African Journal of Agronomy Vol. 1 (2), pp. 017-026, July, 2013. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Evaluation of antifungal and antioxidative potential of hydrolytic products of glucosinolates from some members of Brassicaceae family

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Accepted 22 April, 2013

The primary aim of the present study was to evaluate antioxidative and antifungal potential of hydrolytic products of glucosinolates. These two bioactivities were assessed in the seeds of the rocket salad, Indian rape, cabbage, cauliflower, knol-knol and radish using DPPH and disc diffusion assays. *Alternaria alternata* was used as test pathogen in disc diffusion assay. The antioxidative and antifungal capacities of the water extracts were found to increase with increasing dose. It was observed that Indian rape possessed highest percentage of inhibition, when 100% concentration of extracts were used. In disc diffusion assay, Indian rape and radish gave better results with increasing concentration. Highest percentage of inhibition of the Brassicas can be attributed in part to the maximum concentration of the hydrolytic products of glucosinolates present in them.

Key words: Antioxidative, antifungal, DPPH, disc diffusion assay, hydrolytic products.

INTRODUCTION

Studies have shown that free radicals present in the humans cause oxidative damage to different molecules, such as lipids, proteins and nucleic acids and thus involved in the intiation phase of some degenerative diseases. Factors that induce oxidative stress are poor dietary habits (for example, low intakes of fruits and vegetables) and tobacco smoking which are associated with the development of colon, lung and bladder cancer. Green leafy vegetables, fruits, wheat germ, nuts and vegetable oils are excellent sources of antioxidant components (Weisburger, 1991). Cruciferous vegetables have relatively abundant sources of antioxidant substances with potential anticarcinogenic activity (Kurilich et al., 1999). Both epidemiological studies and experimental research indicated that regular intake of cruciferous vegetables may reduce risk of chronic diseases (Carol et al., 2000; John et al., 1996). Studies have shown that there is inverse relationship between the consumption of cruciferous vegetables and the risk of developing cancer. Cruciferous vegetables are known to contain an important class of phytochemicals known as glucosinolate.

Glucosinolates are organic anionic compounds containing sulphur, nitrogen and a group derived from glucose (Kjaer, 1960; Ettlinger and Kjaer, 1968; Sorensen, 1990). Glucosinolates are found in all parts of the plant (Kjaer, 1976) and up to 15 different types of glucosinolates have been found in the same plant. Seeds contain high amount of glucosinolates (upto 10% of dry weight) as compared to leaves, stem and roots. Concentration of glucosinolates varies according to tissue type, physiological age, plant health and nutrition (Sang et al., 1984; Clossais-Besnard and Larher, 1991; Kirkegaard and Sarwar, 1998). All families containing glucosinolates also contain thioglucoside glucohydrolase enzyme called myrosinase (E.C.3.2.3.1.) (Buchwaldt et al., 1986; Bjergegaard et al., 1994; Bones and Rossiter, 1996; Bjergegaard et al., 2003; Bellostas et al., 2003; Petersen et al., 2003). Glucosinolates and myrosinase come in contact when plant tissue is damaged leading to formation of hydrolytic products of glucosinolates. Myrosinase is not just a single enzyme but is a family or group of enzymes. The breakdown products of

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Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl, **DMSO,** dimethyl sulfoxide, **PDA,** potato dextrose agar, **μmolg^{-1},** micromoles per gram.

glucosinolates when exposed to myrosinase, include isothiocyanates, nitriles, epithionitriles, and thiocyanates. These products known to possess wide array of biological activities ranging from biocidal (Vig et al., 2009) antioxidative (Barillari et al., 2005) antimutagenic (Rampal et al., 2010) and anticancer (Rosea et al., 2005)

Glucosinolates and their hydrolytic products provide classical example of the importance of secondary plant products in the plant-pest interrelationships. Glucosinolate degradation products are bioactive and have the potential to be used as naturally produced pesticides for the control of a number of soil-borne pests such as nematodes, fungi and bacteria (Kirkgaard and Sarwar, 1998; Manici et al., 2000; Smolinska et al., 2003; Kirkegaard and Matthiessen, 2004, 2006; Poulsen et al., 2008). Glucosinolate breakdown products are safer biofumigants in pest control as they are biodegradable and less toxic (Vig et al., 2009). Plants in the genus Brassica are researched as biofumigant crops due to production of secondary compounds from glucosinolate hydrolysis, sometimes referred to as allelochemicals, which can control or suppress soil-borne pests and diseases. Brassica produces 30 to 40 different types of glucosinolates which when combined with the enzyme myrosinase, form breakdown products with the suppressive effects of nematode, fungicidal, etc. These compounds work through interference with pathogen's reproductive cycle, growth inhibition, or feeding deterrence and direct toxicity.

Keeping in view the immense importance of the members of family Brassicaceae, the present study was planned to evaluate the antioxidantive and antifungal potential of glucosinolate hydrolytic products of six Brassica seed extracts viz. two oil-yielding Brassica (Rocket salad and Indian rape) and four vegetables Brassica (cabbage, cauliflower, knol-knol and radish).

