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Identification of Volatile Organic Compounds from *Trichoderma virens* (6011) by GC-MS and Separation of a Bioactive Compound via Nanotechnology

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ABSTRACT

Fungal volatile organic compounds (VOCs) have the potential of being used as biocontrol agents for biotechnological applications in agriculture, industry and medicine. In this research, different VOCs from secondary metabolites of biocontrol fungus *Trichoderma virens* (6011) were separated using n-hexane, n-butanol and methanol solvents and identified by gas chromatography–mass spectrometry (GC-MS) device. According to mass spectra library searching, more than 200 volatile compounds (with spectral match factor higher than 80%) such as alkanes, alkenes, alcohols, organic acids, aromatic compounds, aldehyde, etheric, esteric, phenolic, kenotic derivatives and, sulfur and nitrogen compounds, have been detected. Some of the VOCs such as dibutyl phthalate (DBP) had antifungal activity. The antifungal effect of DBP as a case study was checked and confirmed in *in-vitro* conditions. DBP as a bioactive compound was separated from secondary metabolites using Molecularly Imprinted Polymers (MIPs) as a solid sorbent. Two kinds of the MIPs were synthesized *via* bulk polymerization and precipitation polymerization. Nanoporous MIPs for DBP, with binding capacity ca.462 $mg.g^{-1}$ and the specific surface area $479m^2.g^{-1}$ were synthesized *via* bulk polymerization method while the synthesized MIPs *via* precipitation technique had the binding capacity ca.830 $mg.g^{-1}$ with specific surface area 690 $m^2.g^{-1}$. The synthesized MIPs were evaluated by scan electron microscopy (SEM) device and Brunner Emmett-Teller (BET) analysis. Results showed that, the MIPs nanotechnology can be suggested as a suitable alternative method for separation of the chemical toxins. This study introduces a simple method under laboratory conditions to separate the bioactive compounds from fungal secondary metabolites.

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1. INTRODUCTION

Volatile organic compounds (VOCs) are carbon-based solids and liquids that include several classes of low molecular weight organic compounds with high vapor pressure under ambient conditions. Most of them are lipid soluble and thus have low water solubility [1]. Fungi release wide spectrum of VOCs that belong to several chemical groups with different biochemical origins such as monoterpenes, sesquiterpenes, alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons, ketones, and compounds containing S and N elements [2, 3]. Approximately 479 of VOCs have been identified from fungi which appear as the intermediate and final products of various metabolic pathways [4, 5]. The VOCs emissions play important ecological and physiological roles for many organisms [3]. The VOCs of the biocontrol fungus *Trichoderma* have antibiotic activity against plant pathogenic fungi and also enhance plant growth and systemic resistance in plants. Up to now, different secondary metabolites with antibiotic and antifugal activity have been characterized from *Trichoderma* spp.

In this research, secondary metabolites of *T. virens* (6011) KP671477.1 (that were successful in biological control of different phytopathogens and production of secondary metabolites), have been separated from liquid

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culture using liquid-liquid extraction with three solvents [6, 7]. The extracted materials were identified *via* gas chromatography mass spectrometry (GC-MS) device. Mass spectrometric detection presents the possibility to recognize individual volatiles from complex mixtures. The compound structure is usually identified by comparison of mass spectra with library spectra or by comparison of retention times and spectra with those of known standards [8]. Liquid-liquid extraction method was employed to separate many chemical compounds like methyl acetoacetate from the waste stream [9].

Since, the isolation of the desired compounds by column chromatography is expensive and takes long time, so separation by using nanotechnology (like MIPs technique) was recommended. This technique is widely used in many fields including biosensors, condensed drug, biochemical, membrane separation and extraction of the bioactive ingredients of medicinal plants [10]. Separation of other chemical materials, such as Cu^{2+} and Pb^{2+} was reported by nanoporous adsorbent [11].

Due to the capacity of *Trichoderma* species in crop protection and promoting vegetative growth, the identification of molecules with these biological activities can support the development of new biopesticides, biofungicides and biofertilisers based on *Trichoderma* metabolites [12, 13].

