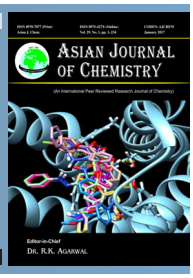
Asian Journal of Chemistry; Vol. 29, No. 2 (2017), 296-300

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2017.20171



Chromatographic Determination of Phenolics in *Brassica juncea*

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Received: 4 July 2016; *Accepted*: 16 September 2016; *Published online*: 30 November 2016; AJC-18148

Brassica juncea (Indian mustard) is an annual growing perennial herb. It contains various secondary metabolites which provide protective effects of the seeds. The objective of the present study was to perform the qualitative and quantitative phytochemical analysis of mustards seeds. Thin layer chromatography analysis identified six different phenolic compounds in all the genotypes. HPLC analysis also observed gallic acid, ferulic acid, sinapic acid, *p*-coumaric acid, *p*-hydroxy benzoic acid and caffeic acids as the major phenolic compounds in *Brassica juncea*. Sinapic acid was predominant compound among all the samples ranged from 13.42 to 15.73 mg/g DW. FA-305, FA-319, FA-337, FA-340, FA-348 showed all the six phenolic compounds with good amount of sinapic acid (14.03-15.73 mg/g DW) and gallic acid (7.26-7.76 mg/g DW). The results achieved will be used as a future source of the neutraceutical compounds and will be helpful to assess the therapeutic potential of seed extract for future pharmacological research.

Keywords: High performance liquid chromatography, *Brassica juncea*, TLC, Phenolic compounds.

INTRODUCTION

Brassica is the most important genus in the Brassicaceae family (syn. Cruciferae) economically and medicinally. It contains many health promoting and potentially protective phytochemicals including folic acid, phenolics, carotenoids, selenium, glucosinolates and ascorbic acids [1]. These endogenous bioactive phenolic compounds provides powerful, broad-spectrum protection against the cancer provoking agent encountered due to the role of phenolic antioxidants in human nutrition and health [2]. Studies have shown that many of these functional compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities [3].

The biosynthesis of phenolic acids starts with the release of ammonia from phenylalanine catalyzed by phenylalanine ammonia lyase, leading to the formation of a carbon-carbon double bond that yields *trans*-cinnamic acid. A hydroxyl group is introduced into the *para* position of the phenyl ring of cinnamic acid *via* catalysis by monooxygenase, yielding *p*-coumaric acid (Fig. 1), which is then hydroxylated further in positions 3 and 5 by hydroxylases and methylated *via* methyl transferases to form caffeic acid, ferulic acid and sinapic acid [4].

Since these compounds possess a phenyl ring and a C3 side chain, they are termed phenylpropanoids [5]. Phenolic acids (Fig. 1) are major chemical constituents of phenylpropanoid pathways and are mainly located in seed cotyledons and only trace amounts are found in seed coats. The determination of phenolic acids is important both for their characterization and to facilitate more efficient uses of the important plant resources.

In order to promote the use of crops with medicinal value, it is important to thoroughly investigate phenolic composition and activity and thus validate their use [6]. There are several chromatographic methods for the quantitative and qualitative determination of phenolic acids in plant. In the last few years, spectroscopic methods have become firmly established as a key technological platform for secondary metabolite profiling in both plant and non-plant species [7,8]. In this study, analytical methods (TLC and HPLC) were established to determine the nonvolatile phenolic compounds present in *Brassica juncea* (Indian mustard). This work will suggest the possible use of rapeseed genotypes with higher phenolics in neutraceutical industry due to its potent bioactive attribute.

EXPERIMENTAL

Pure clean seeds of 35 exotic lines of Rapeseed Mustard of ICAR-DRMR, Bharatpur, India were used in the analysis.

