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Antimicrobial volatile glucosinolate autolysis products from *Hornungia petraea* (L.) Rchb. (Brassicaceae)

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ABSTRACT

Plant samples of *Hornungia petraea* were analyzed for glucosinolate (GLS) autolysis metabolites for the first time. GC–MS analysis of the autolysate and the synthesis of a series (12 compounds) of possible glucosinolate breakdown products revealed/corroborated the presence of glucoaubrietin, glucolimnanthin, glucolepigramin and glucotropaeolin in this species as the most likely "mustard oil" precursors. GLS degradation products identified in the autolysate of *H. petraea*, benzyl isothiocyanate, 3- and 4-methoxybenzyl isothiocyanate, along with several other structurally related compounds were evaluated for antimicrobial activity in order to possibly pinpoint the role of the latter secondary metabolites in the plant tissues. The assays showed a very high antibacterial activity of the tested isothiocyanates against *Sarcina lutea* and an antifungal effect against *Aspergillus fumigatus* and *Candida albicans* with MIC values in the order of 1 μg/ml value.

1. Introduction

Glucosinolates (GLSs), known as mustard oil glucosides, are a class of nitrogen and sulfur-containing natural products distributed in 16 dicotyledonous families of the order Capparales (Al-Shehbaz and Al-Shammary, 1987; Verkerk et al., 2009), but also in the genus *Drypetes* (family Euphorbiaceae) (Rodman et al., 1996). Mostly, glucosinolate-containing genera are clustered within the Brassicaceae, Capparaceae and Caricaceae families (Fahey et al., 2001). Representatives of the Brassicaceae family are of particular importance as vegetables, seasonings and relishes, and sources of vegetable oil. GLSs are recognized as the active constituents responsible for many of the physiological activities proposed for the *Brassica* vegetables (Holst and Williamson, 2004; Verkerk et al., 2009). Up to date, approximately 200 GLSs were described (Clarke, 2010; Fahey et al., 2001; Halkier and Gershenzon, 2006). Upon plant damage, thioglucoside glucohydrolase (E.C.3.2.3.1), or also called myrosinase, hydrolyzes the relatively non-reactive GLS to give an array of biologically active compounds, i.e. isothiocyanates, thiocyanates, nitriles, etc. GLS metabolites have been recognized as antimicrobial agents for many decades (Fahey et al., 2001) and this activity has been proposed to be a part of the crucifers defense against pathogen attack (Radulović et al., 2011). In the past few years isothiocyanates have also been identified as potent cancer-prevention agents (Fahey et al., 2001; Halkier and Gershenzon, 2006; Holland et al., 1995; Zhang and Talalay, 1994). Epidemiological studies have shown that they show a protective effect against cancer – particularly in the bladder, colon and lung (Song and Thornalley, 2007). In addition to their diverse biological and physiological properties, GLSs and their breakdown products are receiving considerable attention of scientists due to their known great chemotaxonomical significance.

The genus *Hornungia* Rchb. (Brassicaceae) comprises approximately 10 species, distributed in Europe, North Africa and west Asia; one species of this genus grows in Australia, south and North Africa. In Serbia, the genus is represented by only one species – *Hornungia petraea* (L.) Rchb. 1837 (syn. *Hutchinsia petraea* (L.) R. Br. 1812; *Lepidium petraeum* L. 1753; Gonešina or grančika, in Serbian) (Jovanović-Dunjić, 1972). In general, a literature survey revealed that the genus *Hornungia* has been poorly investigated. In general, only two previous references on the phytochemistry of *Hornungia* and *Hutchinsia* taxa can be found regarding the analysis of glucosinolates and their breakdown products in *Hutchinsia alpina* (Bennett et al., 2004; Kjaer et al., 1953).

Although there are many articles in the literature dealing with the identification of GLSs, it is often very difficult to identify any consistent results. The difficulty mainly lies in the lack of reliable, standard methods of GLS analysis, their instability and limited analytical data required for the identification. Analysis of volatile GLS autolysis products performed by gas chromatography/mass

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Table 1
Percentage composition of the glucosinolate autolysis volatiles from *Hornungia petraea*.

