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Secondary volatiles and metabolites from *Nigella sativa* L. seed

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Despite Indian nigella being widely used in various herbal preparation across the world, the composition of volatile fraction and seed metabolite content has been poorly investigated. The nigella crop was raised in Coastal Humid Tropics of Andhra Pradesh and the seed collected was used for chemoprofiling. The extraction was done with MTBE solvent for 2 h to extract volatile fraction and 24 h for extracting entire seed metabolite content. The volatile fraction contained 28 different compounds where as the entire seed metabolite content composed of as many as 150 compounds. The thymoquinone content, which is the most important bioactive compound, was 28.70 %, followed by p-cymene which contained 27.8 % in the volatile fraction. The seed metabolites of nigella seed contained fatty acids, volatiles, and other metabolites. The GC-MS profile of fatty acids and related compounds in the seed metabolites contained five fatty acids (Linoleic acid, palmitic acid, cis-13,16-docosadienoic acid, stearic acid, and myristic acid) and one methyl ester of oleic acid (Methyl oleate). A total of 91.39 % in the total seed metabolite content were fatty acid and related compounds. The volatile fraction was only 5.94 %, remaining were other metabolites (2.67 %). Among these, thymoquinone and p-cymene were the most important compounds (≥ 1 %). The seed of nigella is a storehouse of diverse compounds. Research on chemical nature and bio-potent action of seed metabolites other than secondary volatile metabolites is vastly unexplored and needs immense attention.

Keywords: Metabolite, *Nigella sativa* L., Thymoquinone, Volatile oil.

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Introduction

Nigella (*Nigella sativa* L.) is a field crop that belongs to Ranunculaceae, the butter cup family. The seeds or grains resembling onion seed are used both as spice and medicine. The word nigella originated from the Latin word *niger* which means blackish referring to its seed colour. The crop is known by many common names like black-caraway, black-cumin, fennel-flower, nigella, nutmeg-flower, and roman-coriander. It is considered to have originated in the Mediterranean region and subsequently spread to Europe, Asia, and Africa. Seeds of this plant are used both as spice and medicine since a very long time. The seeds are bitter to taste due to the presence of a seed protein, nigellin. The spice is highly regarded for its medicinal values in Greco Arab/Unani Tibb system of medicine, which originated from Hippocrates and other ethno-medicinal systems like *Ayurveda* and *Siddha*[1]. The seeds contain around 30 % fixed and 0.3 to 0.4 % essential oil. Its pharmacological action, such as anti-tumor, anti-diabetic, cardioprotective, gastroprotective, antiasthmatic, nephroprotective, hepatoprotective, antiinflammatory, immunomodulatory, neuroprotective, anticonvulsant, anxiolytic, antioxidant, antinociceptive, antioxytocic, contraceptive, abortifacient, antiimplantation, diuretic, antiurolithiatic, antispasmolytic, antibacterial, antifungal, anti-schistosomiasis and anthelmintic activities were immensely appreciated[2]. The major medicinal components namely thymoquinone and nigellone (a dimer of thymoquinone) were attributed to impart anti-tumour, anti-inflammatory, and anti-diabetic properties[3]. Numerous other important pharmacological effects, which are yet to be understood, might contribute to the above-mentioned effects. Consequently, a snap-shot of the bio-active compounds is required to understand the quality of the herbal products derived from nigella seed[4]. India is the largest producer and exporter of nigella. The Indian origin nigella is used widely in the Mediterranean countries, Europe, and the United States of America[5]. The chemical composition of nigella from different countries is widely reported, however, there is a paucity of data from India, the main exporter of the nigella.

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The objective of the present study was to investigate the chromatographic profile of the volatiles and the seed metabolites in nigella seed of Indian origin. The study of volatile metabolites helps not only in assessing the flavour quality of the seed, but is also useful for chemotaxonomy of the genus *Nigella*. The investigation of occurrence of different metabolites in seed will help in isolation methods for valuable bioactive compounds from the raw seed material.