Based on the low efficiency of chemical toxins for control of soil borne pathogens and the resulting environmental pollutions and little amount of antifungal compounds in metabolites, the MIPs nanotechnology can be introduced as a good method to separate and concentrate these kind of materials.

Meanwhile, this is the first work on the separation of the antifungal compounds from *T. virens* (6011) secondary metabolites by this teqnique.

2. MATERIALS AND METHODS

2. 1. Instrumentation and Reagents Jenway 6305 UV/Visible spectrometer was used to determine amount of the templates in loading process on the polymers in 280 nm wavelength. The porosity was evaluated by nitrogen gas adsorption/desorption analysis using Brouneur Emmet Teller (BET) analysis (PHS1020-China). Surface morphological information of the MIPs was obtained by scan electron microscope (SEM) model VEGA\\TESCAN-XMU (Canada). For the detection of the fungal VOCs, a GC agilent 7890A equipped with an agilent 5975C mass selective detector was used. GC–MS analyses were done with ionization energy of 70 ev using the nonpolar capillary column (DB-5). The VOCs were characterized by comparison of their mass spectra with NIST library spectra.

Potato dextrose agar (PDA) and all of the chemical solvents were purchased from Merck products. Dibutyl phthalate (DBP), 2, 2´ azobisisobutyronitrile (AIBN), trimethylolpropane trimethacrylate (TRIM) and Methacrylic acid (MAA), were prepared from Sigma-Aldrich products.

2. 2. Cultivation of *Trichoderma* Species *T. virens* (6011) KP671477.1 was collected from the Mycology Laboratory, Department of Plant Protection, Gorgan University of Agricultural Sciences & Natural Resources, Gorgan. They were grown on PDA at 28 ± 2 °C for 5 days. Two 7 mm diameter plugs of *Trichoderma* species were inoculated into 1000 ml Erlenmeyer flasks containing 250 ml of sterile one-fifths strength potato dextrose broth (PDB). The stationary cultures were incubated for 30 days at 28 ± 2 °C (12 h darkness, 12 h light). The procedure was replicated three times [14].

2. 3. Extraction Procedure from Secondary Metabolites and Detection of VOCs The culture broth of *Trichoderma* species was extracted by three solvents: methanol (polar solvent), n-hexane (nonpolar solvent) and n-butanol, according to the extraction procedure of Siddiquee et al. [15]. The extracted VOCs were identified *via* GC-MS device.

2. 4. Identification of a Bioactive Compound The Bioactive compounds were identified in *T. virens* (6011) VOCs according to the related references. One bioactive compound with antifungal activity was found. The purified compound was purchased and its antifungal activity tested against two phytopathogenic fungi based on the method of Ahluwalia et al. [16].

2. 5. The MIPs Synthesis Synthesis and evaluation of the MIPs was carried out in three steps, (1) the preparation of fine particles of the MIPs, (2) eluting of the template from particles of the polymers by eluent to achieve the blank MIPs and (3) loading on the blank MIPs by bioactive compound to measure the binding capacity of the MIPs [17].

Two kinds of the MIPs were synthesized *via* bulk and precipitation polymerization and the results were compared together. The bioactive compound (template), MAA and TRIM were dissolved in a glass vial with 18 cm length and 20 mm diameter by 5ml n-hexane in a molecular ratio of 1:20:80 (template: functional monomer: cross-linker) for bulk polymerization [18].

Above mentioned materials dissolved in a round bottom flask by 50 ml toluene in a molecular ratio of 1:4:8 for precipitation polymerization [19]. AIBN as an initiator (for radical reactions) was added and the solution was kept in ice-bath. The pre-polymerization solutions were sonicated by ultrasonic waves and purged with nitrogen gas to remove dissolved oxygen [20]. The reactions were thermally initiated at 60 °C for 24 hours.

2. 6. The NIPs Synthesis According to the same synthetic routes in MIPs synthesis, the non-imprinted polymers (NIPs) were synthesized as a control polymer in the absence of the template molecules.