RI calc.	Component	Compound class	Percentage[a]		Method of identification
			hp1	*hp2*	
965	Benzaldehyde	OTH	0.1	–	RI, MS, Co-GC
978	Phenol	OTH	0.1	–	RI, MS, Co-GC
1106	Nonanal	FAD	0.2	–	RI, MS, Co-GC
1135	**2-Phenylacetonitrile (9)**	**NIT**	**1.2**	**0.2**	**RI, MS, Co-GC**
1361	**Benzyl isothiocyanate (1)**	**ITC**	**8.5**	**4.8**	**RI, MS, Co-GC**
1373	**2-(3-Methoxyphenyl)acetonitrile (11)**	**NIT**	**2.4**	**1.6**	**RI, MS, Co-GC**
1404	Vanillin	OTH	0.1	–	RI, MS, Co-GC
1480	**2-(3-Hydroxyphenyl)acetonitrile (14)**	**NIT**	**1.5**	**–**	**RI, MS**
1599	**3-Methoxybenzyl isothiocyanate (3)**	**ITC**	**25.1**	**34.4**	**RI, MS, Co-GC**
1623	**4-Methoxybenzyl isothiocyanate (4)**	**ITC**	**–**	**tr**	**RI, MS, Co-GC**
1703	**3-Hydroxybenzyl isothiocyanate (13)**	**ITC**	**15.9**	**22.1**	**RI, MS**
1841	Neophytadiene (isomer II)	TER/OTH	0.9	–	RI, MS
1900	Nonadecane	FAD	tr	–	RI, MS, Co-GC
1962	Hexadecanoic acid	FAD	1.1	0.3	RI, MS, Co-GC
2000	Eicosane	FAD	0.2	–	RI, MS, Co-GC
2100	Heneicosane	FAD	0.3	0.1	RI, MS, Co-GC
2141	(*Z,Z,Z*)-9,12,15-Octadecatrienoic acid	FAD	2.6	0.2	RI, MS, Co-GC
2200	Docosane	FAD	0.4	0.1	RI, MS, Co-GC
2300	Tricosane	FAD	0.5	0.1	RI, MS, Co-GC
2400	Tetracosane	FAD	0.3	–	RI, MS, Co-GC
2500	Pentacosane	FAD	0.6	1.4	RI, MS, Co-GC
2600	Hexacosane	FAD	0.5	–	RI, MS, Co-GC
2700	Heptacosane	FAD	1.7	1.2	RI, MS, Co-GC
2800	Octacosane	FAD	0.9	–	RI, MS, Co-GC
2834	Squalene (all *E*)	TER	2.1	0.9	RI, MS, Co-GC
2900	Nonacosane	FAD	12.2	10.8	RI, MS, Co-GC
3000	Triacontane	FAD	2.1	3.0	RI, MS, Co-GC
3100	Hentriacontane	FAD	8.5	4.2	RI, MS, Co-GC
3103	α-Tocopherol	FAD	3.6	3.3	RI, MS, Co-GC
Total			93.6	88.5	

Grouped components	Percentage[a]	
	hp1	*hp2*
Isothiocyanates (ITC)	49.5	61.3
Nitriles (NIT)	5.1	1.6
Terpenoids (TER)	7.0	0.9
Fatty acids and fatty acid derived compounds (FAD)	28.1	21.4
Others (OTH)[b]	3.9	3.3

RI calc. – experimentally determined linear retention indices on an HP-5MS column; *hp1* and *hp2* – samples of *H. petraea* collected from two different locations (for more details see Section 3); tr – trace amounts (<0.05%); RI – retention indices matching with literature data (Adams, 2007); MS – mass spectra matching; Co-GC – co-injection with a pure reference compound; (–) not detected.
Entries in bold letters are compounds believed to be GLS autolysis products.
[a] Values are means of triplicate analyses.
[b] Unclassified constituents, compounds of possible anthropogenic origin.

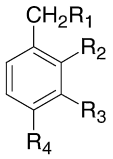
spectrometry (GC–MS) is a simple, rapid and, in most cases, convenient method for the qualitative analysis of glucosinolate-containing plant species.

In connection with our previous studies on the glucosinolate autolysis products (Radulović et al., 2008, 2011), we are interested in the investigations of the chemical composition of volatile autolysis products of Serbian Cruciferae, since these have received almost no attention in this respect. Bearing in mind the scarce literature data on the chemistry of *Hornungia* species, in this study we focused our attention on the glucosinolate autolysis products of *H. petraea*. Another goal of this study was to provide a set of spectral and chromatographic data that would make the detection and identification of a number of GLS breakdown products easier. This was accomplished through the synthesis of a series of regioisomeric compounds of possible GLS origin and their full spectral characterization and accumulation of GC chromatographic retention data (RI).

2. Results and discussion

Plant samples of *H. petraea*, collected at two different locations, were analyzed for glucosinolate autolysis metabolites. GC and GC–MS analyses of the autolysates enabled the positive identification (with GC co-injection of the standards) of 26 compounds and 3 additional tentatively identified according to MS and RI analysis matching in both samples, accounting for 88.5–93.6% of the extracted volatiles based on the areas under the GC chromatograms (Table 1). Seven compounds were detected in the two *H. petraea* samples (*hp1* and *hp2*) as being possible GLS metabolites: 3- (**3**) and 4-methoxybenzyl isothiocyanate (**4**) (25.1 and 34.4%, and trace amounts, respectively), 3-hydroxybenzyl isothiocyanate (**13**) (15.9 and 22.1%), benzyl isothiocyanate (**1**) (4.8 and 8.5%), 2-(3-methoxyphenyl)acetonitrile (**11**) (1.6 and 2.4%), 2-(3-hydroxyphenyl)acetonitrile (**14**) (1.5%) and 2-phenylacetonitrile (**9**) (0.2 and 1.2%).

