modifications. The plant material (seeds and sprouts) was finely powdered in grinder and mixed with hexane (for removal of fats and oil) and incubated for 24 hours overnight on shaker. Water was added to this mixture for hydrolysis in dark for 4 hours and after filtering, methylene dichloride was added. The whole mixture was incubated for 4 hours and methylene dichloride layer was separated from water layer in separating funnel. Methylene dichloride extract was then passed through anhydrous sodium sulfate for 2-3 times to make the extract water free and dried in rotary evaporator (Strike202, Stereo Glass, Italy).

members of family cruciferae contain many health promoting and potentially protective phytochemicals including folic acid, phenolics, carotenoids, selenium, glucosinolates and ascorbic acids [10-13] and offer powerful, broad-spectrum support for protecting against the ubiquitous cancer provoking agent encountered every day in our environment [14]. Considering the significance of plant based compounds in mind, the present investigation was planned to study the hydroxyl radical scavenging activity and in vitro cytotoxicity activity of the extracts of seeds and the sprouts of two different varieties of Indian mustard (viz. RSPR-01 and RSPR-03) by using DNA nicking assay and MTT dye assay using the rat cancer cell line (C6) and three different human cell lines (PC-3, HELA and AS49) respectively. In addition to this, the morphological changes in the cells treated with extracts was observed under confocal microscope.

MATERIALS AND METHODS

Chemicals

Supercoiled plasmid pBR 322 DNA was obtained from Genei, Bangalore. Ethidium bromide, ethylenediaminetetraacetic acid (EDTA), boric acid, ferric chloride (FeCl₃), L-ascorbic acid, sodium chloride (NaCl) and sodium bicarbonate were obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Bromophenol blue, RPMI-1640, DMEM, penicillin, gentamycin, Fetal Bovine Serum (FBS), agarose, dimethylsulfoxide (DMSO), hydrogen peroxide (H₂O₂) were obtained from Sigma Chemicals, USA.

Sample Preparation

Two varieties of Indian mustard (RSPR-01 and RSPR-03) were procured from Sher-i-kashmir University of Agriculture Science and Technology (SKUAST), Jammu. Extracts of seeds and sprouts were made in methylene dichloride (CH₂Cl₂). The seeds were allowed to grow at 25°C under the photoperiod of 16 hours light and 8 hours darkness cycle for 3, 5 and 7 days in the seed germinator. Seeds along with their seedlings were collected gently from the trays and homogenized separately using grinder.

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Table 1. List of Cancer cell lines

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Cell lines</th>
<th>Doubling time (hours)</th>
<th>Origin</th>
<th>Histological type</th>
<th>Medium used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C6</td>
<td>18</td>
<td>Rat(Glioma)</td>
<td>Astrocytoma</td>
<td>DMEM</td>
</tr>
<tr>
<td>2.</td>
<td>PC3</td>
<td>27.1</td>
<td>Human(Prostate)</td>
<td>Carcinoma</td>
<td>RPMI</td>
</tr>
<tr>
<td>3.</td>
<td>HEla</td>
<td>23</td>
<td>Human (cervix)</td>
<td>Adenocarcinoma</td>
<td>DMEM</td>
</tr>
<tr>
<td>4.</td>
<td>A549</td>
<td>22.9</td>
<td>Human (lung)</td>
<td>Non-small cell</td>
<td>DMEM</td>
</tr>
</tbody>
</table>

Confocal Imaging
Confocal imaging was done using method proposed by Ramsay et al [18] with slight modifications. PC-3 cells (1x10^6 cells/ml/well) for 24 hours and before seeding cells cover slips were slipped into each well. The cells were then grown on the surface of coverslips followed by treating cells with extract (1ml/well with the concentration of 5µl/ml) for 3 hours at 37°C and washed with PBS for 3 minutes. Then the cells were fixed with paraformaldehyde (chilled) fixing and washed with chilled PBS. Permeabilization of cells was done in 3% of PBST for 15 minutes so that cells allow the stain to enter. Cells were stained in dark with DAPI and PI (1mg/ml in PBS) and extra stain was removed, by washing it with PBS. Slides were prepared by rinsing in PBS and coverslips were placed over mounting fluid (PBS: Glycerol, 1:1) and were observed under Nikon Air Laser Scanning Confocal Microscope System (Nikon Corp. Japan) and photography was done.