2. 7. Elution of the Template from the MIPs The prepared polymers were eluted by methanol/acetic acid (9:1 V/V) with a magnetic stirrer. This procedure was allowed up to the absorbance of the filtered solution in 280 nm reach to zero. It means that the entire template has been removed from the polymers. The MIPs were centrifuged and washed two times with distillated water. The leached MIPs were dried at 60 ºC overnight for further use.

2. 8. Loading and Measuring Binding Capacity Binding capacity was defined as mg of the absorbed template per 1 gram of the polymer. The binding capacity can be calculated by the Equation (1):

$$Q= (C_i- C_f) * V / W \quad (1)$$

where, C_i is the initial concentration, C_f is the free concentration of DBP in supernatant, V is the volume of the feed with initial concentration of DBP (loading solution) and W represents the mass of polymer in grams in loading procedure.

3. RESULTS AND DISCUSSION

3. 1. Identification of VOCs by GC/MS Analysis According to NIST mass spectra library of the GC–MS analysis, in extracted organic and water phases of *T. virens* (with n-hexane, methanol, and n-butanol solvents), more than 200 volatile compounds were identified. Some alkanes, alkenes, alcohols, organic acids, aromatic compounds, aldehyde, etheric, esteric, phenolic, ketonic derivatives, and sulphur and nitrogen compounds have been detected. Many of the important extracted VOCs were shown in Table 1. Some of the detected compounds have already been ascribed to *Trichoderma* by Siddiquee [4]. These VOCs are shown by "*", in Table 1.

Korpi et. al. [2] reported 2-methyl-1-butanol, ethyl benzene, 1-pentanol, styrene, toluene as the common microbial volatile organic compounds (MVOCs) of fungi and bacteria in the environment.

Jelen et al. demonstrated that *T. atroviride* isolates produced benzene derivatives such as propenyl benzene and most of the *Trichoderma* species produced toluene. They also found 1-pentanol and xylene in *T. koningii* and *T. atroviridae* isolates [21]. Hung et al. reported that low concentrations of 2-methyl-1-butanol yielded a small but significant increase in fresh weight in *Arabidopsis* while 2-ethyl hexanal inhibited spore germination and growth of 2-week-old vegetative plants [22]. Venkata et al. proved alcohols, phenols and phthalates derivatives had antifungal and antibacterial effects [23].

Fatty acids (e.g. Palmitic acid, Octadecenoic acid) are known to possess antibacterial and antifungal activities [24]. We detected these compounds in *T. virens* methanol and n-butanol Phases. Dubey et al. [25] also separated Palmitic acid in *T. virens* and *T. harzianum* .

TABLE 1. Identification of the important extracted volatile compounds from *T. virens* using three solvents.

Category	Volatile metabolites	Phase a
	2,4,6- trimethyl - Azulene	N
	1,2-diethyl- Benzene*	N&M
	1,4-diethyl-Benzene	M
	2,4-dimethyl-1-(1-methylpropyl)- Benzene	M
	(1,1-dimethylpropyl)-Benzene	M
	2-ethenyl-1,4-dimethyl-Benzene	N
	Ethyl Benzene*	N& M
	1-ethyl-2-methyl- Benzene*	N& M
	2-ethyl-1,4-dimethyl-Benzene*	M
	(1-methylethyl) - Benzene *	N& M
	(1-methyl-2-cyclopropen-1-yl)-Benzene	N& M
	(1-methyldodecyl)- Benzene	M
	1-methyl-2-(1-methylethyl)-Benzene*	N& M
	1-methyl-3-(1-methylethyl)-Benzene*	N
	1-methyl-4-(1-methylethyl)-Benzene	M
	1-methyl-1,2-propadienyl-Benzene	N
Aromatic	Propenyl-Benzene	N& M
Compound	Propyl- Benzene*	M
	1,2,3-trimethyl-Benzene*	N
	1,2,3,5-tetramethyl benzene*	M
	tert-butyl-Benzene	N
	Indane*	N& M
	Methyl Indene	N
	Naphthalene	N& M
	decahydro-1,6-dimethyl- Naphthalene*	N
	decahydro-2,6-dimethyl-Naphthalene	N
	1,3-dimethyl-Naphthalene	N
	1,5-dimethyl-Naphthalene*	N& M
	2,6-dimethyl-Naphthalene*	N
	2-methyl-Naphthalene*	N& M
	Phenanthrene	M
	Styrene	N
	Toluene*	N
	Xylenes	N& M
Organic	Palmitic acid	M& B
acids	Octadecenoic acid	N& M
	Bis (2-ethylhexyl) phthalate	N& M
	Butanoic acid, butyl ester *	B
Esters	Dibutyl phthalate *	M
	Phthalic acid, isoporpyl propyl ester	M
	Sulfurous acid, butyl dodecyl ester	N
Ketones	3-methyl-4-Heptanone	B
Halo alkanes	2-Bromo dodecane	N
Epoxides	2-Tolyloxirane	N
Alkyne	Isopropyl-phenyl-acetylene	N
Alcohols	2-methyl-1-Butanol	B
	1-Pentanol	B
Aldehyde	2-ethyl-Hexanal	B