MATERIALS AND METHODS

Seeds of two oil-yielding Brassica (Rocket salad and Indian rape) and four vegetables crops (Cabbage, cauliflower, knol-knol and radish) were obtained from Punjab Agricultural University, Ludhiana. Test fungus (*Alternaria alternata*) was procured from department of microbiology, Guru Nanak Dev University, Amritsar, India and potatoes from local market were used in the present study.

Chemicals

All chemical reagents (DPPH, agar, dextrose, Tween-20 etc.) and all solvents (Methanol, DMSO etc.) were of analytical grade. All stock solutions were prepared from DMSO.

Preparation of seed extracts

Extraction of glucosinolate hydrolytic products was done by the protocol proposed by Charron and Sams (1999) with slight modifications. Crushed seeds were defatted using hexane and the hydrolysis of the sample was carried out by mixing double-distilled water at 1:4 (w/v). This was conserved with parafilm and kept at room temperature as such for 30 min. Volatile hydrolytic products of glucosinolates were isolated with the help of a syringe.

DPPH assay (2,2-diphenyl-1-picrylhydrazyl)

The antioxidantive activity of the six plant extracts was assessed on the basis of the radical scavenging effect of the stable DPPH-free radical scavenging assay as described by Blois (1958). It involves the reduction of a purple coloured solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to diphenylpicrylhydrazine (yellow coloured solution) either by hydrogen or electron abstraction mechanism (Espin et al., 2000). The DPPH solution with solvent was used as control. Different concentrations (25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10 µl/ml) of the extracts were made in DMSO, 2 µl of extract solution was added in 2 ml of DPPH solution. Then mixture was shaken well and absorbance of the resulting solution was measured spectrophotometericaly at 517 nm.

The radical scavenging activity was expressed as the inhibition percentage and monitored as per the equation:

$$\% \text{ DPPH radical scavenging} = (1-A_S/A_C)*100$$

A_C = Absorbance of control, A_S = Absorbance of sample solution.

Statistical analysis

Three replicates of each sample were used for statistical analysis. Analysis of the variance was performed on the original data by one-way analysis of variance (ANOVA) or regression analysis. Differences at $p < 0.05$ were considered significant. The data was also analyzed with the help of Fisher least differential technique through software package (Statistical Package for the Social Sciences).

Disc diffusion assay

Fungicidal activity of the six plants of the Brassicaceae family was carried out by employing disc diffusion method (Bauer et al., 1966). The test fungus *A. alternata* was cultured for the disc diffusion assay by a method proposed by Barnett and Hunter (1972). In disc diffusion assay, sterilized PDA medium was poured in the Petri dishes and allowed to solidify at room temperature. Culture suspensions of selected fungal pathogens were made in Tween 20 sterile saline solution. 100 ml of this fungal suspension was added in 2 ml of soft agar and spread on the PDA medium plates. After 10 min, discs were placed on the Petri dishes, saturated with the sample of different concentrations. The Petri dishes were incubated at 28°C and the inhibitory activity of each extract was examined at intervals of 24 h. The effectiveness of different extracts was evaluated by the formation of inhibition zone.

Inhibitory activity

The inhibitory activity was calculated by employing following formula:

$$\text{Relative magnitude of inhibition} = \frac{\text{Area defined by zone of inhibition}}{\text{Area defined by filter paper disc}}$$

Table 1. Antioxidative potential of oil-yielding Brassica seed extracts.

Concentration of seed extracts (µl/ml)	Rocket salad	Indian rape
	Inhibition (%) ±SE	
0.25	2.43±0.025	1.26±0.005
0.5	24.20±0.056	42.31±0.01
0.75	15.01±0.041	3.07±3E-04
1.0	34.90±0.039	31.10±0.004
2.5	42.21±0.043	23.14±0.008
5.0	38.46±0.042	60.75±0.01
7.5	49.34±0.015	63.11±0.003
10	50.66±0.027	54.43±0.001
Pure extract	74.29±0.281	84.62±0.023

Table 2. Statistical analysis of antioxidative potential of oil-yielding Brassica seed extracts.

Plants	F-ratio	HSD
Rocket salad	15.65594*	0.176005
Indian rape	1005.989*	0.025796

***Indicates the significant values.

RESULTS

Antioxidative potential of seed extracts

The hydrogen donating capacity of different seed extracts of six Brassica was determined by employing DPPH assay. High radical scavenging activity was observed in a dose-dependent manner. The mixture of seed extracts and reagents of DPPH assay was pre-incubated at 37°C for 30 min prior to the DPPH assay. Different concentration of seed extract were made in DMSO *viz.* 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10 (µl/ml) and pure extracts (µl/ml). It was observed that all the extract possessed moderate to high free radical scavenging activity. The strong DPPH scavenging activity can be attributed in part to the glucosinolate hydrolytic products. All the seed extracts exhibited highest percentage of inhibition, when pure extracts were used.