Considering the fact that isomers, in general and especially those possessing an aromatic core, yield upon EI very similar mass spectra (in our case the positional and functional group isomerism of the detected GLS metabolites), and scarce literature GC retention index data of the compounds in question, the detected GLS metabolites and their conceivable isomers were synthesized and GC co-injected with the autolysates in order to acquire an unambiguous corroboration of their identities (Fig. 1).



Compound	R_1	R_2	R_3	R_4
1	NCS	H	H	H
2	NCS	MeO	H	H
3	NCS	H	MeO	H
4	NCS	H	H	MeO
5	SCN	H	H	H
6	SCN	MeO	H	H
7	SCN	H	MeO	H
8	SCN	H	H	MeO
9	CN	H	H	H
10	CN	MeO	H	H
11	CN	H	MeO	H
12	CN	H	H	MeO
13	NCS	H	OH	H
14	CN	H	OH	H

Fig. 1. Structures of the synthesized/identified benzylic isothiocyanates and thiocyanates as well as of the phenylacetonitriles.

Due to the limited literature data, and the difficulties encountered in the synthesis of the chemical instable 3-hydroxybenzyl isothiocyanate (**13**) and 2-(3-methoxyphenyl)acetonitrile (**14**), the presence and identification of these compounds in *H. petraea* autolysate were concluded based on MS data, while the distinction of the regio isomers were made by a comparison of their retention times with those reported by Olsen and Sorensen (1980) (only available GC data for these compounds) (Fig. 2). A very nice fit (correlation coefficient $R = 0.99878$) was observed for the compounds in common to this work and the present one.

Identification of the above-mentioned compounds (**1**, **3–4**, **9**, **11**, **13–14**; Fig. 1) in the autolysates suggests the occurrence of benzyl (glucotropaeolin), 3-methoxybenzyl (glucolimnanthin), 3-hydroxybenzyl-(glucolepigramin) and 4-methoxybenzyl glucosinolate (glucoaubrietin) in this species as the possible "mustard oil" precursors (Fig. 3).

Previous studies on the glucosinolates in *H. alpina*, a species closely related to *H. petraea*, the only studied from this genus,

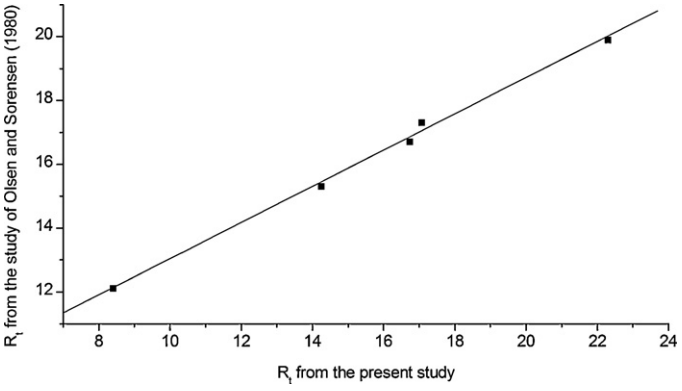
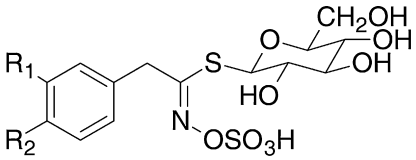


Fig. 2. A plot showing the correlation between GC retention times (R_t, min) of 2-phenylacetonitrile (**9**), benzyl isothiocyanate (**1**), 2-phenethyl isothiocyanate, 2-(3-hydroxyphenyl)acetonitrile (**14**) and 3-hydroxybenzyl isothiocyanate (**13**), respectively, from the present study and those reported by Olsen and Sorensen (1980) demonstrating the matching of the retention data utilized in the identification procedure with a correlation coefficient of $R = 0.99878$. The following compounds were plotted, R_t in our study, R_t previously reported: **9**, 8.44, 12.1; **1**, 14.25, 15.3; 2-phenethyl isothiocyanate (synthesized compound in our lab, but not the part of this study), 16.73, 16.7; **14**, 17.08, 17.3 and **13**, 22.30, 19.9.