[a] "N", "M" and "B" mean that compound was extracted *via* n-hexane, methanol solvent and n-butanol solvents.

The antifungal activities of DBP, has been already proved [26], so we were interested in separating it from *T. virens* (6011) secondary metabolites.

Since this compound was found in methanol phase, GC–MS chromatogram of the extracted VOCs in that phase was shown in Figure 1.

According to wide spectrum of the fungal VOCs and biocontrol properties of *Trichoderma*, the usage of a suitable procedure for separation is important. Extraction of special chemical compounds *via* organic solvents seems to be simple, cheap and convenience. In this method, the related materials will be separated from other organic compounds based on their distribution coefficient, then identified by GC–MS device.

3. 2. Identification of a Bioactive Compound DBP is one the bioactive chemical compound in secondary metabolites of *T. virens* (6011) KP671477.1 that was chosen as a case study template in this research. Its antifungal effectiveness was confirmed in *in-vitro* conditions.

3. 3. Binding Capacity of the MIPs Consideration of the relationship between binding capacity vs. concentration of the template was carried out in different concentrations. In each loading process, 10 mg of the polymers with 20 ml of different concentration of the feed were treated. Loading processes were summarized in Table 2. In this table, P-NIPs and B-NIPs are introduced as the synthesized NIPs *via* precipitation and bulk polymerization methods, respectively.

The results showed that, during increasing of the concentration in loading solution (up to 500ppm), the binding capacities were increased (Figures 2 and 3). The utmost binding capacity of the synthesized MIPs in both polymerization techniques, have been observed in 500 ppm concentration of the template.

The binding capacity values were decreased for concentration of the template more than 500ppm. It means that, binding capacitiy is depended on the quantity of the polymers.

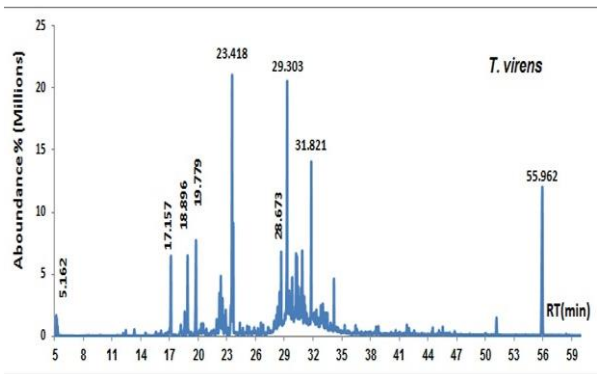

Figure 1. GC–MS chromatogram of *T. virens* VOCs.

TABLE 2. The binding capacity results for polymers.