Material and Methods

Plant material

Nigella sativa 'Ajmer Nigella-1' (Plate 1) was cultivated during the 2013-14 winter season at Lam, Guntur, Andhra Pradesh, India with the recommended

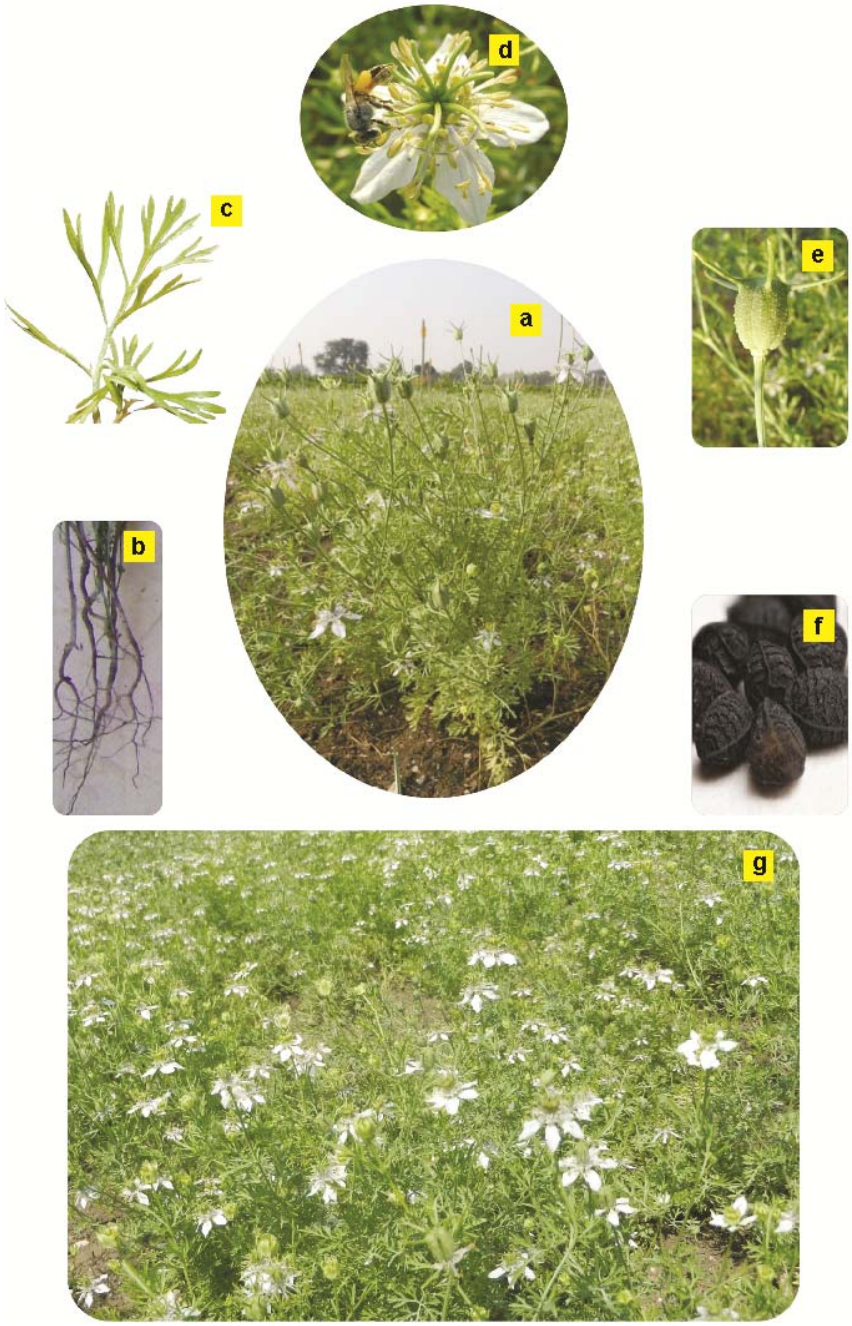

Plate 1 — Description of the variety Ajmer Nigella-1 (a:plant, b:roots, c:leaf, d: flower, e: fruit, f: seed, g: field view of the crop)

package of practices. The variety has been developed by National Research Center on Seed Spices, Ajmer, India and is popular in black cumin growing areas of India. The cultivar contains approximately 0.35 % volatile oil, which was determined by hydrodistillation. The produce obtained from this crop was used for experimental evaluation. The plant came to flowering in 35-40 days. The fruits set, matured by 55 days and ready for seed extraction (Plate 2). The freshly harvested seed was dried to constant weight (residual moisture content approximately 7 %) in a drying cabinet at 35 ^{o}C and were used for carrying out the assays.