Oil-yielding Brassica exhibited highest antioxidative potential (84.62 and 74.29), when pure extracts were used. The percentage of inhibition was increased in a concentration dependent manner and least values (2.43 and 1.26) of percentage of inhibition were recorded at lowest concentration. From the results, it was concluded that Indian rape is a strong antioxidant and more potent in scavenging free radical than rocket salad at different concentrations. In statistical analysis, it was found that both Indian rape and rocket salad's values are significant. The percentage of inhibition of different extracts, F-ratio and HSD are represented in Tables 1 and 2, Figures 1 and 2 illustrate the graphical representation of percentage inhibition vs. concentration of two plant extracts.

DPPH assay revealed the antioxidative potential of hydrolytic product of glucosinolates of Brassica and categorized as least, moderate and strong antioxidative activity. Cauliflower and radish came under strong category, cabbage in moderate and knol-knol was found as a weak antioxidant. Cauliflower possessed maximum antioxidative potential (82.72) when pure extract was used and same trend was followed by radish, cabbage and knol-knol. The lowest percentage of inhibition namely, cauliflower (1.51), radish (3.40), cabbage (3.61) and knol-knol (2.51) was recorded at minimum concentration of extracts. In statistical analysis it was found that all the values were significant except cauliflower. The percentage inhibition of different extracts, F-ratio and HSD are represented in Tables 3 and 4, Figures 3, 4, 5 and 6 illustrates the graphical representation of percentage inhibition vs. concentration of four plant extracts.

Antifungal potential of seed extracts

The antifungal effect of hydrolytic products of glucosinolates of six plants mentioned earlier was evaluated by employing disc diffusion assay. All concentrations namely, 30, 50, 70 and 100% for disc diffusion assay were prepared from water. Antifungal activity of extracts was investigated using test fungus *A. alternata*. The inhibition zone was calculated by relative magnitude of inhibition (mm). The antifungal activity was observed in a concentration-dependent manner. It was observed that all the extracts exhibited moderate fungicidal potential and antifungal response of these

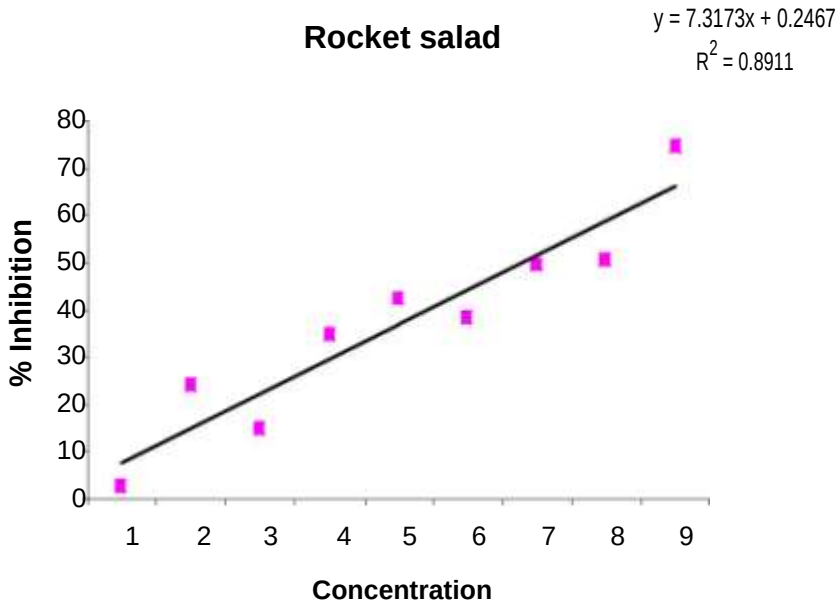

Figure 1. Concentration vs. percentage of inhibition in rocket salad.