Kind of polymer	C° (ppm)	C (ppm)	Q (mg/g)	Imprinting Factor(IF)
B-MIPs	125	110	30	1.1
B-MIPs	250	157	186	1.2
B-MIPs	500	269	462	1.2
B-MIPs	750	592	316	1.5
B-NIPs	125	112	27	-
B-NIPs	250	172	156	-
B-NIPs	500	307	386	-
B-NIPs	750	641	217	-
P-MIPs	125	93	64	3.5
P-MIPs	200	165	70	2.9
P-MIPs	250	205	90	3.4
P-MIPs	400	166	468	2.3
P-MIPs	500	85	830	2.7
P-MIPs	600	250	700	3.5
P-MIPs	750	675	150	3.7
P-NIPs	125	116	18	-
P-NIPs	200	182	24	-
P-NIPs	250	237	26	-
P-NIPs	400	300	200	-
P-NIPs	500	350	300	-
P-NIPs	600	500	200	-
P-NIPs	750	730	40	-

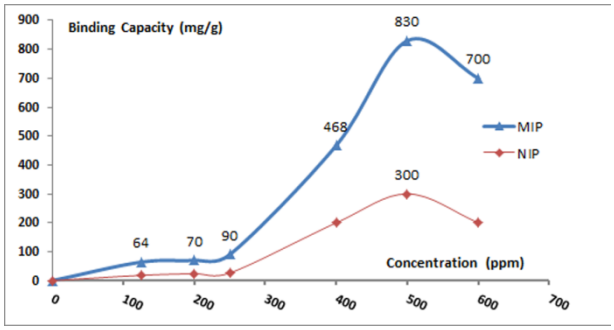

Figure 2. The binding capacity for DBP in precipitation polymerization.

The related curves indicated that the binding capacity of the synthesized polymers *via* precipitation polymerization method (P-MIPs) is highly more than the synthesized MIPs in bulk polymerization technique (B-MIPs) in 500 ppm concentration. Meanwhile, the binding capacities of the MIPs in both methods for all of the feed concentration were more than NIPs. Since the MIPs particles were imprinted, the related results were expected.

Zhu et al. synthesized MIPs on the silica surface by methacrylic acid (MAA) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as cross linking agent with ratio 6:3:15 (template: functional monomer: cross-linker) *via* precipitation method. The related MIPs showed a binding capacity as equal 8.940 mg/g for DBP [27].

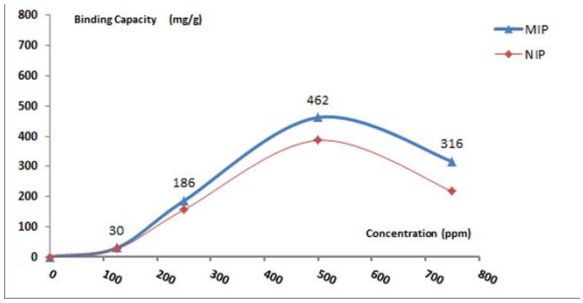

Figure 3. The binding capacity for DBP in Bulk polymerization.

3. 4. Porosity Studies in Polymers The porosity was characterized by nitrogen gas adsorption measurements using BET technique. The results indicated that the specific surface area of the MIPs in both polymerization methods were clearly more than NIPs. It means that in both methods, nonoporous MIPs were successfully synthesized for DBP. As shown in Table 3, the porosity and the specific surface area of the synthesized MIPs in precipitation polymerization method (P-MIPs) are highly more than the synthesized MIPs in bulk polymerization method (B-MIPs).

Because of the imprinting process in MIPs in both polymerization methods, the average pore diameter of the MIPs was less than NIPs (Table 3). It means that the nano pores in MIPs have obviously been created more than NIPs. High porosity in MIPs causes the binding capacity to be increased.

3. 5. Morphological Studies Surface morphological information of the MIPs was obtained by scan electron microscope (SEM). Figures 4 and 5 show the morphology of B-MIPs and P-MIPs, respectively.

In bulk polymerization method, the template is captured inside of the polymer matrices and it occupies the space. After removal of the template, the leached MIPs will be cancellous and fragile. Since in bulk polymerization method, the polymer matrices must be grinded to the fine particle, so after crushing by pestle, it is expected that the particles to be amorphous.

