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Assessment of bioactive compounds in *Brassica juncea* using chromatographic techniques

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Abstract
The investigation was carried out to determine the phytochemical components from methanolic extract of Indian mustard using High performance liquid chromatography (HPLC) and gas-chromatography mass spectrometry (GC-MS). GC-MS analysis of the extract led to the identification of more than 5 compounds in all the genotypes together. The major compounds in these mustard genotypes were found to be methallyl cyanide; allyl isothiocyanate; Oxime-methoxy-phenyl; 1-butene-4-isothiocyanato; 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl; 2-Furancarboxaldehyde; 5-(hydroxym-ethyl)-methyl-beta-d-galactopyranoside. Moreover, the genotype also showed allyl isothiocyantes in small percentage. Fatty acid analysis of Indian mustard also showed the presence of good amount of oleic acid, linolic acid and palmitic acid. The detection of these compounds in seeds of mustard genotypes highlights the pharmaceutical properties of this crop. The results inferred that these genotypes contain variable patterns of bioactive compounds and could be used as natural antioxidant source for various herbal formulation as anti-inflammatory, analgesic, antipyretic, cardiac tonic and antiasthamatic.

Keywords: Indian mustard, GC-MS analysis, methanolic extract

Introduction
Brassica juncea (L.) Czern & Coss., belongs to plant family Brassicaceae (cruciferae). However, the bioactive phytochemicals present in them have not received attention as that of fruits and vegetables, despite they contain a unique blends of bioactive phytochemicals. Continuous improvement in rapeseed-mustard has resulted in nutritionally superior edible oil, and meal as an important source of protein in animal feed [1, 2]. They also possess antioxidant properties as they contain phenolic compounds [3, 4]. Nutritional quality of rapeseed-mustard seed is determined by oil content and its fatty acid constituents and various anti-nutritional factors including glucosinolates, phytic acid, sinapine etc [5, 6]. These factors are also very important as it can be used to provide defense response in plants [7, 8]. Quality characteristics of rapeseed-mustard oil have also been reported by earlier workers for various nutritional & antinutritional factors [9, 10, 11]. These quality characteristics are important as these can further be used for breeding programs. In spite of these pharmacological potential of seeds is not fully exploited yet. Phytochemicals in brassica are the most important compounds because of their nutraceutical potentials such as antioxidant, anti-inflammatory, anti-carcinogenic, antimicrobial, anti-diarrheal, antiulcer, and anti-cardiovascular properties [12]. The determination of phenolic acids is important both for their characterization and to facilitate more efficient uses of the important plant resources.

Many plants emit substantial amounts of phytogenic volatile organic compounds which include Alkanes, Alkenes, Alcohols, Aldehydes, Ethers, Esters and Carboxylic acids [13]. The synergistic effects of the chemical constituents present at different concentrations are the major factors for the biological activities of these volatile compounds [14]. These volatile compounds can be identified using gas chromatography mass spectrophotometry in a short time span with high accuracy.

HPLC techniques are preferred for efficient separation of metabolites, good selectivity and sensitivity of detection, together with the capability of providing on-line structural information [15]. At present, various plants have been analyzed with respect to phenolics by HPLC [16].

The present work aimed to compare the phytochemicals of Indian mustard with high oil content (>38%) through GC-MS assay of the methanolic extract by Soxhlet extraction. Further the phenolic acid characterization was done by HPLC and fatty acid analysis was done by GC. These techniques are less time consuming and highly sensitive. This research explores the possible medicinal value of the brassica through identification of the essential compounds present in the crops using highly sensitive and accurate techniques.

Material and Methods

Plant Material

Pure clean seeds (50 gm) of Indian mustard seeds were procured from ICAR-DRMR, Bharatpur, Rajasthan, India were used in the analysis.