Compound	R_1	R_2
Glucotropaeolin	H	H
Glucolimnanthin	MeO	H
Glucolepigramin	OH	H
Glucoaubrietin	H	MeO

Fig. 3. Structures of the glucosinolates glucotropaeolin, glucolimnanthin, glucolepigramin and glucoaubrietin, believed to be the precursors of the identified "mustard oils" in *H. petraea* autolysate.

revealed the presence of β-phenethyl isothiocyanate and of a second, unknown isothiocyanate (Kjaer et al., 1953). Recently, benzyl-, 3-hydroxybenzyl-, 4-hydroxybenzyl-, 2-methoxybenzyl- and 3-methoxybenzyl glucosinolates were found in the same species (Bennett et al., 2004). The same authors investigated GLS profiles of a number of GLS-containing species, and noticed a clear subdivision of the crucifers based on their GLS content into four groups: (i) short- to medium-chain-length aliphatic; (ii) long-chain-length aliphatic; (iii) simple aromatic and (iv) highly substituted aromatic ones. According to this classification, these two *Hutchinsia* species could be recognized as crucifers with the predominance of simple aromatic GLSs. From the aforementioned observations, an obvious similarity of the GLS profile of the two *Hutchinsia* species and of their phylogenetically close *Hornungia* taxon exists. This provides support for the suggestion of Appel and Al-Shehbaz (1997) to reduce the genus *Hutchinsia* (=*Pritzelago*) to synonymy of *Hornungia*, i.e. to treat *H. alpina* as a species of the genus *Hornungia*.

The synthesized compounds **1–5**, **7–9** were evaluated for antimicrobial activity in microdilution assays against eight pathogenic bacterial and two fungal strains. The microorganisms listed in Table 2 were selected according to two criteria: GLS breakdown products were previously found to possess an effect on the growth of such and related strains of human pathogenic bacteria and fungi (Radulović et al., 2011) and some of these may be present in the soil associated with the plant species and hence could represent direct plant pathogens (e.g. *Aspergillus fumigatus*, O'Gorman et al., 2008). The tested compounds were showed to be active against all of tested microorganisms, with the activity ranging from 1 to 1250 μg/ml for inhibitory and 1 to 5000 μg/ml for microbicidal activity (Table 2). Among the tested compounds, the most active were the ones bearing an isothiocyanate group – benzyl isothiocyanate (**1**) against *A. fumigatus* (MIC = 9 μg/ml; MBC = 10 μg/ml), 2- (**2**) and 3-methoxybenzyl isothiocyanate (**3**) against *Sarcina lutea* (MIC = MBC = 5 μg/ml and MIC = MBC = 2 μg/ml, respectively) and 4-methoxybenzyl isothiocyanate (**4**) against *C. albicans* (MIC = 1 μg/ml; MBC = 2 μg/ml) and *A. fumigatus* (MIC = MBC = 1 μg/ml). This significant and non-selective both antibacterial and antifungal activities of the tested isothiocyanates, connected with their mode of release by mechanical stress, suggests that isothiocyanates (and their precursor glucosinolates) play a significant role in the defense of the plants against various phytopathogens.

3. Experimental procedures

3.1. General experimental procedures

All chemicals were commercially available and used as received (Aldrich, USA; Merck, Germany; Fluka, Germany), except that the

Table 2
Antimicrobial activity of the synthesized benzyl isothiocyanates **1–4** and thiocyanates **5**, **7–8** as well as of the phenylacetonitrile **9** – minimal inhibitory (MIC) and minimal bactericidal/fungicidal concentrations (MBC/MFC).

Bacterial strain	Compound (mg/ml)																Tetracycline (mg/ml)
	1		**2**		**3**		**4**		**5**		**7**		**8**		**9**		
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MBC
S. aureus (isolate 1)	0.07	0.07	0.15	0.15	0.15	0.15	0.15	0.60	0.60	1.25	0.15	0.30	0.60	0.60	0.60	0.60	0.025
S. aureus (isolate 2)	0.01	0.03	0.03	0.03	0.03	0.03	0.01	0.30	0.30	0.60	0.07	0.15	0.30	0.30	0.30	0.30	0.025
S. enteritidis	0.01	0.01	0.07	0.30	0.03	0.07	0.03	0.07	0.07	0.07	0.07	0.07	0.03	0.07	0.03	0.07	0.025
K. pneumoniae	0.03	0.03	0.03	0.07	0.03	0.07	0.01	0.01	0.07	0.15	0.03	0.15	0.07	0.07	0.07	0.07	0.012
S. lutea	0.01	0.01	0.005	0.005	0.002	0.002	0.03	0.07	0.15	0.30	0.07	0.07	0.15	0.15	0.15	0.15	0.002
E. coli	0.07	0.15	1.25	5.00	0.30	0.30	0.30	0.60	1.25	1.25	0.30	0.30	0.60	0.60	0.60	0.60	0.025
Shigella sp.	0.03	0.15	0.60	1.25	0.60	1.25	1.25	1.25	1.25	1.25	0.15	0.30	0.60	1.25	0.60	1.25	0.025
B. cereus	0.01	0.03	0.15	0.60	0.15	0.60	0.62	1.25	0.07	0.15	0.01	0.07	0.07	0.60	0.07	0.60	0.006