Sample preparation: Samples were extracted with a slight modification of the method of Rehman [9] and Demiray *et al*. [10] using aqueous methanol. The brassica seeds were homogenized in pestle and mortar at room temperature with aqueous methanol (methanol:water, 70:30 v/v). Extracts were

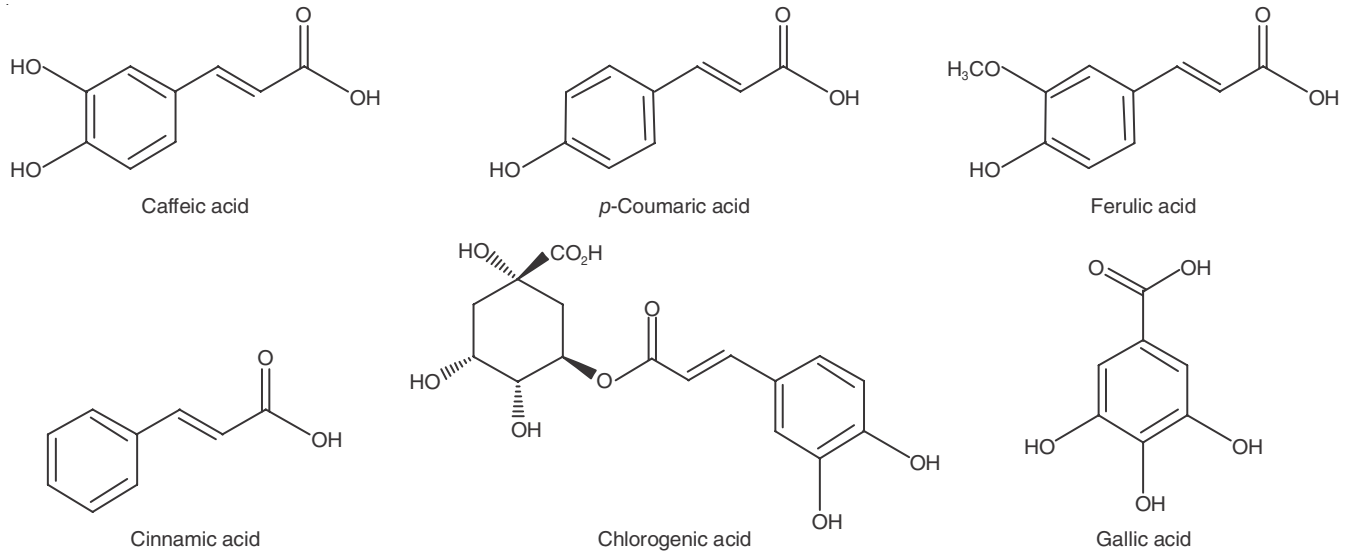

Fig. 1. Chemical structure of phenolic acids in mustard

centrifuged at 4000 rpm for 0.5 h and the residues were re-extracted under the same conditions. Supernatants were pooled and combined and evaporated with a rotary evaporator. Extracts were stored at 4 °C for biochemical studies. To maintain the quality of the data, all the experiments were carried out in triplicates and were validated by using standards wherever required.

Estimation of polyphenols: Extraction of polyphenols from defatted seed flours were performed according to the slight modification of the optimum polyphenols extraction that had been studied previously [11]. Briefly, defatted seed flours were extracted with a mixed solvent of methanol:acetone: water (7:7:6, v/v) using a Soxhlet apparatus at 70 °C for 0.5 h. The sample to solvent ratio was 1:10 (g/mL). The slurries were centrifuged at 13.000× g for 15 min to remove the solid materials and the supernatants were stored at 4 °C prior to analysis. After 1:2 dilution with methanol, this extract (5 %) was subjected to analysis.

Thin layer chromatography separation: Thin layer chromatography was used for the conformation of the different secondary metabolites on analytical plates. 10 µL of the extract and standards were loaded on the analytical plate (2.5 cm above from the bottom) and dried on air for 0.5 h. The spotted plates were kept in a previously saturated developing chambers containing mobile phase and allowed to run 3/4th of the height of the prepared plates [12]. The solvent system contains petroleum ether:benzene:methanol (16:3:2) as mobile phase. The different bands of chromatograms were observed under visible light and photographed.

Here different solvents were used for identification of secondary metabolites with different spraying solutions (Table-1). The spots were marked with lead pencil and their R_f values were calculated using the formula:

$$R_f = \frac{\text{Distance (cm) traveled by the spot from the origin}}{\text{Distance (cm) traveled by the solvent from the origin}}$$

R_f values of standards are given in Table-1.

