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Chemical Composition of Bioactive Compounds by GC-MS Screening and Anti-fungal Properties of the Crude Extracts of Cabbage Samples

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ABSTRACT

The chemical composition of the methanolic crude extract of the leaves of cabbage samples collected from the local market in Dhaka Metropolitan City (DMC), Dhaka, Bangladesh was analysed by GC-MS. It was determined that 44 compounds, which represented 66% of organic compounds, were present in the methanolic crude extract. The methanolic crude extract contained the major compounds mainly phenanthrene, anthracene, oxalic acid, napthalene, β-pinene, 2-octen-3-ol, 3-octamol and 3,4-dihydroxymendelic acid. Thus, the extract was found different types of compounds such as caffice acid and mono, di and tri terpenes and their respective hydrocarbons. Methanol extract of cabbage samples and the derived fractions of hexane, chloroform and ethyl acetate were tested for anti-fungal activity, which was determined by disc diffusion and Minimum Inhibitory Concentration (MIC) determination methods. The methanol extract and the derived fractions of methanol showed great potential of anti-fungal activity as a mycelial growth inhibition against the tested some fungi such as *Sclerotium rolfsii*, *Rhizoctonia solani*, *Aspergillus niger* and *Aspergillus fumigatus* in the range of 54.6-68.0% and minimum inhibitory concentration ranging from 500-1000 μg mL^{-1}.

Key words: Cabbage samples, chemical composition, constituents, anti-fungal activity, MIC

INTRODUCTION

Experimental and clinical as well as population studies confirmed the benefits of diet rich in fruits and vegetables in prevention of cardiovascular diseases, cancer, hypertension, diabetes and obesity. In particular, several epidemiological studies report an inverse correlation between consumption of Brassicaceae and risk of cancer (Verhoeven *et al.*, 1996; Geber and Bowerman, 2001; Ambrosone *et al.*, 2004; Barbara *et al.*, 2008). Cruciferous vegetables such as cabbage are among the most important dietary vegetables consumed in Europe owing to their availability in local markets, cheapness and consumer preference. The mechanism of chemopreventive action of cruciferous vegetables is still not fully clarified, however many animal and human intervention studies suggest that the substances present in these plants, especially glucosinolates (GLS) and products of their decomposition, are able to modulate activity of phase I and II enzymes.

As a class of well known carcinogenic compounds originating from incomplete combustion (IARC, 1983; Harvey, 1991), polycyclic aromatic hydrocarbons (PAHs) are among the most important environmental contaminants in China (Dong *et al.*, 1999). Located at the fastest growing

coastal area of China, suffers particularly from severe contamination of PAHs from various sources (TEPB, 1996, 2001). The PAHs occur as contaminants in various food categories including vegetables, which have been documented to be one of the important contributors to human intake of PAHs (Dennis *et al.*, 1983).

This is particularly true in China given the fact that vegetables are basic food in China as well as in Bangladeshi diet. It has been reported that plants uptake of PAHs is primarily from atmosphere through gas and particle-bound depositions and relative importance of these two mechanisms is driven by the gas/particle partitioning of the compound (Simnonish and Hites, 1995). A framework for identifying the major uptake process of semivolatile organic compounds based on octanol-air partition coefficient (K_{OA}) was developed and two separate tools for interpretation of plant uptake behavior for either gas or particle-bound chemicals were established (Simnonish and Hites, 1995). However, knowledge gap still remains for quantitative relationship between the plant accumulation and the level in the air. In this study, we examined the chemical composition of the methanolic crude extract isolated from the whole cabbage sample by GC-MS; (b) and investigated the anti-fungal activity of methanolic extract of cabbage samples and its derived fractions of hexane, chloroform and ethyl acetate against some fungi causing destruction in the foods.

MATERIALS AND METHODS

Chemicals and reagents: All solvents used for chromatography were methanol and dichloromethane (GC grade), obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or GC grade. Anhydrous sodium sulphate (Merck, Germany) was cleaned by heating at 200°C before use. Silica gel (60-120 mesh, Merck, Germany) activated at 400°C for 12 h prior to use.