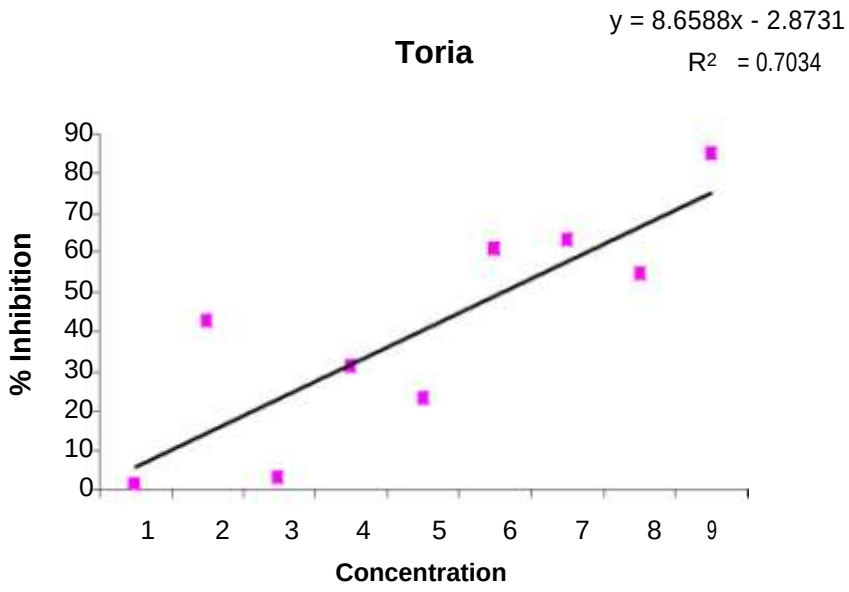

Figure 2. Concentration vs. percentage of inhibition in Indian rape.

extracts could be attributed in part to the hydrolytic products of glucosinolates.

The effect of oil-yielding Brassica was categorized as least (0.0 to 1.0 mm), moderate (2.0 to 2.55 mm) and strong (4.25 to 5.75 mm). Table 5 illustrates the effect of different concentrations of seed extracts on pathogen *A. alternata*. It was observed that Indian rape and rocket salad gave strong inhibition that is, 5.75 and 2.0 mm at 100% respectively. However, Indian rape showed least inhibition at 30% concentration of seed extract and rocket salad did not show any inhibition at 30%. The relative magnitude of inhibition was declined with decrease in the concentration of extracts. Indian rape and rocket salad possessed antifungal potential and gave better results with increased in concentrations of extract. The results are represented in Table 5 and Figure 7.

In vegetable Brassica, it was observed that radish gave strong inhibition that is, 2.55 mm at 100% followed by knol-knol, cabbage and cauliflower. However, radish exhibited 2.5 and 2.0 mm zone of inhibition at 70 and

Table 3. Antioxidative potential of cauliflower, radish, knol-knol and cabbage extracts in DPPH assay.

Concentration of extracts (µl/ml)	Cabbage	Cauliflower	Knol-Knol	Radish
	Inhibition (%) ±SE			
0.25	3.61±0.033	1.51±0.037	2.51±0.021	3.40±0.003
0.5	11.77±0.031	19.39±0.016	17.79±0.013	6.98±0.003
0.75	12.12±0.044	24.24±0.05	23.30±0.007	24.68±0.054
1.0	25.40±0.059	26.06±0.031	25.82±7E-04	19.77±0.012
2.5	34.96±0.03	37.72±0.01	47.08±0.007	42.69±0.023
5.0	41.37±0.075	47.27±0.024	51.49±0.004	60.70±0.040
7.5	62.4±0.140	59.54±0.031	56.37±0.061	78.21±0.118
10	79.13±0.02	70.15±0.004	61.73±0.002	80.54±0.024
100	79.83±0.066	82.72±.034	70.55±6E-04	81.98±0.065

Table 4. Statistical analysis of antioxidative potential of oil-yielding Brassica seed extracts.

Plants	F-ratio	HSD
Cabbage	22.85729*	0.268838
Cauliflower	1.001257	227.8672
Gand- Gobee	196.1059*	0.056932
Radish	70.16392*	0.162863

*****Indicates the significant values.

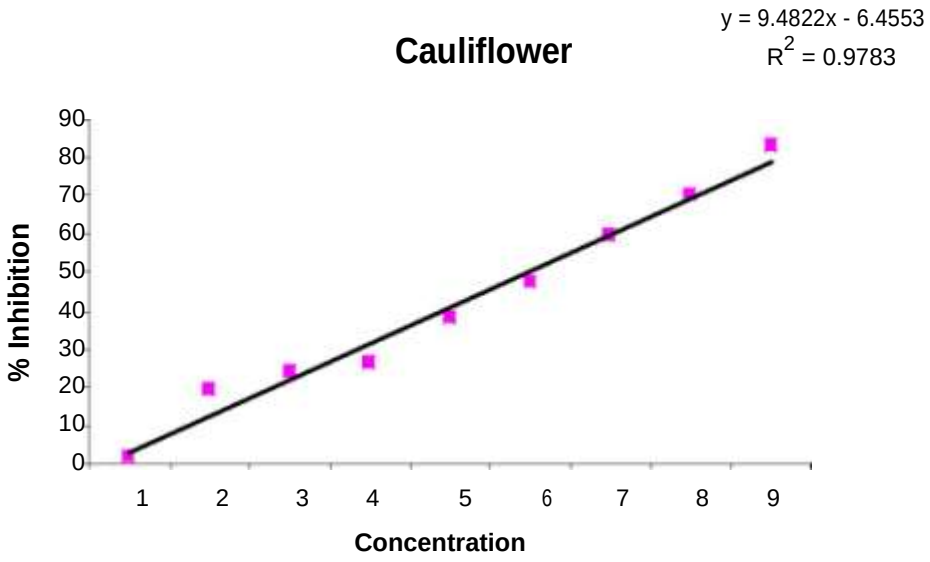

Figure 3. Concentration vs. percentage of inhibition in cauliflower.