HPLC analysis: The methanolic extracts of the samples were filtered using pore size 0.45 µm, Millipore filters. Samples (20 µL) were injected into a loop injection valve of HPLC (Waters HPLC system) equipped with photodiaode detector and analog pump connected to controller. 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. Gallic acid (GA), caffeic acid (CA), sinapic acid (SA), ferulic acid (FA), *p*-coumaric acid (*p*-Cou-A) and hydroxyl benzoic acid (BA) were used as standards. Phenolic compounds present in the sample were identified by comparing retention time (Rt) of standards, *e.g.*, gallic acid (retention time 2.06 min), sinapic acid (retention time 2.88 min), ferulic acid (retention time 3.42 min), *p*-coumaric acid (retention time 4.22 min), caffeic acid (retention time 4.71 min) and hydroxyl benzoic acid (retention time 5.45 min). The HPLC of samples was run at 290 nm using a reverse phase C-18 column. During the run, a flow rate of 1 mL/min was maintained using isocratic mode for 10 min. Triplicate samples were analyzed for statistical validation of results.

For quantitative determination of various peaks, the integration area values of different standard phenolic acids with

TABLE-1
R_f VALUES OF SOME OF THE EXOTIC LINES OF BRASSICA

Solvent mixtures	Spraying solutions	Colour appeared	R_f values						
			CA	FA	SA	*p*-Cou-A	FA-305	FA-319	FA-348
Benzene:glacial Acetic acid:water	Fuming with ammonia	Yellow and brown spots	–	0.69	0.67	0.73	0.69, 0.67, 0.73	0.73, 0.69, 0.67	0.73, 0.67, 0.69
Methanol:water	Fuming with ammonia	Brown spots	0.72	0.77	0.75	0.79	0.77, 0.75, 0.72, 0.79	0.77, 0.75, 0.72, 0.79	0.74, 0.77, 0.75, 0.72
Chloroform:water	Diazotized *p*-nitro aniline	Blue spot under UV	0.24	0.54	0.59	0.63	0.63, 0.55	0.23, 0.54	0.63, 0.57

known concentration were compared with the sample peaks and the phenolic content was calculated accordingly. Integrations and data storage were carried out by means of a Pentium-III computer.

Data expression and analysis: All the experiments were repeated thrice for their validation. The results are expressed as the mean ± standard deviation of the mean (SD).

RESULTS AND DISCUSSION

A large number of potential classes of phytochemicals with antibacterial, antifungal and anticancerous activity have already been reported in *Brassica oleracea* and *Brassica napus* [13]. The phenolic profile of mustard seeds is less complex as compared to mustard greens and most of the hydroxycinnamic acids except sinapic acid derivatives were not reported in *Brassica juncea*. Therefore, in the present study, phenolic acid profiling in *Brassica juncea* extract was carried out.

Thin layer chromatography qualitative profile showed the presence of various phytoconstituents: The fingerprinting of extract by TLC was carried out to detect the presence of various phytoconstutients that could be present in the extract that are reported to be antioxidants and carcinoprotective [14]. A total of six to eight distinct bands were observed under different spraying conditions with R_f 0.69, 0.67, 0.70, 0.72, 0.73, 0.75, 0.77 and 0.79. Based on R_f values of standard phenolic acids, various types of phenolic acids *viz.* sinapic acid, gallic acid, ferulic acid, caffeic acid, 4-hydroxybenzoic acid and *p*-coumaric acid were identified in all genotypes (Table-1). These phenolic acid were already reported as strong antioxidants [2].

From present data, the chromatogram shows the presence of coloured spots *i.e.* yellow, orange and brown which confirms the presence of phenolic compounds [15]. When ammonia fumes were applied on the sample running TLC plates as spraying solutions (mobile phase is benzene:glacial acetic acid:water) then three spots have been observed in all the genotypes. The chromatogram shows two dark brown and one light brown spot in most of the cultivars. The dark brown spot authenticates the presence of ferulic acid (R_f value 0.69) and sinapic acid (R_f value 0.67) (Fig. 2a). Similarly, light brown colour indicates the presence of coumaric acid (R_f value 0.73). These coloured bands reflects the presence of common phenolic acids *e.g.* sinapic acid, ferulic acid, coumaric acid in this solvent system.