Plant material: The cabbage samples (Brassica oleracea var. capitata f. alba) were collected from the local market of Dhaka Metropolitan City (DMC) at Dhaka in Bangladesh, in January 2006 and initially identified by morphological features and database present in the library at the herbarium of the Department of Biology, Dhaka University, Dhaka, Bangladesh.

Preparation of crude extracts: The air-dried of the leaves of cabbage samples were pulverized into powdered form. The dried powder (50 g) was extracted three times with methanol (200 mL x3) at 120°C and the solvents from the combined extracts were evaporated by Kuderna-Danish evaporator. The methanol extract (5.3 g) suspended in water and extracted successively with hexane, chloroform and ethyl acetate to give hexane (1.97 g), chloroform (0.93 g) and ethyl acetate (0.78 g) and residual methanol fractions (0.58 g), respectively.

Clean-up procedure to remove vegetables fats, oil and lipids: The cleanup column (i.d. = 1 cm) was filled with cotton in the bottom. An activated silica gel (17 g) soaked with dichloromethane was loaded into the cleanup column (5 cm), which was then topped with 1.5 cm of anhydrous sodium sulfate. Five milliliters of dichloromethane was added to wash the sodium sulfate and the silica gel. The dried 1 mL methanolic crude sample was then transferred into the column, the vessel was rinsed twice with 2 mL dichloromethane, which was also added to the column. Sixty milliliters of dichloromethane was added to the column and allowed to flow through the column at a rate of 3-5 mL min^{-1} and the eluent was collected. The collected eluent from the cleanup procedure was reconcentrated to 0.5 mL with K-D concentrator.

GC-MS analysis: The GC-MS analysis of the methanolic crude extract of cabbage samples was performed using a Varian GC-MS (Model Varian CP 3800) equipped with a VF-5 fused silica capillary column (30 m x0.25 i.d., film thickness 0.25 μm). For GC-MS detection an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. Injector and mass transfer line temperature were set at 250 and 280°C, respectively. The oven temperature was programmed from 50 to 200 at 8°C min^{-1} then held isothermal for 20 min and finally raised to 280°C at 10°C min^{-1}. Diluted samples (1/100, v/v, in methanol) of 0.2 μL was manually injected in the splitless mode. The relative percentage of the methanolic crude extract constituents was expressed as percentage by peak area normalization.

Identification of compounds of the methanolic crude extract was based on GC retention time on VF-5 capillary column, computer matching of mass spectra with those of standards (Mainlab, Replib and Tutorial data of GC-MS systems) and when ever possible, by co-injection with authentic compounds (Elad, 1991).

Microorganisms: The fungal species used in the experiment were *Sclerotium rolfsii* (BJ 35), *Rhizoctonia solani* (BJ 68), *Aspergillus niger* (BJ 403) and *Aspergillus fumigatus* (BJ 598). The fungal cultures were obtained from the Department of Biotechnology, Daegu University, Republic of Korea. Cultures of each fungal species were maintained on Potato-Dextrose-Augar (PDA) slants and stored at less than 4°C.

Preparation of spore suspension and test samples: The spore suspension of *Sclerotium rolfsii* Molds, *Rhizoctonia solani* Molds, *Aspergillus niger* Molds and *Aspergillus fumigatus* Molds were obtained from their respective 10 days cultures, mixed with sterile distilled water to obtained a homogenous spore suspension 1×10^{7} spore mL^{-1}. Methanol extract of the leaves of cabbage samples and its derived fractions of hexane, ethyl acetate and chloroform were dissolved in methanol separately to prepared the stock solution with their respective known weight, which were further diluted to prepare test samples.

Determination of anti-fungal activity of methanolic crude extract of cabbage samples: Petri dishes (9 cm diameter) containing 20 mL of PDA medium were used for anti-fungal activity assay, performed in solid media by disc diffusion method (Hoffman, 1987). Sterile Whatman paper discs of 6 mm diameter were pierced in the agar plates, equidistant and near the border, while the methanolic extract and its derived fractions samples 7.5 μL (1500 ppm) were used separately. A disc of fungal inoculum 6 mm in diameter was removed from a pregrown culture of all the fungal strains tested and placed upside down in the centre of the Petri dishes. The plates were incubated at less than 30°C for 7 days, time by which the growth of control would have reached the edges of the plates. Growth inhibition of each of the fungal strains was calculated as the percentage of inhibition of radial growth relative to the control along with anti-fungal effect on fungal mycelium. The plates were used in triplicates for each treatment.