Extraction of volatile oils and seed metabolites

The extraction of volatile secondary metabolites was carried as per the procedure[6] mentioned in Botnick *et al*. One gram of the seeds was weighed and frozen in liquid N_2. The seeds were then ground with a mortar and pestle. Extraction of the volatile fraction was made by adding a 3 to 1 ratio (v/w) of tert-butyl methyl ether (MTBE). After a short vortex, the ground seeds were shaken for 2 h at room temperature (25-30 ^{o}C) for extracting volatile fraction. For extracting entire seed metabolites, the grounded seeds were incubated in the rotary shaker for 24 h. Then, the extract was passed through a sodium sulfate column (Pasteur pipette) to remove excess water. The extract was then stored at 4 ^{o}C in amber coloured vials for GC-MS analysis.

GC-MS analysis

After sample extraction, 1 µL of the extract was injected into a GC-MS (CLARUS 600, Perkin Elmer, USA) equipped with a TG-5MS capillary column (30 m x 0.25 mm x 0.25 µm). The inlet was set to 280 °C with a split ratio of 25. Helium at a flow rate of 1.0 mL/min was used as carrier phase. The initial temperature was 40 °C and temperature gradient was programmed to 5 °C/min till 190 °C and then 15 °C/ min to 300 °C and held for 10 min. The temperature of quadrupole detector was set to 280 °C and scanning range was between 40–650 m/z. Kováts retention indices (KIs) of the components were calculated by interpolation of retention indices of C8-C22 series for volatile extract and C8-C40 series of seed metabolites[7]. The components were identified by i) comparing their mass spectra with reference MS from commercial libraries (NIST 08 and NIST 08s), ii) the pherobase[8], iii) literature data and data of authentic compounds, and iv) comparing their calculated KIs with those of authentic standards and the data published in the literature. The contents (%) of compounds were determined from the peak areas in chromatograms, without using correction coefficients.

Results and Discussion

The extraction conducted for 2 h could successfully extract the volatile fraction for GC-MS analysis. The characterization of volatile secondary metabolite profile was carried out by GC–MS detection (Fig.1) and is listed in Table 1. A total of 28 compounds were identified from the profile. The thymoquinone content, which is the most important bioactive compound, was 28.70 %, followed by p-cymene which was 27.8 % in the volatile fraction. The chemoprofile of the volatile fraction of black cumin was reported to vary by origin of the plant and produce[9], season and time of cultivation[10], crop management aspects such as nutrient and irrigation management[11] and extraction method[12]. The

Plate 2 — Developmental changes in *N. sativa* L. fruit and seed

presence, yield, and composition of the volatile metabolites in plants from their formation in the plant to their final isolation are influenced by environmental factors[13].

Nigella essential oil is very rich in terpenic compounds such as hydrocarbons, alcohols, ethers, ketones, aldehydes, and phenols. All these compounds are more or less known for their