RESULTS AND DISCUSSION
Cancer is now a day is the major problem of the world. Sporn and Suh [19] reported that DNA damage and mutations are the major causes of cancer. Cancer therapies which are available are least effective and exhibit dangerous side effects. Chemoprevention is a new pharmacological branch which deals with reversal, suppression and prevention of cancer. Dietary agents include both nutritive and non nutritive agents which can pause the factors that can trigger the cancer. Glucosinolates are one of such non nutritive plant secondary metabolites and chemically it is β-thioglycoside N-hydroxy sulphates. In plants, they are present in their inactive state but on hydrolysis, it results into a number of hydrolytic products, like nitriles, epinitriles, isothiocyanates, allylisothiocyanates etc and these products are known to possess a wide array of biological activities. So, keeping this in mind, the present study was aimed to explore the bioactivities of Indian mustard that might be due to the presence of glucosinolates. For this study, the seeds and sprouts of two varieties RSPR-01 and RSPR-03 of Indian mustard were studied. As glucosinolates are volatile they are tough to extract therefore, one of the most effective method of extraction with dichloromethane was used [20, 15]. Seeds were used as the starting material for the extraction as they are known to be the richest source of parent glucosinolates [21]. Four different solvents such as methylene dichloride, ethyl acetate, chloroform and hexane were compared for extraction of sulforaphene from Indian mustard seeds and it was found that best results of extraction were seen with methylene dichloride [22].

Figure 1. Effect of extracts of B. juncea (RSPR01) seeds and different days sprout on of supercoiled pBR322 DNA against hydroxyl radicals in plasmid nicking assay.

Figure 2. Effect of extracts of B. juncea (RSPR03) seeds and different days sprout on of supercoiled pBR322 DNA against hydroxyl radicals in plasmid nicking assay.
Table 2. Densitometric analysis of different forms of DNA after treatment with B. juncea RSPR-01 seeds and different days sprouts.

<table>
<thead>
<tr>
<th>Form of DNA</th>
<th>Lane-1</th>
<th>Lane-2</th>
<th>Lane-3</th>
<th>Lane-4</th>
<th>Lane-5</th>
<th>Lane-6</th>
<th>Lane-7</th>
<th>Lane-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Rutin</td>
<td>FR</td>
<td>Rutin+FR</td>
<td>Seeds</td>
<td>3days</td>
<td>5days</td>
<td>7days</td>
</tr>
<tr>
<td>Form I</td>
<td>72.9</td>
<td>50.9</td>
<td>0</td>
<td>24.2</td>
<td>65.5</td>
<td>27.3</td>
<td>19.3</td>
<td>29.2</td>
</tr>
<tr>
<td>Form II</td>
<td>27.1</td>
<td>49.9</td>
<td>34.5</td>
<td>45.2</td>
<td>14.7</td>
<td>55.6</td>
<td>5.19</td>
<td>27.1</td>
</tr>
<tr>
<td>Form III</td>
<td>0</td>
<td>0</td>
<td>65.5</td>
<td>30.6</td>
<td>19.8</td>
<td>17.1</td>
<td>21.6</td>
<td>43.6</td>
</tr>
</tbody>
</table>

DNA Nicking Assay
The hydroxyl radical protective effect of different extracts of Indian mustard was done by using DNA nicking assay. Hydroxyl radicals are one of most unstable and harmful free radicals which can react with almost all bio-molecules [23] resulting in DNA damage [24]. In biological system, hydroxyl radicals are produced as the byproduct in a number of processes like phagocytosis, hormone biosynthesis, enzyme decomposition etc. The seed and sprout extracts reduced the hydroxyl radicals and thus provided the protection to DNA by preventing the conversion of Form I i.e. supercoiled DNA to Form II i.e. nicked and Form III i.e. linear DNA. Rutin, a standard antioxidant compound was also loaded along with extracts to compare their activity. Quantification for the different forms of DNA was done by using Gel Doc system. Fig. 1 and Table 2 depict the protective activity of seeds and sprouts i.e. 3 days, 5 days and 7 days of variety RSPR-01 and RSPR-03 variety seeds. These all four extracts were compared at the concentration of 10mg/ml. It was observed, that seed extracts effectively protected DNA and recovered it from Form II and Form III to Form I. The densitometric analysis showed that, the amount of supercoiled DNA was 65.5% at the concentration of 10µl/ml (Table 2). However, it was observed that sprout extracts were less effective in defending supercoiled DNA from damaging effect of hydroxyl radicals. In case of sprouts, the amount of supercoiled DNA was less, i.e. 27.3%, 19.3% and 29.2% in 3 days, 5 days and 7 days respectively. This variable pattern in activity can be explained on the basis of presence and absence of some compounds and their relative quantities in sprouts in comparison to seeds. Fig. 2 and Table 3, explains the protective ability of seed extracts of RSPR-03 in comparison to the extracts of three different days sprouts i.e. 3 days, 5 days and 7 days. It was observed that extracts of seeds in comparison to sprouts was comparatively more effective (28.9%) in protecting the DNA damage. Furthermore, the densitometric analysis depicts that, the extracts of RSPR-01 was able to recover supercoiled DNA more effectively than RSPR-03 variety (Table 3). It was also observed that the amount of supercoiled DNA was maximum in case of 7 days old sprouts followed by 5 days and 3 days.