50%, and knol-knol, cauliflower and cabbage has 2.0 and 1.75 mm zone of inhibition at 70 and 50% concentration respectively. Three vegetable Brassica showed no inhibition at all at 30% except cauliflower that is, 1.0 mm. It is concluded from results that vegetables possessed moderate antifungal potential and in concentration-dependent manner. The results are represented in Table 6 and Figures 8.

DISCUSSION

The glucosinolates are found in high concentration in many crucifers and it is well established that their breakdown products induce endogenous antioxidant defences such as quinine reductase and glutathione-S-transferase in cells (Vig et al., 2009). In present work, the antioxidative potential of glucosinolates hydrolytic

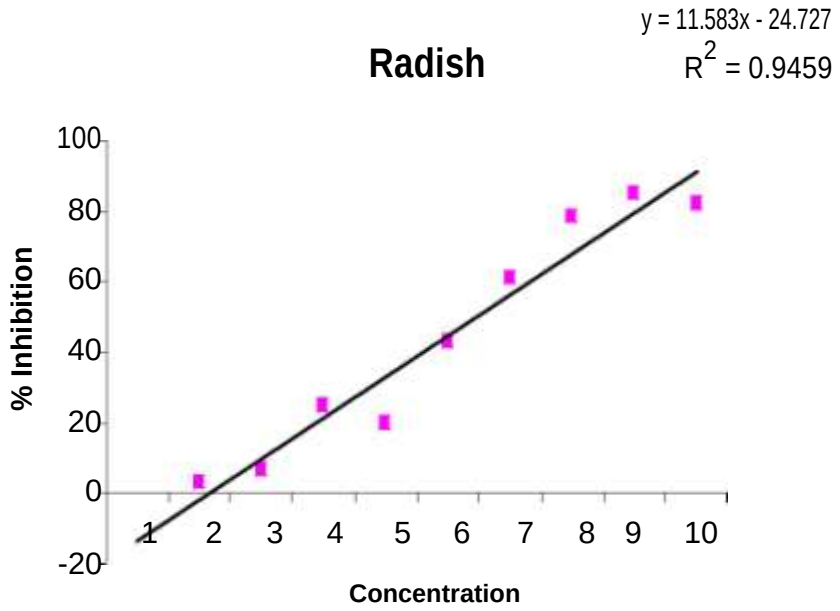

Figure 4. Concentration vs. percentage of inhibition in cauliflower.

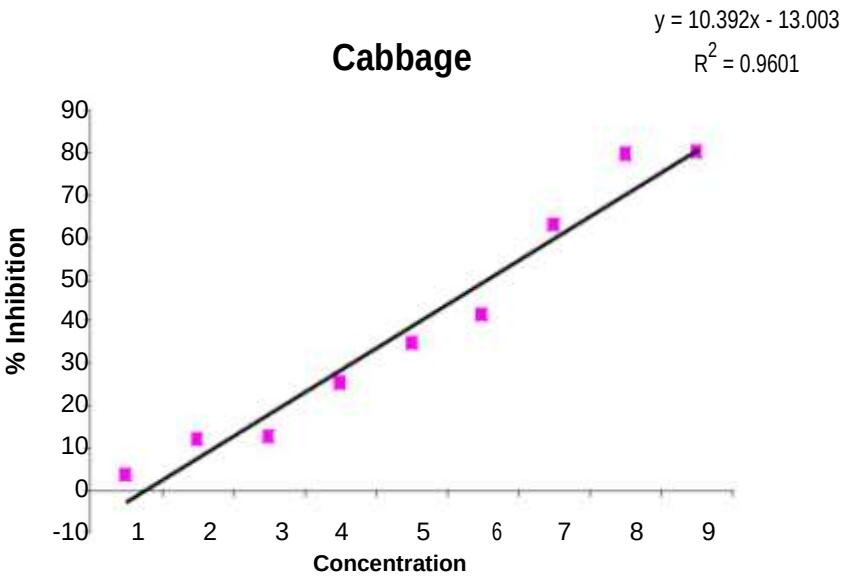

Figure 5. Concentration vs. percentage of inhibition in cauliflower.

products obtained from Brassica was assessed by employing DPPH assay. It was estimated that biological activity of the plant may be related to the presence of isothiocyanates in the plant parts (Razavi and Nejad-Ebrahimi, 2009). Sulforaphane has been promoted as a putative chemopreventive agent to reduce cancer with the induction of phase II xenobiotic metabolism enzymes via an activation of the *Keap1/Nrf2* antioxidant response pathway (Zhou et al., 2007).