Preparation of extract

Forty grams finely ground seeds of Indian mustard genotypes were soaked in 100 ml of 80 per cent methanol overnight and then filtered through whatmann filter paper No. 41. The filtrate was concentrated by removing methanol using rotary vapor at 68.7°C until the final volume of 1 ml. Samples were extracted for HPLC analysis by a slight modification of the method of Demiray [17] using methanol as extraction solvent.

Gas chromatography mass spectrometric assay (GC-MS)

The obtained extracts were analyzed for gas-chromatography mass spectrometry to identify the number of compounds. Agilent 6890 gas chromatograph and 5975B mass spectrometer in trace ion detection mode with a programmable temperature vaporizer injector (PTV) was used to characterize secondary metabolites and oleo/ aromatic compounds. One microliter of the extract was injected in the split mode at 280ºC (Agilent#5183-2037). The oven programming was set according to Medini [18]. All the mass spectra of the identified peaks were compared with the spectra from the NIST'05, WILAY spectral library and F.A.M.E mix (C8:C24). The results (quality match > 90%) for individual compounds were only reported as their percentage of the total area of peaks in the total ion chromatogram. Total GC running time was 50 minutes.

Interpretation of the mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST) which consists of more than 62,000 patterns.

High performance liquid chromatography (HPLC)

The methanolic extracts of the samples were filtered using pore size 0.45 µm, Millipore filters. 20 µl of the samples were injected into a loop injection valve of HPLC (Waters HPLC system). Running conditions included mobile phase methanol-0.4 % acetic acid (80:20, v/v), flow rate 1.0 ml/min, injection volume 5 µl and detection at 290 nm. GC-MS and HPLC chromatograms were obtained through outsourcing at Directorate of Agricultural Marketing, New Delhi.

Results and Discussion

We identified more than fifteen chemical constituents from the methanolic extract by GC, GC-MS, HPLC. Use of these techniques can easily identify the major volatiles, fatty acids and secondary metabolites in any crop with accuracy. These can be used for the identification of bioactive compounds in short time period.

The details of all identified compounds by GC-MS were grouped according to their chemical nature and are presented in Tables 1. Data showed the presence of non-polar phyto-components such as aromatic phenolic compounds, alcohols, aldehydes, alkenes (Figures 1). Major compounds identified were Methallyl cyanide, Acetic acid, Allyl Isothiocyanate, Oxime-, methoxy-phenyl-, 1-Butene, 4-isothiocyanato-, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 2-Furancarboxaldehyde, 5-(hydroxym ethyl)- and methyl .beta.-d-galactopyranoside. However the compound present in this genotype in highest amount is methyl .beta.-d-galactopyranoside with 23.21%. The compounds Methallyl cyanide, Acetic acid, and 2-Furancarboxaldehyde, 5-(hydroxym ethyl)-were also present in good amount. However allyl isothiocyanate, a potent anticancerous compound, is also present. This clearly depict that Indian mustard have pharmacological and industrial application (Table 6). Fatty acid analysis of these genotypes also showed the presence of higher amount of oleic acid and linoleic acid in the investigated extracts.

The reported saturated fatty acids oleic acid and linoleic acid (9,12-Octadecadienoic acid (Z,Z)-, methyl ester) are the most common fatty acids found in animals and plants and are primarily used to regulate blood pressure, blood clotting, blood lipid levels, the immune response, and the inflammation response to injury infection etc [19, 20]. Oleic acid, which is an omega–9 fatty acid as the major fatty acid also is equally important having all the health benefits of linoleic acid. In cases of reduced availability of omega-6-fatty acids, omega-9-fatty acids are converted to omega-6-fatty acids [21]. High amount of unsaturated fatty acids *viz.*, linoleic and oleic have been reported in Indian mustard earlier by Kunyanga [22]. The presence of high amounts of these essential fatty acids suggests that these food ingredients are highly nutritious, due to their ability to reduce serum cholesterol. Similarly, eicosenoic acids are reported to have antimicrobial and cytotoxic activity [23].