On the other hand, when sample loaded TLC plates were kept in methanol:water solvent system for 3-4 h and after 0.5 h these were sprayed with ammonia fumes, four major spots were observed in most of the genotypes (Fig. 2b). Among these four spots, three were of light brown in colour and the last one is of light yellow-brown and these spots were considered as caffeic acid (R_f value 0.72), sinapic acid (R_f value 0.75), ferulic acid (R_f value 0.77) and coumaric acid (R_f value 0.79).

Another solvent used for development of spots on silica gel TLC plates for identification of phenolic compounds was chloroform: water and spraying material was diazotized *p*-nitroaniline, two greenish blue coloured spots were identified under UV light, however these were not visible with the naked eyes and the R_f value of these spots were aligned with the R_f value of coumaric acid and ferulic acid (Fig. 2c). Hence, from the present investigation, it is clear that a large number of phenolic compounds are present in the exotic lines of rapeseed mustard. These results are in agreement with Lavid *et al.* [16]. They reported that the green phenol moiety consists mainly of polyphenols, hydrolyzable tannins and gallic acid derivatives in *Nymphaea*.

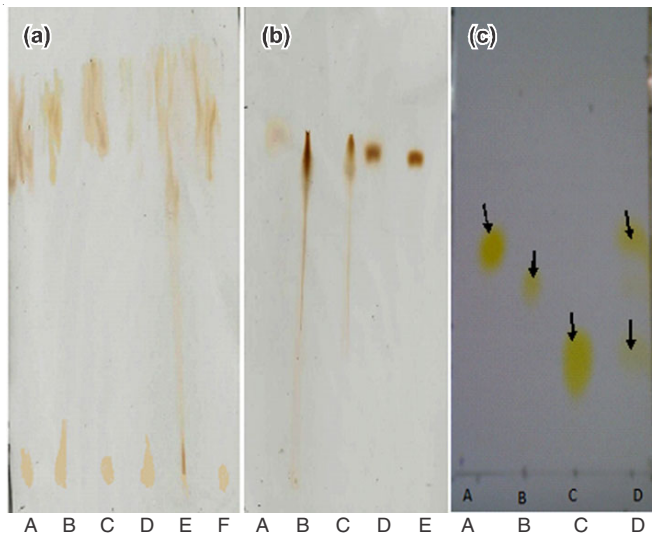

Fig. 2. (a) TLC of polyphenol content of exotic lines with methanol:water and then applied ammonia fumes; A: Coumaric acid, B: Ferulic acid, C: Sinapic acid, D: FA-319, E: FA-323, F: Coumaric acid; (b) TLC of polyphenol content of exotic lines with benzene:acetic acid:water and then applied ammonia fumes; A: Caffeic acid, B: Sinapic acid, C: Ferulic acid D: Coumaric acid, E: FA-319l; (c) Thin layer chromatography of polyphenol content of exotic lines with chloroform:water and then applied diazotized *p*-nitro aniline; A: Caffeic acid, B: Sinapic acid, C: Ferulic acid D: FA-319

Separation of crude extract by solid phase extraction (HPLC) confirms the presence of various phytochemicals: Quantitative analysis of polyphenol contents was carried out by HPLC using two solvent systems at a concentration of 60:40 with a flow rate of 1 mL min^{-1} at 320 nm wavelengths. The peaks were analyzed by comparing the retention time of the standard phenolic compounds with methanolic extracts of all the genotypes. Six major phenolic acids (Fig. 3) have been identified and quantified in all the brassica genotypes. Brassicaceous plants have characteristic hydroxycinnamate conjugates such as hydroxyl benzoic acids, ferulic acids, caffeic acids and sinapic acids *etc.* [17].