Growth inhibition of treatment against control was calculated by percentage, using the following formula:

$$\text{Inhibition ratio (\%)} = \frac{\text{1-mycelium growth of treatment (mm)}}{\text{Mycelium growth of control (mm)}} \div 100$$

Minimum Inhibitory Concentration (MIC): The Minimum Inhibitory Concentration (MIC) of methanol crude extract and its derived fractions was determined by two-fold dilution method against *Sclerotium rolfsii*, *Rhizoctonia solani*, *Aspergillus niger* and *Aspergillus* fumigatus (Mistscher *et al*., 1987). Samples were dissolved in methanol according with the respective known weight. The solutions were serially diluted with methanol and were added to PDA to final concentrations of 125, 250, 500 and 1000 µg mL, respectively. A 10 µL spore suspension of each test strains was inoculated in the test tubes in PDA medium and incubated for 5-7 days at less than 28°C. The control tubes containing PDA medium were inoculated only with fungal suspension. The minimum concentration at which no visible growth was observed was defined as the MIC, which was expressed at µg mL^{-1}.

Statistics: Values are given as the Mean±SD of triplicate experiments. Statistical analysis was done by Student's t-test.

RESULTS

Chemical composition of methanol extract: The GC-MS analyses of the methanol extract led to the identification of 44 different organic compounds, representing 66% of the total extract. The identified compounds are listed in Table 1 according to their elution order on a VF-5 capillary column.

The methanol crude extract contains a complex mixture consisting of mainly caffice acid, oxygenated mono, di and triterpenes and mono and sesqueterpene hydrocarbons. The major compounds detected in the methanol crude extract from the cabbage samples were phenanthrene, anthracene, oxalic acid, napthalene, β-pinene, 2-octen-3-ol, 3-Octamol and 3,4-dihydroxymendelic acid, as shown in Table 2. Mono-and sesqueterpenes hydrocarbons were the characteristic constituents of the cabbage samples. 7-Pentadecyne, cis-3-hexen-1-ol, benzane acetic acid, hexan-1-ol and caffice acid also found to be the minor components of cabbage sample in the present study.

Anti-fungal activity of crude extracts: The crude methanol extract and it derived fractions exhibited a moderate to high anti-fungal activity against all the tested fungi except *Aspergillus fumigatus*. At the concentration of 7.5 µL (1500 ppm), crude MeOH extract showed potent inhibitory effect on the growth of *Sclerotium rolfsii* (65.5%), *Rhizoctonia solani* (60.3%) and *Aspergillus niger* (68.0%), as shown in Table 3. Also, the methanol derived fractions (1500 ppm) showed moderate anti-fungal activity against some of the some fungi but not for all. Hexane fraction showed anti-fungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* (54.5-53.3%), whereas, chloroform fraction showed comparatively better anti-fungal effect against *Rhizoctonia solani* and *Aspergillus niger* (59.2-63.4%) than ethyl acetate fraction (54.6-58.3%).

Minimum Inhibitory Concentration (MIC): According to the results shown in the Table 4, MIC of methanol extract was found more effective against *Sclerotium rolfsii* (500 µg mLc) as compared to those of *Rhizoctonia solani* and *Aspergillus niger* (1000 µg mL^{-1} for each). As control, methanol did not affect the growth of samples strains at the concentration used in this study. The sub-inhibitory concentrations defined as the lowest concentrations of methanol extract against *Rhizoctonia solani* and *Aspergillus niger* (1000 µg mL^{-1}) and *Sclerotium rolfsii* (500 µg mL^{-1}) and