These compounds indicate their potential use for curing various diseases in traditional systems. The presence of these chemical compounds in Indian mustard demonstrate their potential as therapeutic, functional food and variation for these compounds can be capitalized for development of varieties with enhanced levels of such nutritional compounds.

Table 1: GC-MS Profiling

RT± 0.5 (min)	Components	Per cent Area (SD)
3.693	Methallyl cyanide	11.14
3.883	Acetic acid	10.98
5.151	Allyl Isothiocyanate	1.39
5.947	Oxime-, methoxy-phenyl-	0.38
6.732	1-Butene, 4-isothiocyanato-	4.19
9.850	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	1.97
11.386	2-Furancarboxaldehyde, 5-(hydroxym ethyl)-	15.42
17.061	Methyl .beta.-d-galactopyranoside	23.21
19.32	3,4-Altrosan	8.97

Table 2: Major compounds with their activity

S. No	Name of the compound	Molecular Formula	Nature of the compound	Activity
1.	Allyl Isothiocyanate	C_4H_5NS	Synthetic mustard oil	pungent taste , defense against herbivores
2.	2-furancarboxaldehyde, 5-(hydroxyethyl)-	$C_6H_6O_3$	5-(Hydroxyethyl) furfural	Inhibits the formation of sickled cells in the blood. Antimicrobial, Preservative
3.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	1. DDMP-4-one	Good immunomodulator, Antibacterial agent, Antineoplastic agent
4.	Oxime-, methoxy-phenyl-	RC(=NOH)(NRR')	Aldoximes/ Ketoximes	Antidotes for nerve agents, reactivate acetylcholinesterase
5.	3,4-Altrosan	$C_6H_{10}O_5$	3,4-Anhydrohexopyranose	Bacteriostat Fungicide
6.	Methyl .beta.-d-galactopyranoside	• $C_7H_{14}O_6$	Methylgalactoside	Antioxidant, antibacterial
7.	Methallyl cyanide	C_5H_7N	3-Butenenitrile	Biopesticide, cross-linking agent in some polymers