It was found that *p*-hydroxy benzoic acid, gallic acid and sinapic acid are the most common phenolics present in almost all the genotypes. Other phenolic acids such as caffeic acid, ferulic acid, coumaric acid (Fig. 3) were also present in some genotypes in detectable amount. In this study HPLC analysis showed that FA-305, FA-316, FA-319, FA-337, FA-340, FA-345 and FA-348 possessed considerable amount of all studied phenolic acids other than ferulic acid (Table-2). *p*-Hydroxy benzoic acid is a popular antioxidant and is isomeric with 2-hydroxy benzoic acid which is known as salicylic acid, a precursor to aspirin. 4-Hydroxy benzoic acid has estrogenic activity both *in vitro* and *in vivo* [18] and stimulates the growth of human breast cancer cell lines [19]. In this report FA-319

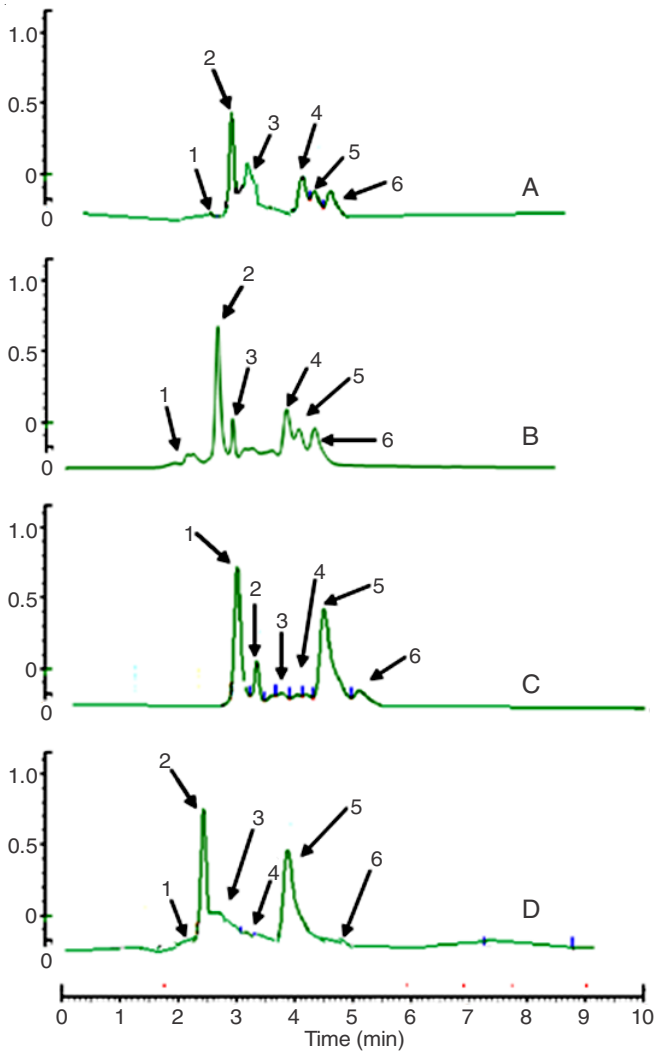

Fig. 3. Graph no. A: FA-305, B: FA-319, C: FA-340, D: FA-348; Peaks # 1. Gallic acid (GA), 2. Sinapic acid (SA), 3. Ferulic acid (FA), 4. *p*-Coumaric acid (*p*-Cou-A), 5. Caffeic acid (CA), 6. *p*-Hydorxy benzoic acid (BA)