Fungal strain	Compound (mg/ml)																Nystatin (mg/ml)
	1		**2**		**3**		**4**		**5**		**7**		**8**		**9**		
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MFC
C. albicans	0.03	0.60	0.15	1.25	0.15	0.15	0.001	0.002	1.25	1.25	0.60	0.60	0.60	5.00	0.60	5.00	0.025
A. fumigatus	0.009	0.01	0.07	0.15	0.03	0.15	0.001	0.001	0.15	0.15	0.30	0.60	0.15	0.30	0.15	0.30	0.002

solvents were purified by distillation. The GC–MS analyses were performed on a Hewlett-Packard 6890 N gas chromatograph equipped with fused silica capillary column HP-5MS and coupled with a 5975B mass selective detector from the same company as detailed under Section 3.4. Preparative medium pressure liquid chromatography (MPLC) separations were made on pre-packed silica gel (40–63 μm) polypropylene cartridges under gradient conditions using hexane–ether as eluent at a flow rate of 2.5 ml/min using a Büchi system (C 610, Büchi Labortechnik, Switzerland). The IR measurements were carried out using a Thermo Nicolet model 6700 FTIR instrument (Waltham, USA) – ATR: attenuated total reflectance. The ^{1}H and ^{13}C NMR spectra have been recorded on a GEMINI-200 spectrometer operating at 200 and 50.3 MHz, respectively. All NMR spectra were measured at 25 °C in $CDCl_3$ with tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in ppm (δ) and referenced to TMS. The purity and identity of the synthesized compounds was checked by TLC, GC–MS and spectroscopic techniques (MS, IR, ^{1}H and ^{13}C NMR) and confirmed by comparison of their spectral data to those existent in the literature (Asghari et al., 2010; Kiasat and Fallah-Mehrjardi, 2008; Kitamura et al., 1990; Stevens et al., 2009; Vaughn et al., 1996).

3.2. Plant material

The plant samples (aerial and underground parts) of *H. petraea* utilized in this work were collected from natural populations from rocky and calcareous soils: (i) in Prizrenska Bistrica river gorge (Prizren, Serbia) at the end of March 2007 (sample *hp1*), and (ii) on Stara Planina Mountain (Southeast Serbia) in April 2007 (sample *hp2*). Voucher specimens were deposited at the Herbarium collection of the Faculty of Science and Mathematics, University of Niš, under the accession numbers MD0207 (*hp1*) and MD0307 (*hp2*).

3.3. Isolation of hydrolysis products

Hydrolysis of GLSs was catalyzed by the endogenous myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) according to the procedure of Radulović et al. (2008).

Autolysis experiments (three repetitions for each sample) were performed on homogenized fresh plant material (10 g of samples – aerial and underground parts together), which were mixed with a sufficient quantity of distilled water to make a paste (25 ml). No separation of the roots was made due to the small size of the plants (around 5 cm in height) and its minor contribution to the bulk of the plant material. According to a modified procedure reported by Vaughn and Berhow (2005), Et_2O (10 ml) was added to each sample, the all-glass vessels sealed, and the flasks placed in an incubator shaker set at 25 °C and 200 rpm for 8 h. Following hydrolysis, sodium chloride (6 g) was added and mixed thoroughly. The ether layer was then separated from the brine solution and filtered through a Whatman No. 1 filter paper and the residual plant mass was extracted an additional three times with excess Et_2O. The combined crude ether extracts were dried over dry magnesium sulfate, evaporated to dryness on a rotary evaporator at 30 °C, and redissolved in Et_2O (1 ml) (Radulović et al., 2008).