In Vitro Cytotoxicity Activity of Extracts
Extracts of both the varieties of Indian mustard were evaluated against different cancer cell lines at five different concentrations viz. 6.25, 12.5, 25, 50 and 100µl/ml in MTT dye assay. MTT assay is based on the ability of cells to change the soluble tetrazolium bromide (MTT), into an insoluble formazan precipitate. Twentyman and Luscombe [25] explored the optimal use of tetrazolium (MTT) based colorimetric assay for cell growth and chemosenstivity. The effectiveness of the extracts was measured on the basis of growth inhibiting effects of extracts on the cancer cell lines. It was seen that, all the extracts showed dose dependent inhibition of cancer cells. Seeds and different days sprouts were compared with each other and their IC50 values were calculated, in order to determine the effective cytotoxic concentration. In MTT assay, viable cancerous cells are known to convert tetrazolium salts to purple colored formazan crystals and measured spectrophotometrically.

![Figure 3. Graph showing concentration dependent growth inhibition (%) of C6 cell line on treatment of RSPR-01 and RSPR-03 extracts.](http://ijps.aizonepublishers.net/content/2013/5/ijps343-349.pdf)
concentrations. Seed extracts were more effective in inhibiting the growth of cancer cells. The inhibition pattern has been observed also vary with different cell lines. Among four cell lines, best results were seen with C6 (rat brain cancer) and A549 (human lung cancer) cell lines.

Fig. 4. Graph showing concentration dependent growth inhibition (%) of A549 cell line on treatment of RSPR-01 and RSPR-03 extracts.

Fig. 5. Graph showing concentration dependent growth inhibition (%) of PC3 cell line on treatment of RSPR-01 and RSPR-03 extracts.

Fig. 6. Graph showing concentration dependent growth inhibition (%) of HELA cell line on treatment of RSPR-01 and RSPR-03 extracts.

In C6 cell line, at lowermost concentration i.e. 6.25μl/ml, seeds of RSPR-01 and RSPR-03 were effective upto 48% and 25% respectively and at their higher concentration i.e. 100 μl/ml, the seed extracts were effective upto 82% and 75% respectively (Fig. 3). In A549 cell line, cell growth inhibition at lowermost concentration was upto 34% and 38% and at higher concentration it was 79% and 74% respectively for RSPR-01 and RSPR-03 (Fig. 4). Results of cell growth inhibition in case of PC-3 (human prostrate cancer) and HELA (Human cervix) cell lines were also effective, at higher concentration of seeds extract of RSPR-01. Fig. 5 and 6, depicts that, both the cell lines showed insignificant inhibition at lower concentrations. However, more than 50% inhibition was seen in case of seed extracts of RSPR-01 against C6, PC-3, HELA and A549 at 50μl/ml of concentration. C6 cell line showed effective IC50 value of 8.838 mg/ml with seed extract of RSPR-01 and least effective IC50 (186.936mg/ml) with 7 days sprouts of RSPR-03 (Table 3). In both the cell lines i.e. HELA and A549, the IC50 value of 19.13mg/ml was observed with seed extract of RSPR-01 variety. However, the PC-3 cell line, the IC50 value of 29.859mg/ml was observed which was lower than that of other three cell lines (Table 4). However difference in the activity of different extracts may be related to the chemical nature and also showed great variability in presence and absence of one other volatile compound in these extracts. The studies in literature by Boivin and Coworkers [26], on antiproliferative activities of different vegetable juices antiproliferative activities explained that large differences exist among the cytotoxic potential of different vegetables. It was found that cauliflower extract they significantly inhibited the growth of PC3 (prostrate), AGS (stomach), U-87 (glioblastoma), A-549 (lung) and MCF (Breast) cell line and much less effective against Panc-1 (Pancreas) cell line.