Table 1: Chemical composition of the methanol extract of cabbage leaves

No.	R_t (min)[a]	Compound[b]	Composition (%)
1	5.505	Pivalic acid	0.03
2	5.573	p-Nitro carbanilic acid	0.08
3	5.620	Acetamide	0.21
4	5.629	Cyanic acid	0.43
5	6.897	Ethylphosphonic acid	0.65
6	6.98	Hexanal	1.07
7	7.01	Trans-2-hexanal	0.98
8	7.08	Cis-3-hexen-1-ol	0.01
9	7.11	Hexan-1-ol	0.05
10	7.16	Cis-4-Heptenal	0.78
11	7.28	Heptenal	0.97
12	7.32	Benzaldehyde	0.99
13	7.35	α-Pinene	0.72
14	7.49	Camphene	1.65
15	7.51	Cis-2-Octenal	1.98
16	7.54	2-Octen-3-ol	8.22
17	7.64	β-Pinene	2.10
18	8.22	2-Amylfurane	0.56
19	8.74	Cis-(2-(2-pentenyl)furane	1.11
20	8.99	3-Octamol	1.92
21	9.69	3,4-Dihydroxymendelic acid	1.65
22	12.62	Cyclohexasiloxane	0.67
23	13.98	Napthalene	5.82
24	15.38	Oxalic acid	3.22
25	15.39	Carbonic acid	0.23
26	16.919	Benzofuranone	0.88
27	17.85	Benzoic acid	1.10
28	19.05	4-Hydroxy-4-methylhex-5-enoic acid	0.92
29	19.92	Benzane acetic acid	0.13
30	20.80	Anthracene	6.39
31	20.81	Phenanthrene	9.44
32	21.10	3,7-Dimethyl-6-nonen-1-ol acetate	1.12
33	21.08	7-Pentadecyne	0.03
34	22.21	Cyclopentane	2.09
35	22.26	3-Methyl-5-(1,4,4-trimethylcyclohex-2-en	0.65
36	22.40	6,11-Dimethyl-2,6-dodecatrien-1-ol	1.33
37	22.41	9-Octadecyne	0.55
38	22.42	8-Hexadecyne	0.77
39	24.55	Di-N-butylphthalic acid	0.51
40	23.64	Phthalic acid	1.02
41	25.66	Eupatorin	0.99
42	31.34	Caffice acid	0.19
43	54.64	Tetratetracone	1.00
44	54.55	Hentriacotane	0.72

[a]Retention time (as minutes). [b]Compounds are listed in order of elusion from VF-5 capillary column

but no inhibition was observed against *Aspergillus fumigatus*. Chloroform fraction was resulted in low inhibition of visible growth against *Rhizoctonia solani* and *Aspergillus niger* but it did not

Table 2: Major organic compounds detected in the cabbage leaves

Sl. No.	Name of compound	Composition (%)
1	Phenanthrene	9.44
2	Anthracene	6.39
3	Oxalic acid	3.22
4	Napthalene	5.82
5	β-Pinene	2.10
6	2-Octen-3-ol	8.22
7	3-Octamol	1.92
8	3,4-Dihydroxymendelic acid	1.65

Table 3: Anti-fungal activity of methanol extract and its derived fractions 7.5 µL (1500 ppm) of leaves of cabbage samples

	Mycelial growth inhibition[a]							
	CME		HAF		EAF		CHF	
Fungal strains	mm	%	mm	%	mm	%	mm	%
Sclerotium rolfsii	15.5±0.5	65.5±0.5	20.1±1.2	54.5±1.3	18.8±0.5	58.3±1.3	nd	nd
Rhizoctonia solani	17.9±0.7	60.3±1.4	19.0±0.4	56.3±0.5	nd	nd	18.7±0.5	59.2±1.1
Aspergillus niger	14.5±0.8	68.0±1.7	nd	nd	20.3±0.5	54.6±0.7	16.6±0.4	63.4±1.1
Aspergillus fumigatus	nd	nd	nd	nd	nd	nd	nd	nd

CME: Crude methanol extract, HAF: Hexane fraction, EAF: Ethyl acetate fraction, CHF: Chloroform fraction, nd: No detection of anti-fungal activity. [a]Values are represented as the Mean±SD of three experiments