3.4. Gas chromatography/mass spectrometry (GC–MS)

The chemical composition of *H. petraea* autolysates were analyzed by GC and GC–MS immediately upon completion of the hydrolysis and separation procedure described above. The GC–MS analyses (three repetitions) were performed on a Hewlett-Packard 6890 N gas chromatograph equipped with a fused silica capillary column HP-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 °C and 320 °C, respectively. Oven temperature was raised from 70 to 315 °C at a heating rate of 5 °C min^{-1} and then isothermally held for 10 min. As a carrier gas helium at 1.0 ml min^{-1} was used. The samples, 1 μl of the solutions in Et_2O (10 mg in 1 ml of Et_2O), were injected in a pulsed split mode (the flow was 1.5 ml/min for the first 0.5 min and then set to 1.0 ml/min throughout the remainder of the analysis; split ratio 40:1). The mass selective detector was operated at the ionization energy of 70 eV, in the 35–650 amu range and scanning speed of 0.32 s. GC (FID) analyses were carried out under the same experimental conditions using the same column and same gas chromatograph type as described for the GC–MS. The percentage composition was computed from the total ion chromatogram peak areas without the use of correction factors. Qualitative analysis of the autolysate constituents was based on the comparison of their linear retention indices relative to retention times of C_7–C_{32} *n*-alkanes on the HP-5MS column (Van den Dool and Kratz, 1963) with those reported in the

literature (Adams, 2007), and by comparison of their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST05, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances, and wherever possible, by GC co-injection with an authentic sample.

3.5. General procedure for the synthesis of benzylic isothiocyanates 1–4 (Tsogoeva et al., 2005)

The benzyl isothiocyanates **1–4** were synthesized by the reaction of the corresponding amine and carbon disulfide in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) according to a modified procedure reported by Tsogoeva et al. (2005).

Carbon disulfide (12.5 ml) and DCC (6.3 g, 30.6 mmol) were added to a solution of the appropriate amine (33 mmol) in dry CH_2Cl_2 (40 ml) at −10 °C. With stirring, the reaction mixture was allowed to warm slowly to room temperature over a period of 3 h and then was stirred for a further 5 h at room temperature. The reaction was monitored by TLC. After the separation of the precipitated thiourea by filtration, the solvent was removed under vacuum. The residue was taken up in ether and more of the precipitated thiourea was removed by filtration. Evaporation of the solvent gave a crude product which was further purified by MPLC to afford the desired isothiocyanates of sufficient purity for spectral analysis.

3.5.1. Benzyl isothiocyanate (1)

Yellowish liquid; 3.73 g (75%), R_t (HP-5MS) 14.25 min, RI 1362; FTIR (ATR) cm^{-1}: 3063 (ar. C–H), 3031 (ar. C–H), 2166 (vs, N=C=S), 2067 (N=C=S), 1495, 1454, 1346, 1301, 1200, 1072 (C–N), 813, 696 (N=C=S), 573; ^{1}H NMR spectral data ($CDCl_3$): δ 4.72 (2H, *s*, H-7), 7.25–7.45 (5H, *m*, Ar-H); ^{13}C NMR spectral data ($CDCl_3$): δ 48.6 ($\underline{C}H_2$), 126.9 (C-2, C-6), 128.4 (C-4), 129.0 (C-3, C-5, N$\underline{C}$S), 134.3 (C-1); EIMS, 70 eV, *m*/*z* (rel. int.): 149 (23), 92 (7), 91 (100), 90 (2), 89 (6), 65 (15), 63 (6), 51 (5), 50 (3), 39 (5).

3.5.2. 2-Methoxybenzyl isothiocyanate (2)

Yellow liquid; 0.81 g (69%), R_t (HP-5MS) 19.22 min, RI 1569; FTIR (ATR) cm^{-1}: 2927, 2853, 2170 (vs, N=C=S), 2071 (N=C=S), 1693, 1602 (ar. C=C), 1492, 1462, 1342, 1246 (ar. C–O–al. C), 1287, 1118, 1026 (ar. C–O–al. C), 750 (C–S), 671 (N=C=S); ^{1}H NMR spectral data ($CDCl_3$): δ 3.85 (3H, *s*, CH_3), 4.69 (2H, *s*, 7-H_2), 6.80–7.01 (2H, *m*, H-3, H-5), 7.24–7.37 (2H, *m*, H-4, H-6); ^{13}C NMR spectral data ($CDCl_3$): δ 44.3 ($\underline{C}H_2$), 55.3 ($\underline{C}H_3O$), 110.4 (C-3), 120.6 (C-1), 122.4 (C-5), 127.9 (C-4),[1] 128.2 (C-6),[1] 129.7 (N$\underline{C}$S), 156.5 (C-2); EIMS, 70 eV, *m*/*z* (rel. int.): 179 (13), 122 (9), 121 (100), 93 (8), 92 (8), 91 (97), 78 (13), 77 (12), 65 (16), 51 (12).