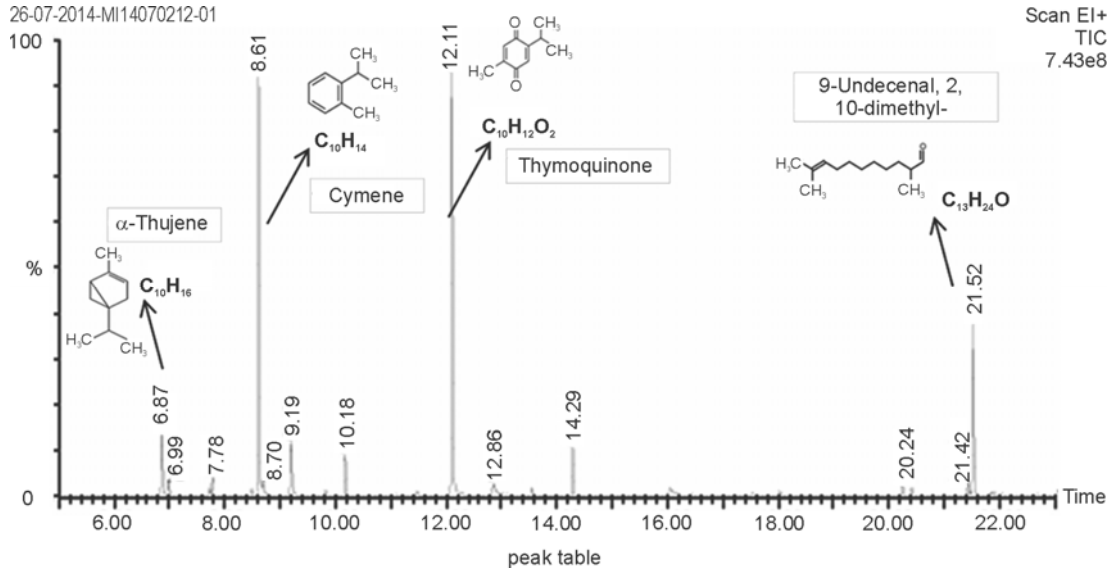

Fig. 1 — Mass spectrum with depiction of important volatile

Table 1 — Composition of volatile fraction of *N. sativa* L. seeds

Compound	Volatile secondary metabolites	RT	KI	Area %
1	α-Thujene	6.872	925	5.09
2	α-Pinene	6.991	935	1.00
3	Sabinene	7.725	965	0.44
4	β-Pinene	7.776	972	1.31
5	Terpinolene	8.492	1023	0.56
6	p-Cymene	8.612	1018	27.80
7	α-Terpinene	9.192	1050	5.23
8	cis-4-Methoxythujane	9.823	1090	0.44
9	trans-4-Methoxythujane	10.181	1110	3.62
10	2E,4Z- Decadienal	10.915	1290	0.14
11	Terpinen-4-ol	11.102	1170	0.21
12	(+)-Dihydrocarvone	11.477	1187	0.58
13	Thymoquinone	12.109	1223	28.70
14	Carvacrol	12.859	1283	2.44
15	Acetamide, N-(2-hydroxy-3-pentenyl)-	13.098	1560	0.22
16	Longipinene	13.558	1350	0.61
17	Longifolene	14.292	1400	3.24
18	Thymohydroquinone	16.049	1525	2.40
19	Nonyl allyl oxalate	16.441	1750	0.24
20	Sulfurous acid, 2-ethylhexyl isohexyl ester	17.533	1919	0.32
21	1. 2,3-epoxy-gerianial	18.027	1871	0.45
22	2-methyl hexanoic acid	19.852	1750	0.16
23	4-Tridecene	20.245	1780	0.92
24	citronellyl n-butyrate	20.415	2100	0.88
25	cis-11,14-Eicosadienoic acid methyl ester	21.422	2310	0.81
26	9-Undecenal, 2,10-dimethyl-	21.524	2075	11.54
27	2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene	21.899	2125	0.47
28	2,4-Octadienal, (E,E)-	22.053	2225	0.15

RT, Retention time; KI, Kováts indices, relative to C8–C22 n-alkanes on TG-5MS capillary column.

bioactivity. The oils with high concentrations (terpenes, terpenoids, and molecules with an aromatic ring) are highly bioactive[14], as is the case of nigella. Essential oils with aldehydes or phenols as major components are the most effective, followed by essential oils containing terpene alcohols[15]. Essential oils with ketones or esters possess lower activity[16]. Compounds possessing aromatic hydroxyl and acetoxyl group, such as carvacrol and thymol, had a more potent activity than aspirin, which is well known as a remedy for thrombosis[17] and these compounds were reported to have valuable bacteriostatic action[18].

The other major phenolic compounds thymoquinone, dithymoquinone, thymohydroquinone are widely known for their bioactivity. The most abundant one, thymoquinone, has been reported to exhibit antioxidant[19], anti-inflammatory[20], chemosensitization and chemopreventive potential effects[21], neuroprotective activity[22], anti-rheumatism activity[23], cardioprotective nature[24], hepatoprotective[25], and anti-cancer drug[26].