(7.12 µg/g) has the highest amount of 4-hydroxy benzoic acid followed by FA-338 (5.42 µg/g), FA-305(5.06 µg/g), FA-336 (4.99 µg/g) and FA-348 (4.95 µg/g). Similarly here our data clearly shows that FA-307 showed significantly higher content of gallic acid (8.17 µg/g). Other genotypes *e.g.* FA-305, FA-319, FA-345 also possess good amount of gallic acid (7.26, 7.61 and 8.00 µg/g) respectively. Gallic acid (3,4,5-trihydroxy-benzoic acid), a naturally occurring low molecular weight triphenolic compound, has emerged as a strong antioxidant and an efficient apoptosis inducing agent [20]. They observed that gallic acid derivatives have diverse biological and pharmacological activities, including radical scavenging, interfering with the cell signaling pathways and apoptosis of cancer cells. This simple polyphenol has potential balance between its antioxidant and prooxidant potential. However perusal of data clearly indicates that in this study sinapic acid is the major phenolic compound, which is present in all the genotypes of exotic lines of brassica. Out of all genotypes FA-337 (15.73), FA-316 (15.05) has the highest amount of sinapic acid. Whereas FA-315 (15.13), FA-327 (15.10), FA-331 (15.22) and FA-319 (14.73) also have promising amount. Our results are in agreement with Mayengbam *et al.* [21], where they observed that sinapic acid is the major phenolic content in *Brassica napus*. Siger *et al.* [22] identified and quantified sinapic acid derivatives in the crude extracts of *Brassica napus L.* seeds and indicated a high amount of total phenolics. Milkowski and Strack [23] showed that sinapic acid is synthesized *via* shikimate/phenylpropanoid pathway and its conversion to O-ester conjugates includes multiple enzymes. They also speculated that this choline ester might work as a storage vehicle for the biosynthesis of phosphatidyl choline thereby strengthening the cell membrane. This it can be hypothesized that these high sinapic acid lines have more protection from biotic and abiotic stresses. In this study ferulic acid was detected only in 10 genotypes (*e.g.* FA-316, FA-319, FA-323 *etc.*) whereas coumaric acid was noticed in 11 genotypes (FA-305,

TABLE-2
QUANTIFICATION OF PHENOLIC ACIDS USING HPLC *e.g.* GALLIC ACID (GA), FERULIC ACID (FA), SINAPIC ACID (SA), *p*-COUMARIC ACID (*p*-Cou-A), *p*-HYDROXY BENZOIC ACID (BA) AND CAFFEIC ACIDS (CA)

Genotypes	BA	GA	CA	SA	FA	*p*-Cou-A
FA-305	5.06 ± 0.09	7.26 ± 0.72	1.39 ± 0.25	14.20 ± 0.13	0.13 ± 0.08	0.23 ± 0.08
FA-307	4.85 ± 0.05	8.37 ± 0.39	1.33 ± 0.06	15.06 ± 0.06	ND	ND
FA-311	3.85 ± 0.19	7.47 ± 0.09	1.44 ± 0.17	15.07 ± 0.11	0.27 ± 0.11	ND
FA-312	3.94 ± 0.12	7.39 ± 0.18	ND	14.96 ± 0.07	ND	0.70 ± 0.08
FA-313	4.14 ± 0.12	7.95 ± 0.06	1.44 ± 0.07	15.02 ± 0.18	ND	ND
FA-315	3.94 ± 0.11	7.28 ± 0.17	1.40 ± 0.02	15.13 ± 0.11	ND	ND
FA-316	4.73 ± 0.16	7.84 ± 0.15	1.48 ± 0.16	15.05 ± 0.09	0.13 ± 0.12	0.94 ± 0.08
FA-317	3.91 ± 0.03	7.78 ± 0.11	1.44 ± 0.17	15.10 ± 0.02	ND	ND
FA-318	4.02 ± 0.18	7.90 ± 0.12	1.51 ± 0.08	14.81 ± 0.23	ND	ND
FA319	7.12 ± 0.10	7.61 ± 0.16	1.43 ± 0.06	14.73 ± 0.09	0.17 ± 0.07	0.90 ± 0.21
FA-321	3.70 ± 0.16	7.56 ± 0.11	1.39 ± 0.03	14.96 ± 0.04	ND	ND
FA-322	4.96 ± 0.06	ND	ND	14.92 ± 0.21	ND	ND
FA-323	ND	8.05 ± 0.17	ND	13.87 ± 0.12	0.27 ± 0.23	ND
FA-324	5.07 ± 0.05	8.05 ± 0.07	ND	14.01 ± 0.03	ND	ND
FA-326	4.73 ± 0.11	ND	ND	14.99 ± 0.09	ND	ND
FA-327	4.82 ± 0.12	8.17 ± 0.18	1.19 ± 0.14	15.10 ± 0.02	ND	ND
FA-328	ND	7.45 ± 0.11	ND	14.01 ± 0.16	ND	1.04 ± 0.40
FA-329	4.07 ± 0.08	ND	ND	14.91 ± 0.19	ND	ND
FA-330	4.48 ± 0.07	7.72 ± 0.05	1.20 ± 0.03	14.98 ± 0.10	0.28 ± 0.16	ND
FA-331	4.79 ± 0.08	7.35 ± 0.24	ND	15.22 ± 0.19	ND	ND