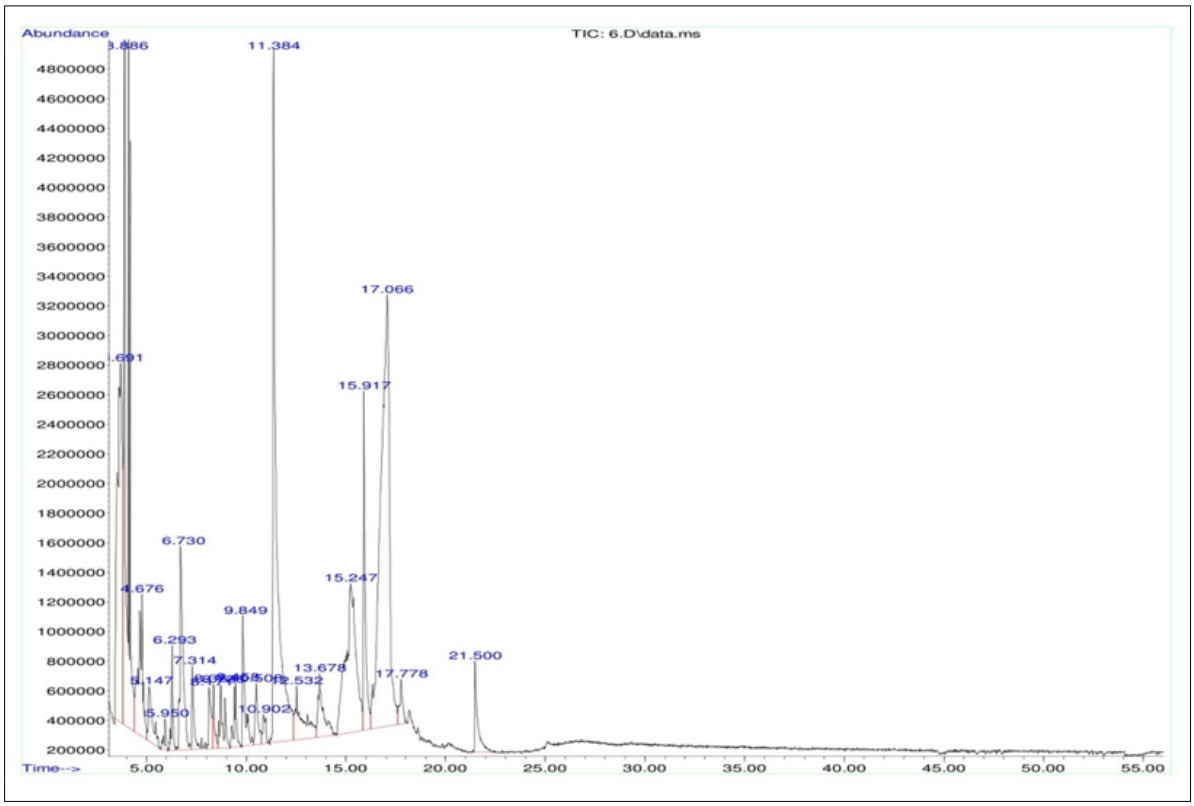

Fig 1: GC-MS analysis of methanolic extract of Indian mustard

HPLC analysis

The methanolic extracts of the samples were filtered using pore size 0.45 µm, Millipore filters. 20 µl of the samples were injected into a loop injection valve of HPLC (Waters HPLC system) with mobile phase methanol-0.4 % acetic acid (80:20, v/v), flow rate 1.0 ml/min, injection volume 5 µl and detection at 290 nm. Qualitative and quantitative estimation was carried out with integration area of standard and sample. Tannic (TA), gallic (GA), caffeic (Caf-A), ferulic (FA), and chlorogenic (Chl-A) were used as standards. The HPLC of samples was run at 290 nm using a reverse phase C-18 column.

HPLC analysis showed that methanolic extract possessed considerable amount of all studied phenolic acids other than cinnamic acid (Table 1). It showed significantly higher content of gallic acid (13.22 µg/g). Since gallic acid is highly anti-inflammatory in high amount, it is advisable for its daily consumption to those suffering from joint pains or inflammation of the body. Apart from this ferulic acid, chlorogenic acid, caffeic acid was also detected in the methanolic extract. Similarly, Sharma[7] have reported ferulic, p-coumaric and cinnamic acids as the major phenolics in finger millet which are known to express high antioxidant activity. Here also it has been reported that caffeic acid, ferulic acid and chlorogenic acid imparts high chelating and antioxidant activity. Few peaks remained unidentified in the chromatograms, which could be flavonoids, anthocyanins or even anthocyanidins, besides phenolics.

Table 3: Major phenolic compounds identified in Indian Mustard

Name	RT (min)	Std area	Phenolic acid (µg/g)
Caffeic acid	3.894	2515	0.41
chorogenic acid	3.282	2732	0.11
Gallic acid	4.145	2036	13.22
Sinapic acid	7.137	4393	1.09
Ferulic acid	5.068	3010	0.09

This is the first report showing the presence of an array of phenolic compounds and other phytochemicals involved in defense mechanism and antioxidant activity in rapeseed mustard plants.

Conclusion

In the present study, *Brassica juncea* were studied for the presence of volatile compounds and fatty acids. We identified more than fifteen chemical constituents from the methanolic

extract by GC, GC-MS, HPLC. Use of these techniques can easily identify the major volatiles, fatty acids and secondary metabolites in any crop with accuracy. These can be used for the identification of bioactive compounds in less time. The presence of allyl isothiocyanates and other bioactive compounds highlights the medicinal and pharmaceutical usage of Indian mustard. Further studies of medicinal properties of brassica are under progress. Fatty acid profile of the methanolic extract indicates that Indian Mustard has good edible value. Therefore, this study depicts the potential of brassica as pharmaceutical crop. Validation of the research is under progress.

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Conflict of interest: There is no conflict of interest.

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