The antioxidant capacity of red cabbages, white cabbages, savoy cabbages and brussel sprouts was due to their glucosinolate and polyphenol contents. The extract containing glucosinolates degradation products was capable of scavenging O_2, DPPH and $ABTS^+$ radicals and inhibited lipid peroxidation in a linoleic acid emulsion (Podsedek et al., 2006). Kim et al., (2006), evaluated the antioxidant capacity of the radish sprout extracts and claimed that the treated samples had significantly higher DPPH free radical scavenging activity than that of the control. The flower buds of turnip were revealed to be the most active part, followed by leaves, stem and seed (Ferreres et al., 2006; Vrchovska et al., 2006). The

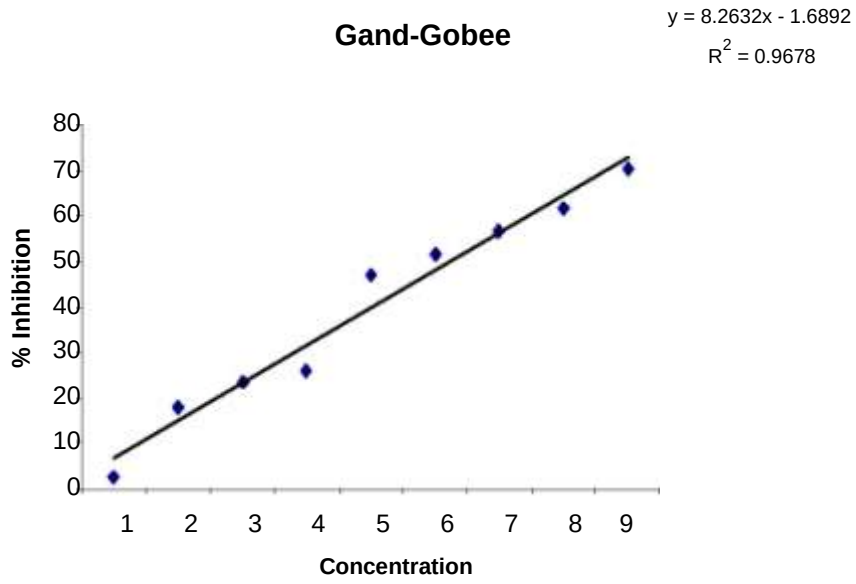

Figure 6. Concentration vs. percentage of inhibition in cauliflower.

Table 5. Antifungal potential of different concentration of hydrolytic products of glucosinolates of oil-yielding brassica (mm).

S/N	Name of plant extract	Concentrations of extract (%)			
		100	70	50	30
1.	Rocket salad	2.0	1.75	1.0	-
2.	Indian rape	5.75	4.75	4.25	2.0

samples with higher phenolic and organic acid contents displayed the major antioxidant potential (Vrchovska et al., 2006). The antioxidative activity of extracts is strongly dependent upon the solvent, due to different antioxidative potential of compounds with different polarity (Marinova and Yanishlieva, 1997; Soong and Barlow, 2004). The antioxidative activity of two purified desulfo-GSLs from leaves of rocket salad was measured and these results demonstrated that side chain of the parent glucosinolate was important for its antioxidative activity (Kim and Ishii, 2004). Yoshiki et al. (2004) have claimed that the photon intensity showed a high correlation with the chemopreventive activity against H_2O_2 and DPPH radicals in tea and cabbage. Plant phenolics present in plants parts have received considerable attention because of their potential antioxidant activity (Dziedzic and Hudson, 1983 and Lopez-Velez et al., 2003). Germano et al. (2002) reported that the antioxidant activities of the methanolic extract of *Capparis spinosa* were related to the high levels of phenolic and glucosinolates constituents of plant. In a similar manner it was found that cauliflower byproduct extracts showed significant free radical scavenging activity and the antioxidant activity was linearly correlated with the glucosinolates degradation products content, (Llorach et al., 2003).

Plants are attacked by a broad array of herbivores and pathogens. In response, plants deploy an arsenal of defensive traits. As an alternative to conventional synthetic pesticides, interest has focussed on evaluating plant extracts as potential insecticides on the promise that these materials are less specific in their mode of action. In addition, plant extracts are mostly biodegradable which suggests that their application would be more environmentally acceptable and compatible with Integrated pest management programmes. The methanol extracts of cabbage showed great potential of antifungal activity against the tested fungi such as *Sclerotium rolfsii, Rhizoctonia solani, Aspergillus niger* and *Aspergillus fumigates* in the range of 54.6 to 68.0% and minimum inhibitory concentration ranging from 500 to 1000 µg ml^{-1} (Hossain and Rahman, 2011). The volatile samples containing glucosinolate degradation products were evaluated for antimicrobial activity using the disc diffusion method with calculated minimum inhibitory concentrations (MIC) and expressed a wide range of growth inhibition activity against both Gram-positive and Gram-negative bacteria and fungi. The minimum inhibitory concentrations varied between 0.008 and 0.115 mg/ml (Blazevic et al., 2010). Handiseni (2009) had applied seed meal amendments of *Brassica napus, Brassica juncea* and *S. alba* at high rates as 4 Mt