Genotypes	BA	GA	CA	SA	FA	*p*-Cou-A
FA-332	4.25 ± 0.06	7.71 ± 0.22	ND	13.74 ± 0.34	0.14 ± 0.08	ND
FA-333	4.75 ± 0.03	7.44 ± 0.16	ND	13.42 ± 0.51	ND	1.13 ± 0.46
FA-334	4.91 ± 0.09	7.68 ± 0.25	ND	13.47 ± 0.57	ND	ND
FA-335	ND	ND	ND	14.29 ± 0.13	ND	0.78 ± 0.16
FA-336	4.99 ± 0.09	7.62 ± 0.17	1.68 ± 0.30	14.49 ± 0.36	ND	ND
FA-337	3.82 ± 0.09	7.69 ± 0.20	1.30 ± 0.01	15.73 ± 0.08	0.15 ± 0.08	0.75 ± 0.04
FA-338	5.42 ± 0.21	7.43 ± 0.24	1.44 ± 0.08	13.35 ± 0.15	ND	ND
FA-339	4.74 ± 0.05	7.42 ± 0.03	1.44 ± 0.16	14.19 ± 0.22	ND	ND
FA-340	4.44 ± 0.09	7.28 ± 0.11	1.42 ± 0.04	14.48 ± 0.30	0.18 ± 0.10	1.00 ± 0.24
FA-341	4.38 ± 0.14	7.78 ± 0.10	1.39 ± 0.25	13.88 ± 0.30	ND	ND
FA-342	3.95 ± 0.06	7.23 ± 0.12	1.14 ± 0.07	12.84 ± 0.21	ND	ND
FA-343	4.28 ± 0.13	7.59 ± 0.30	1.15 ± 0.11	13.69 ± 0.14	ND	ND
FA-345	4.27 ± 0.13	8.00 ± 0.22	1.39 ± 0.24	13.23 ± 0.29	ND	1.03 ± 0.09
FA-347	ND	7.55 ± 0.33	1.31 ± 0.22	14.46 ± 0.19	0.28 ± 0.16	ND
FA-348	4.95 ± 0.05	7.72 ± 0.27	1.13 ± 0.08	14.03 ± 0.05	0.14 ± 0.07	0.99 ± 0.16

ND = Not detected

FA-319, FA-337, FA-345, FA-348 *etc*.). Ferulic acid has been reported to have many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis and anticancer activities [24,25]. Ferulic acid and coumaric acid are known to express high antioxidant activity in cereals [3]. In this study, few peaks remained unidentified in all the chromatograms, which could be flavonoids, anthocyanins or even anthocyanidins, besides phenolics (Fig. 2a,b,c). Further, our results are in agreement with Marton *et al*. [26], as they also mentioned the higher amount of phenolic compounds in brassica species. Present workers also observed that among all the studied phenolics, sinapic acid was present in higher quantity in all the genotypes. These are the similar results as shown by Mayengbam *et al*. [21] and Karamac *et al*. [27]. It has been already reported that sinapic acid is the major phenolic acid followed by ferulic acids [28], caffeic acid and gallic acid [29] in rapeseed mustard extracts. Therefore it can be concluded that FA-305, FA-319, FA-337, FA-340, FA-348 showed all the six phenolic compounds with good amount of sinapic acid (14.03-15.73 mg/g DW) and gallic acid (7.26-7.76 mg/g DW). These genotypes can be further used as a good source of antioxidants.

Conclusion

In this study, since all rapeseed mustard genotypes contain sinapic acid, which is natural antioxidant in high amount. It is advisable for its daily consumption to minimize the aging process of the body. This is the first report showing the presence of an array of phenolic compounds and their determination in rapeseed mustard genotypes. These results clearly indicates that rapeseed mustard is a very good source of antioxidants, which could alleviate the wide spread diseases in India. This work will definitely provides a scientific rationale for the use of brassica as a therapeutic and health building food.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge ICAR-Directorate of Rapeseed Mustard Research, Bharatpur, India for providing the necessary research facilities.

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