The bioactivity of the aforementioned compounds is quite pronounced in biological systems, even though they are less soluble in water. Essential oil of nigella comprised of a large number of compounds. It is likely that their mode of action involves several targets in bacterial cell[27]. The relative position of the hydroxyl group and the nature of bonds in the compounds could change the bioactivity of the compounds to a large extent; nonetheless stereochemistry has an influence on the bioactivity[28]. The biological activity of nigella may be due to the presence of major components, the role of minor components in synergistic, additive, and antagonistic effects cannot be ruled out. The essential oil of nigella and its components are potent food preservatives, must be explored for potential use in food industry[29].

The long incubation time (24 h) allowed the solvent to penetrate the macerated tissue more and many non-volatile metabolites dissolved in the solvent. Particularly, the fatty acids and related compounds were readily accumulated in the solvent along with other seed metabolites. The contents of p-cymene and thymoquinone, the major volatile components in *N. sativa* were seen in representative quantities in volatile fraction as well as in the entire seed metabolite contents. The seed metabolite composition indicated that as many as 150 compounds from the extract were identified by GC-MS detection compared to 28 compounds in the volatile fraction. The GC-MS profile of fatty acids and related compounds (Table 2, Fig. 2) in the seed metabolites indicated that the MTBE extract contained five fatty acids (Linoleic acid, palmitic acid, cis-13,16-docosadienoic acid, stearic acid, and myristic acid) and one methyl ester of oleic acid (Methyl oleate). A total of 91.39 % in the total seed metabolite content were fatty acid and related compounds. The volatile fraction was only 5.94 %, remaining were other metabolites.

Table 2 — Fatty acids and related compounds in seed metabolites in *N. sativa* L. seeds

Compound	Fatty acids and related compounds in seed metabolites	RT	Area %
1	Linoleic acid	19.838	68.14
2	Palmitic acid	18.078	12.14
3	Methyl oleate	23.719	6.61
4	cis-13,16-docosadienoic acid	21.199	2.39
5	Stearic acid	19.958	1.90
6	Myristic acid	15.938	0.21
	Total	-	91.39

RT, Retention time

The GC-MS profile of the volatile fraction indicated the presence of 19 major compounds in the MTBE extract (Table 3). Among these, thymoquinone and p-cymene were the most important compounds (≥ 1 %). The proportions of these compounds changed with the period of incubation indicating differential response of these compounds to the period of incubation. Such variation might be due to the better tissue penetration of solvent during long incubation. Further, the compounds that might be bound in tissues, which are trapped in the pores of the matrix, in different mechanisms might have caused differences in the proportions of the compounds in the final extract. However, in the present study the content of p-cymene was higher than that of thymoquinone endorsing the efficiency of extraction procedure. It was reported that thymoquinone, dithymoquinone, thymohydroquinone, and thymol were the major phenolic compounds in Indian samples extracted with Supercritical CO_2[30]. The use of MTBE as an extraction solvent with 24 h incubation shaking was found to give an elaborate picture of the seed metabolites. The procedure could be used where detailed information is needed as in case herbal medicines prepared from whole grains of nigella.