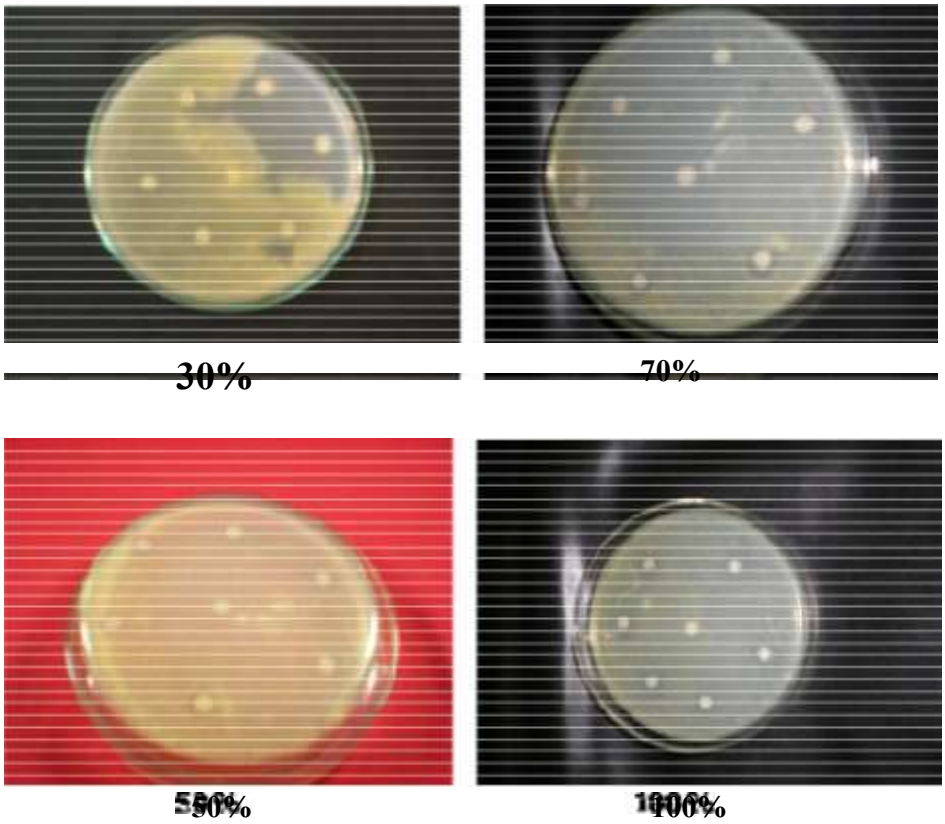

Figure 7. Results of disc diffusion assay at different concentration: Indian rape.

Table 6. Antifungal potential of different concentration of hydrolytic products of glucosinolates of vegetable brassica (mm).

S/N	Name of plant extract	Concentrations of extract (%)			
		100	70	50	30
1.	Radish	2.55	2.5	2.0	-
2.	Gandgobee	2.5	2.0	1.75	-
3.	Cauliflower	2.4	2.0	1.75	1.0
4.	Cabbage	2.5	2.0	1.75	-

ha^{-1} in tomato and peppers seedling production systems and found that such high seed meal application rates manage weeds or soil borne pathogens without limiting its seedling emergence and growth. However, when recommending the use of Brassicaceae seed meal, perhaps climatic conditions such as temperature, must be considered specified. *B. napus and B. juncea* both showed good fungicidal efficacy against *Pythium ultimum* These mechanisms may be directly or indirectly associated with glucosinolate hydrolysis products. The differences observed between seed meal type might be accounted for by the fact that different AGs of *Rhizoctonia solani* have different degrees of sensitivity to allyl glucosinolate (Smith and Kirkegaard, 2002).

All seed meal amendments provided some level of protection to the wheat seedlings with respect to percentage of infected seminal roots and disease score ratings. These results are in agreement with the findings of Mazzola and Zhao (2007) who reported that all seed meals suppressed root infection by native *Rhizoctonia* spp. The acitivity of glucosinolates isolated from oilseed and rapeseeds against three fungal species were tested *in vitro* with disc diffusion assay and found that glucosinolates present in the medium had not totally inhibited the growth of the fungi, but considerably confined the area of colonies of 2 out of 3 fungal species (Waligora et al., 2002). In Brassicaceae, the glucosinolates-myrosinase complex is a sophisticated two-component system to ward off opponents. Glucosinolates are vacuolar defense compounds of quantitative value (Wink and Schimmer, 1999; Rosential and Janzen, 1979) which are effective against