3.5.3. 3-Methoxybenzyl isothiocyanate (3)

Yellow liquid; 0.76 g (67%), R_t (HP-5MS) 19.92 min, RI 1598; FTIR (ATR) cm^{-1}: 2929, 2852, 2202 (vs, N=C=S), 2089 (N=C=S), 1692, 1601 (ar. C=C), 1490, 1452, 1336, 1261 (ar. C–O–al. C), 1150, 1039 (ar. C–O–al. C), 773 (C–S), 700 (N=C=S), 544; ^{1}H NMR spectral data ($CDCl_3$): δ 3.81 (3H, *s*, CH_3), 4.68 (2H, *s*, H-7), 6.78–6.98 (3H, *m*, H-2, H-4, H-6), 7.29 (1H, *t*, H-5); ^{13}C NMR spectral data ($CDCl_3$): δ 48.5 ($\underline{C}H_2$), 55.2 ($\underline{C}H_3O$), 112.3 (C-4), 113.7 (C-2), 118.9 (C-6), 129.3 (C-5), 130.0 (N$\underline{C}$S), 135.6 (C-1), 159.9 (C-3); EIMS, 70 eV, *m*/*z* (rel. int.): 179 (32), 122 (9), 121 (100), 91 (28), 78 (15), 77 (14), 65 (8), 63 (6), 51 (8), 39 (6).

3.5.4. 4-Methoxybenzyl isothiocyanate (4)

Yellowish liquid; 2.76 g (77%), R_t (HP-5MS) 20.53 min, RI 1623; FTIR (ATR) cm^{-1}: 3291, 2928, 2835, 2183 (vs, N=C=S), 2075 (N=C=S), 1685, 1610 (ar. C=C), 1510, 1438, 1341, 1246 (ar. C–O–al. C), 1174, 1029 (ar. C–O–al. C), 815, 751, 668, 545; ^{1}H NMR spectral data ($CDCl_3$): δ 3.79 (3H, *s*, CH_3), 4.61 (2H, *s*, H-7), 6.87 (2H, *m*, H-3, H-5), 7.21 (2H, *m*, H-2, H-6); ^{13}C NMR spectral data ($CDCl_3$): δ 48.1 ($\underline{C}H_2$), 55.2 ($\underline{C}H_3O$), 114.2 (C-3, C-5), 126.2 (C-1), 128.7 (C-2, C-6, N$\underline{C}$S), 159.5 (C-4); EIMS, 70 eV, *m*/*z* (rel. int.): 179 (9), 122 (8), 121 (100), 91 (6), 89 (3), 78 (11), 77 (11), 63 (3), 52 (3), 51 (5).

[1] Interchangeable.

3.6. General procedure for the synthesis of benzylic thiocyanates 5–8

The benzyl thiocyanates **5–8** were prepared by the reaction of the corresponding aryl chlorides with potassium rhodanide in dimethylformamide (DMF) at room temperature (Suzuki et al., 1979).

A solution of the appropriate aryl chloride (15.8 mmol) and potassium thiocyanate (31.5 g, 32 mmol) in DMF (150 ml) was stirred at room temperature for 4 h, according to a modified procedure reported by Suzuki et al. (1979). The reaction mixture was then diluted with water (300 ml) and extracted with Et_2O (3× 50 ml). The combined ether extracts were washed with water and dried over dry $MgSO_4$. Evaporation of the solvent gave the desired thiocyanates of sufficient purity for spectral analysis.

3.6.1. Benzyl thiocyanate (5)

White crystals; 2.04 g (89%), R_t (HP-5MS) 13.63 min, RI 1342; FTIR (ATR) cm^{-1}: 3030 (ar. C–H), 2992, 2181 (SC≡N), 2050, 1598 (ar. C=C), 1492, 1454, 1426, 1243, 1203, 1159, 1074, 1027, 918, 804, 767–565 (ar. C–H and ar. C–C); ^{1}H NMR spectral data ($CDCl_3$): δ 4.15 (2H, *s*, H-7), 7.25–7.44 (5H, *m*, Ar-H); ^{13}C NMR spectral data ($CDCl_3$): δ 38.3 ($\underline{C}H_2$), 111.9 (S$\underline{C}$N), 128.8 (C-4), 128.9 (*two overlapped signals*, Ar-H), 129.1 (*two overlapped signals*, Ar-H), 134.3 (C-1); EIMS, 70 eV, *m*/*z* (rel. int.): 149 (3), 92 (8), 91 (100), 89 (4), 65 (15), 63 (6), 51 (4), 50 (2), 45 (2), 39 (7).