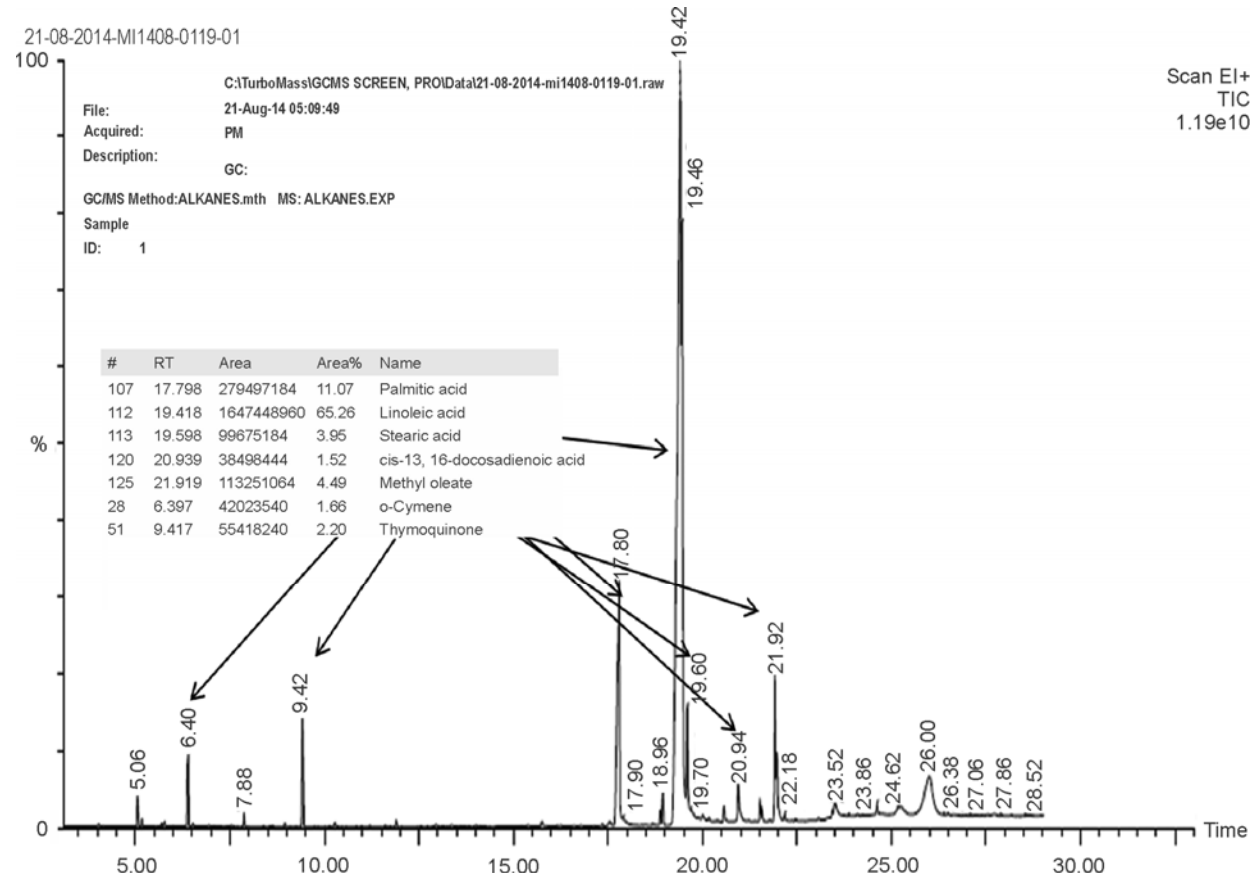

Fig. 2 —Mass spectrum of seed metabolites

Table 3 — Composition of secondary volatiles in *N. sativa* L. seed metabolites

Compound	Volatile secondary metabolites	RT	KI	Area %
1	α-Thujene	5.117	928	0.54
2	α-Pinene	5.237	936	0.12
3	Sabinene	5.777	972	0.08
4	β-Pinene	5.837	976	0.16
5	α-Terpinolene	6.617	1029	0.07
6	p-Cymene	6.477	1019	1.62
7	α-terpinene	7.037	1057	0.31
8	cis-4-Methoxythujane	7.617	1095	0.03
9	trans-4-Methoxythujane	7.957	1118	0.19
10	trans-Verbenol	8.477	1154	0.02
11	Terpinen-4-ol	8.757	1174	0.02
12	(+)-Dihydrocarvone	9.057	1194	0.05
13	Thymoquinone	9.577	1232	1.29
14	Isobornyl acetate	10.217	1278	0.01
15	Carvacrol	10.478	1297	0.23
16	α-Longipinene	11.318	1351	0.03
17	Longifolene	12.018	1411	0.14
18	Thymohydroquinone	13.618	1549	0.31
19	9-Undecenal, 2,10-dimethyl-	19.098	2084	0.72
	Total			5.94
	Other metabolites (> 0.01 %, 31 compounds)			2.67
	Trace compounds in seed metabolites (< 0.01 %)			87 compounds

RT, Retention time; KI, Kováts indices, relative to C8–C40 n-alkanes on TG-5MS capillary column.

Conclusion

The bioactivity of nigella seed was attributed to the high content of antioxidants such as thymoquinone and other active components. The aromatic compounds thymol, carvacrol, and p-cymene contribute essentially to the flavour of the seed, though thymol and carvacrol are known to have high bioactivity. Though, *N. sativa* seed contains more than 100 compounds, only a few dozen compounds were known or studied. The preservative and biopotent action of seed metabolites other than secondary volatile metabolites is vastly unexplored and needs immense attention.

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