Confocal studies

The extracts of both varieties of Indian mustard were tested in PC-3 cell line for the morphological changes using confocal imaging. Different seed and sprout extracts of RSPR-01 and RSPR-03 were tested and to ascertain their mode of action, the cells were studied to know the mode of cell death. For this purpose, two contrasting dyes i.e. DAPI (4′-diamidino-2-phenylindole) and PI (Propidium iodide) were used. DAPI which is a blue fluorescent dye, has strong tendency to bind with A-T rich regions that can pass through alive as well as dead cell and further counter stained with PI. PI is a red fluorescent dye which has a tendency to bind to RNAs and DNA both. DAPI binds to DNA which appears blue in colour and PI binds to RNA as well as DNA and the cells appear red in colour. It was observed that on treatment with the extracts, there were many changes with respect to the shape of cells and size of cells. Also in some cells early chromatin condensation occurred and furthermore shrinkage of cell was seen in case of cells treated with seed extracts. The concentration of 50μl/ml was used to study the apoptosis and morphological changes in the cells. Fink and Cookson [27] demonstrated apoptosis as an active and a programmed mechanism of cell death that avoids eliciting inflammation, where as necrosis is passive and accidental cell death resulting from environmental stress. One of the remarkable features of apoptosis is stereotyped morphology showing condensation of
nuclear heterochromatin, cell shrinkage and disorganization of organelles in cytoplasm [28-29]. The presence of apoptotic bodies confirmed that the extracts triggered cell death by apoptosis. The seed extracts were more effective than sprout extracts and also caused early cell death (Fig. 7-10). Confocal images afforded the determination of structural and biochemical events occurring during apoptosis. DNA in cells is generally stained by using DAPI (4’-diamino-2-phenylindol) for fluorescent microscopy [30] and when the cells were stained with DAPI, DNA under ultraviolet (UV) illumination appears as Blue-White and also the positions of cells nuclei and nucleoids can also be determined [31]. PI is membrane impermeable and thus it is excluded from viable cells, used as counter stain in multicolor fluorescent techniques. As PI binds to the nucleotide pair of guanine and cytosine, therefore stains both DNA and RNAs [32]. So, the combination of both the stains PI and DAPI were used to study the apoptosis and condition of the cells after the extract treatment on PC3 cell line. In the present study, seed extracts of both varieties were effective in causing early cell death and in some cells chromatin condensation was also observed. This study, on the whole demonstrated that seed extracts of *Brassica juncea* have more potential as chemopreventive and chemotherapeutic agents than sprout extracts.

### Table 4. IC50 values of RSPR-01 and RSPR-03 seeds and sprouts against C6, PC3, HELA and A549 cell lines.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>C6 cell line</th>
<th>PC3 cell line</th>
<th>HELA cell line</th>
<th>A549 cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 Value (mg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seeds</td>
<td>RSPR-01</td>
<td>RSPR-03</td>
<td>RSPR-01</td>
<td>RSPR-03</td>
</tr>
<tr>
<td>29.859</td>
<td>35.9109</td>
<td>8.838</td>
<td>26.693</td>
<td>19.685</td>
</tr>
<tr>
<td>3 days sprouts</td>
<td>38.471</td>
<td>58.294</td>
<td>17.492</td>
<td>62.682</td>
</tr>
<tr>
<td>5 days sprouts</td>
<td>67.522</td>
<td>60.902</td>
<td>61.8822</td>
<td>85.0326</td>
</tr>
<tr>
<td>7 days sprouts</td>
<td>81.2309</td>
<td>134.737</td>
<td>32.826</td>
<td>186.936</td>
</tr>
</tbody>
</table>

### CONCLUSION

This study determines the variation in anticaner and hydroxyl radical scavenging activity of *B. juncea* (L.) Czern., which may be due to the phytochemicals present in extracts especially glucosinolates. The hydroxyl radical scavenging activity and cytotoxicity potential of extracts of seeds and different day sprouts increased in a dose dependent manner. The mechanism of cell death by extracts was confirmed by confocal imaging.

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### REFERENCES


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