TABLE 3. BET analyses of the MIPs and NIPs

Kind of polymer	The BET specific surface area (m^2/g)	The Langmuir specific surface area (m^2/g)	Micropore specific surface area (m^2/g)	Average pore diameter of MP model (nm)
B-MIPs	479	730	215	0.465
B-NIPs	467	708	211	0.496
P-MIPs	690	1059	1192	0.303
P-NIPs	427	644	89	0.521

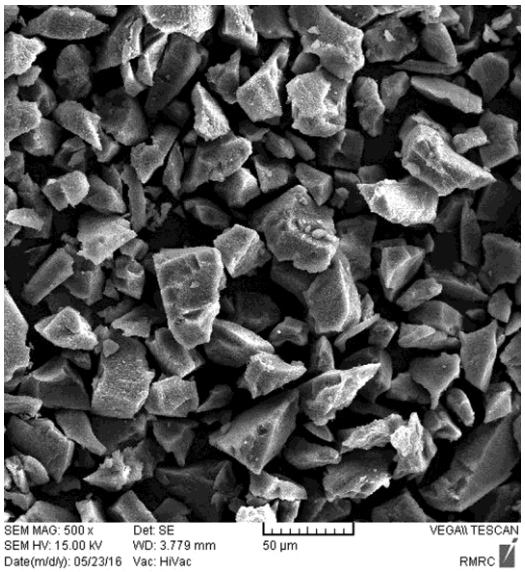

Figure 4. SEM imaging for the B-MIPs.

While in precipitation method it is not necessary to carry out each grinding process, so P-MIPs have the same spherical shape with nano dimensions. After removal of the template, the nanoparticles of P-MIPs will have the first shapes which were synthesized. According to precipitation polymerization technique, the spherical shape of the polymers is expected while in this research, in spite of dispersion of the polymers before SEM, we could not prepare the spherical P-MIPs, separately. SEM imaging of the P-MIPs indicated that the accumulated nanoparticles have been created (Figure 5).

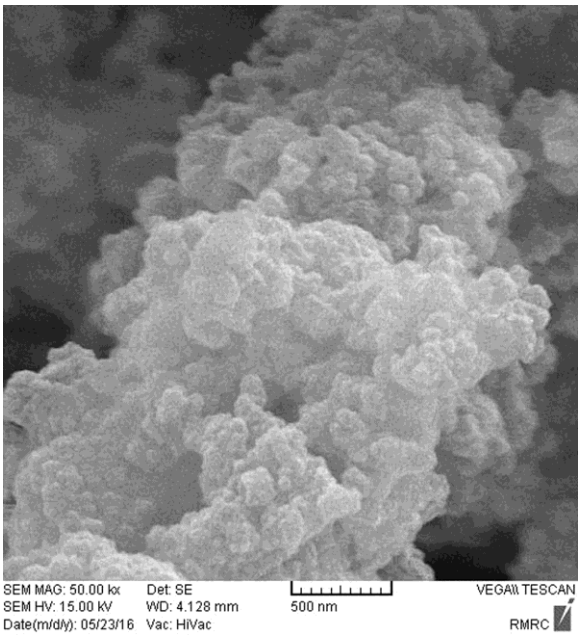

Figure 5. SEM imaging for the P-MIPs.