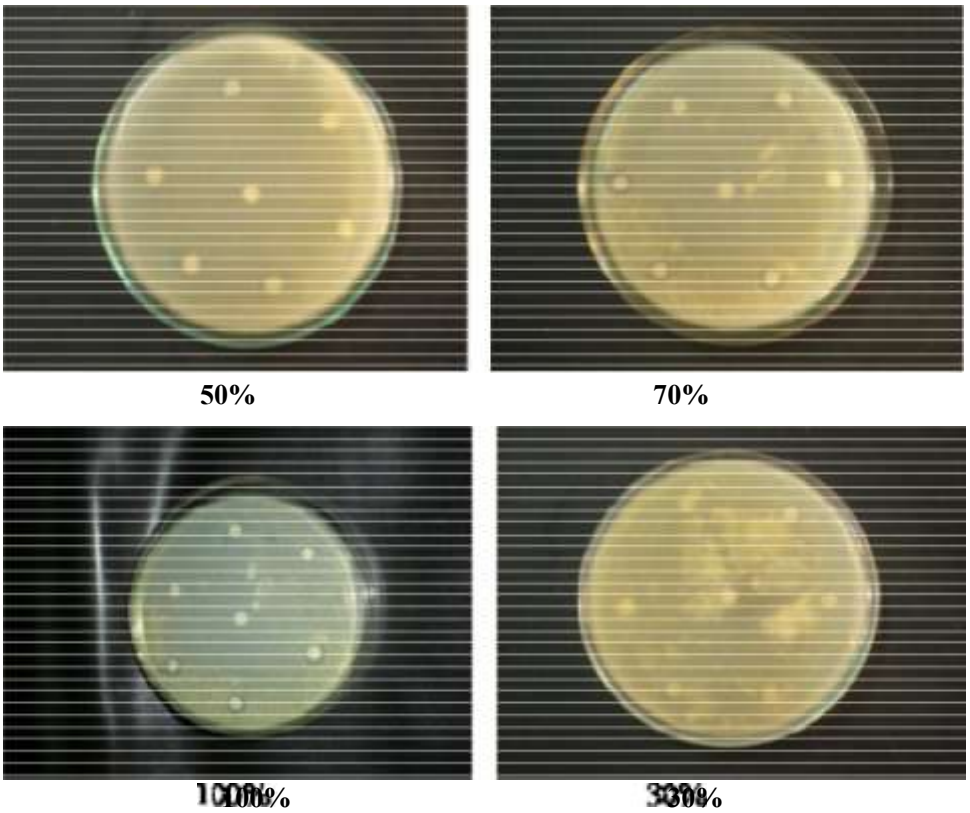

Figure 8. Results of disc diffusion assay at different concentration: Cabbage.

fungi and may retard multiplication of spores (Drobnica et al., 1967). Manici et al. (1997) have reported rankings of *in vitro* toxicity of some ITCs to *Fusarium culmorum*. In another study Smonlisnka et al. (1997) reported that the extracts from autoclaved meal of *B. napus*, in which myrosinase was denatured, has a low glucosinolate content and resulted little disease reduction and had less impact on mycelial growth (In experiments where only the volatiles released from hydrolysis of Brassica tissues, contact the test fungus, tissues dominant in more volatile types such as PrITC were more toxic (Angus 1994; Kirkegaard et al., 1996; Mayton et al., 1996). A variety of plant pests are suppressed by the decomposition of glucosinolates into toxic products. Arenas that contained soil amended with 30 g defatted winter rapeseed meal kg^{-1} soil and found that late instar wireworms were completely repelled (Brown et al., 1991).

A series of antifungal sulphur-containing indole derivatives, which appear to be related to glucobrassicin, are produced as phytoalexins in *Raphanus sativus* and *Brassica campestris* in response to fungus (Devys et al., 1990; Harborne et al., 1989). The cytotoxicity of ITCs is generally attributed to their reaction with sulfydryl, disulphide and amine groups present in proteins and amino acids (Kawakishi and Kaneko, 1987). Similarly, where aqueous extracts were tested, the activity of more soluble ITCs will be favoured and the toxicity of less soluble forms may be under estimated. Mithen et al. (1986) estimated that glucosinolate hydrolytic products were toxic for the growth of *Leptosphaeria maculans* and presence of indole glucosinolate was considered for the antifungal properties.

The metabolic enzyme and differences in fungal sensitivity might not be related to the cytotoxicity only but to the ability of the ITCs to penetrate cells also (Wood, 1975), while in experiments using ITCs dissolved in agar, aromatic ITCs were up to 20 times more toxic (Drobnica et al., 1967). In practical agricultural and horticultural situations, it is feasible that different seed meals can be blended so that a combination of weeds or other pests can be effectively controlled.

Conclusion

A high radical scavenging and antifungal activities were observed in all seed extracts of oil-yielding and vegetables Brassica in a dose-dependent manner. A highest radical scavenging and antifungal activities were observed in Indian rape extract that is, (84.6) and (5.75 mm) respectively. Glucosinolate and their hydrolytic products appear to act as chemical signals that are perceived differentially by different members of the ecological community and therefore are important in

driving coevolution between plants, their pest and disease organisms.

ACKNOWLEDGEMENT

The authors are grateful to the Head of Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar for providing the necessary facilities and guidance.

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