Table 4: Minimum inhibitory concentrations of methanol extract and its derived fractions of cabbage leaves against tested fungi

	MIC (µg mL^{-1})			
Fungal strains	CME	HAF	EAF	CHF
Sclerotium rolfsii	500	nd	500	nd
Rhizoctonia solani	1000	nd	nd	500
Aspergillus niger	1000	nd	nd	1000
Aspergillus fumigatus	nd	nd	nd	nd

CME: Crude methanol extract, HAF: Hexane fraction, CHF: Chloroform fraction, EAF: Ethyl acetate fraction, nd: no detection of anti-fungal activity

show any inhibition against *Sclerotium rolfsii* and *Aspergillus fumigatus*. Ethyl acetate fraction showed the inhibition only against *Sclerotium rolfsii* with the MIC value 500 µg mL, whereas, hexane fraction did not show desirable results against all the fungi tested. Crude methanol extract of cabbage samples and its chloroform fraction were found more susceptible than did hexane and ethyl acetate fractions against the tested fungi (Table 4).

DISCUSSION

Ancient traditional use of plants as medicines provide the basis for indicating which plant extracts may be useful for specific medical conditions. Historically, many plant extracts have been used as topical antiseptics, or have been reported to have anti microbial properties (Morris *et al*., 1989; Murray *et al*., 1995).

It is important to investigate scientifically those plants, which have been used in traditionally medicines as potential sources of novel anti-microbial compounds (Niklas, 1992). Also, the

resurgence of interest in natural control of fungi and increasing consumer demand for effective, safe and natural products means that quantitative data on plant extracts are required. Various publications have documented the anti-fungal activity of essential oils and plant extracts including rosemary, peppermint, bay, basil, tea tree, celery seed and funnel (Yousef and Tawil, 1980; Adam, 2001).

The cabbage powder was extracted three times with methanol at 120°C and the solvents from the combined extracts were evaporated by Kuderna-Danish evaporator. The methanol extract suspended in water and extracted successively with hexane, chloroform and ethyl acetate and residual methanol fractions, respectively. The methanol extract was found mainly contained caffice acid and mono, di and tri terpenes, polycyclic aromatic hydrocarbons and their respective hydrocarbons.

The methanolic crude extract of cabbage samples showed remarkable anti-fungal effect against three out of four fungi tested. This activity could be attributed to presence of phenanthrene, anthracene, oxalic acid, napthalene, β-pinene, 2-octen-3-ol, 3-Octamol and 3,4-dihydroxymendelic acid, which significantly (65% and above) inhibited the growth of all the fungal tested and/or other major and minor oxygenated mono-and sesquiterpenes present in the extract.

Certain plant extracts with their derived fractions and phytochemicals acts in many ways on various types of disease complex and may be applied to the crop in the same way as other agriculture chemicals. Cabbage samples can also be used as a leading factor in a wide range of activities against many phytopathogens, where the pathogens have developed resistance against the specific fungicides (Adam, 2001).

In this study, methanol extracts and its different organic fractions showed varying anti-fungal activity against some fungi. This activity could be attributed to presence of β-pinene, phenanthrene, napthalene, anthracene, oxalic acid, 2-octen-3-ol, 3-octamol and 3,4-dihydroxymendelic acid, which significantly (65% and above) inhibited the growth of all the fungal tested and/or other major and minor oxygenated mono-and sesquiterpenes present in the extract (Zacchino *et al*., 1999; Hammer *et al*., 2003; Filipowicz *et al*., 2003; Kouam *et al*., 2007; Yang *et al*., 2008). Therefore, it would also be interesting to study the effects of essential oil of crude extracts of cabbage samples on medicinally important fungi for development of new anti-fungal agents for preventive treatment of serious fungal disease infections in animals and human beings. In this regard we have started a program aimed at the evalution of anti-fungal activity of methanolic extract cabbage samples and its derived fractions of hexane, ethyl acetate and chloroform, in hope to find out new natural products to be used in the biocontrol against fungi.

According to the results of this study, we can suggest that the cabbage samples could become an alternative to synthesis fungicides for using in food industries to control fungi causing destruction to foods.

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