4. CONCLUSION

Volatile metabolites have been involved in different biological processes such as biocontrol or communication between fungi and their living environment. Some VOCs are widely reported to have antibiotic and immunosuppressant activities. Therefore, it offers the requirement for monitoring fungal VOC profiles. This study successfully separated nonpolar and medium polar compounds from other volatile compounds using three kinds of solvents (n-hexane, n-butanol, and methanol). After extraction and GC separation with nonpolar capillary column (DB-5), the constituents of complex mixtures of VOCs detected *via* mass spectrometry (MS) by comparison of mass spectra with library spectra searching. According to NIST mass spectra library, more than 200 volatile compounds have been detected. Most of these compounds have not previously been reported. These results improve our knowledge about none and medium polar chemical compounds produced by *T. virens* (6011) KP671477.1 in different polarity of solutions. This is a simple method to perform the above mentioned procedure, under laboratory conditions. Since, isolation of the bioactive compound by column chromatography is expensive and takes long time, so nanotechnology by using Molecularly Imprinted Polymers (MIPs) was used. The MIPs results indicated that nanoporous MIPs is a good candidate for extraction of the special bioactive compound from *Trichoderma* species in solid phase extraction. The nanoporous MIPs can be introduced as a novel method to separate the antifungal bioactive from metabolite produced by *Trichoderma* species. Because of a little amount of the bioactive compounds in secondary metabolites, it is reasonable to find a suitable method to extract them from natural products.

5. ACKNOWLEDGMENT

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Identification of Volatile Organic Compounds from *Trichoderma virens* (6011) by GC-MS and Separation of a Bioactive Compound via Nanotechnology

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چکیده

ترکیبات آلی فرار قارچی (VOCs) دارای استعداد بالقوه‌ای هستند که می‌توانند به‌عنوان عوامل کنترل زیستی جهت کاربردهای بیوتکنولوژیکی در کشاورزی، صنعت و داروسازی مورد استفاده قرار گیرند. در این تحقیق، ترکیبات آلی فرار مختلفی از متابولیت‌های ثانویه قارچ بیوکنترل *Trichoderma virens* (6011) با استفاده از حلال‌های ان- هگزان، ان- بوتانول و متانول جداسازی و به‌وسیله‌ی دستگاه گاز کروماتوگرافی جرمی (GC-MS) شناسایی شدند. براساس جستجو در طیف‌های جرمی کتابخانه‌ای دستگاه، بیش از ۲۰۰ ترکیب فرار نظیر آلکان‌ها، الکل‌ها، اسیدهای آلی، ترکیبات آروماتیک، آلدئیدها، مشتقات اتری، استری، فنلی، کتونی و ترکیبات حاوی نیتروژن و گوگرد (با فاکتور تطبیق طیفی بالاتر از ۸۰٪)، تشخیص داده شدند. برخی از ترکیبات آلی فرار نظیر دی بوتیل فتالات، فعالیت ضدقارچی داشتند. اثر ضدقارچی دی بوتیل فتالات به‌عنوان مطالعه موردی در شرایط آزمایشگاهی، بررسی و تأیید گردید. دی بوتیل فتالات به‌عنوان یک ترکیب زیست‌فعال با استفاده از پلیمرهای قالب مولکولی(MIPs) به‌عنوان یک جاذب جامد، از متابولیت‌های ثانویه جداسازی شد. از طریق پلیمریزاسیون توده‌ای و رسوبی، دو نوع پلیمر قالب مولکولی سنتز شد. پلیمرهای قالب مولکولی نانومتخلخل برای دی بوتیل فتالات با ظرفیت اتصال $٤٦٢ mg.g^{-1}$ و سطح ویژه $٤٧٩ m^2.g^{-1}$ ازطریق روش پلیمریزاسیون توده‌ای سنتز شدند، در حالی‌که پلیمرهای قالب مولکولی سنتز شده با تکنیک پلیمریزاسیون رسوبی، دارای ظرفیت اتصال $٨٣٠ mg.g^{-1}$ و سطح ویژه $٦٩٠ m^2.g^{-1}$ بودند. پلیمرهای قالب مولکولی سنتز شده با استفاده از دستگاه میکروسکوپ الکترونی روبشی (SEM) و آنالیز برونر- امت- تلر (BET) مورد ارزیابی قرار گرفتند. نتایج نشان داد که نانو فناوری پلیمرهای قالب مولکولی می‌تواند به‌عنوان یک جایگزین مناسب برای جداسازی سموم شیمیایی پیشنهاد شود. این تحقیق در شرایط آزمایشگاهی، روش ساده‌ای را برای جداسازی ترکیبات زیست فعال از متابولیت‌های ثانویه قارچی، معرفی می‌نماید.

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