3.6.2. 2-Methoxybenzyl thiocyanate (6)

Yellow liquid; 0.24 g (84%), R_t (HP-5MS) 18.53 min, RI 1540; FTIR (ATR) cm^{-1}: 2941, 2637, 2366, 2149 (SC≡N), 1600 (ar. C=C), 1492, 1462, 1438, 1291, 1247 (ar. C–O–al. C), 1180, 1107, 1025 (ar. C–O–al. C), 883, 853, 751, 641; ^{1}H NMR spectral data ($CDCl_3$): δ 3.87 (3H, *s*, CH_3), 4.17 (2H, *s*, H-7), 6.89–6.99 (2H, *m*, H-3, H-5), 7.26–7.39 (2H, *m*, H-4, H-6); ^{13}C NMR spectral data ($CDCl_3$): δ 33.8 ($\underline{C}H_2$), 55.4 ($\underline{C}H_3O$), 110.7 (C-3), 112.8 (S$\underline{C}$N), 120.7 (C-1), 122.9 (C-5), 130.4 (C-4, C-6), 157.2 (C-2); EIMS, 70 eV, *m*/*z* (rel. int.): 179 (9), 122 (9), 121 (100), 93 (10), 92 (8), 91 (98), 78 (14), 77 (9), 65 (14), 51 (9).

3.6.3. 3-Methoxybenzyl thiocyanate (7)

Yellow liquid; 0.22 g (98%), R_t (HP-5MS) 19.31 min, RI 1572; FTIR (ATR) cm^{-1}: 2936, 2836, 2356, 2153 (SC≡N), 1600 (ar. C=C), 1489, 1453, 1435, 1265 (ar. C–O–al. C), 1153, 1037 (ar. C–O–al. C), 875, 782, 735, 700, 595, 568; ^{1}H NMR spectral data ($CDCl_3$): δ 3.82 (3H, *s*, CH_3), 4.13 (2H, *s*, H-7), 6.81–6.98 (3H, *m*, H-2, H-4, H-6), 7.23–7.34 (1H, *m*, H-5); ^{13}C NMR spectral data ($CDCl_3$): δ 38.3 ($\underline{C}H_2$), 55.2 ($\underline{C}H_3O$), 111.9 (S$\underline{C}$N), 114.3 (C-4),[1] 114.4 (C-2),[1] 121.1 (C-6), 130.1 (C-5), 135.7 (C-1), 159.9 (C-3); EIMS, 70 eV, *m*/*z* (rel. int.): 179 (33), 121 (100), 91 (30), 89 (6), 78 (16), 77 (13), 65 (7), 63 (7), 51 (8), 39 (6).

3.6.4. 4-Methoxybenzyl thiocyanate (8)

Yellow liquid; 2.02 g (82%), R_t (HP-5MS) 20.04 min, RI 1603; FTIR (ATR) cm^{-1}: 2936, 2836, 2356, 2153 (SC≡N), 1600 (ar. C=C), 1489, 1453, 1435, 1265 (ar. C–O–al. C), 1153, 1037 (ar. C–O–al. C), 875, 782, 735, 700, 595, 568; ^{1}H NMR spectral data ($CDCl_3$): δ 3.79 (3H, *s*, CH_3), 4.12 (2H, *s*, H-7), 6.88 (2H, *m*, H-3, H-5), 7.27 (2H, *m*, H-2, H-6); ^{13}C NMR spectral data ($CDCl_3$): δ 38.1 ($\underline{C}H_2$), 55.2 ($\underline{C}H_3O$), 112.1 (S$\underline{C}$N), 114.3 (C-3, C-5), 126.1 (C-1), 130.2 (C-2, C-6), 159.8

(C-4); EIMS, 70 eV, *m*/*z* (rel. int.): 135 (1), 121 (100), 107 (21), 94 (1), 79 (3), 77 (7), 69 (1), 64 (1), 51 (5), 41 (1).

3.7. General procedure for the synthesis of phenylacetonitriles 9–12 (Adams and Thal, 1922)

The phenylacetonitriles **9–12** were prepared from the corresponding chlorides with potassium cyanide in refluxing ethanol (Adams and Thal, 1922).

A mixture of powdered sodium cyanide (2.6 g, 53 mmol) and water (3 ml) was warmed on a water bath in order to dissolve most of the cyanide. Then, under reflux, a solution of the appropriate aryl chloride (34.8 mmol) in 95% ethanol (4 ml) was added dropwise over a period of 30 min. After heating under reflux for a further 3.5 h (the reaction was monitored by TLC), the resulting mixture was cooled to room temperature, filtered with suction to remove most of the sodium chloride, and the precipitate was washed with a small portion of ethanol. After evaporation of ethanol, the residue was extracted with Et_2O (3× 50 ml). The combined Et_2O extracts were dried over dry $MgSO_4$ and concentrated at reduced pressure. The resulting crude product was further purified by MPLC to afford the desired nitriles of sufficient purity for spectral analysis.

3.7.1. 2-Phenylacetonitrile (9)

Yellow liquid; 3.45 g (85%), R_t (HP-5MS) 8.44 min, RI 1136; FTIR (ATR) cm^{-1}: 2940, 2837, 2354, 2250 (C≡N), 1600 (ar. C=C), 1493, 1463, 1416, 1292, 1249, 1180, 1108, 1026, 832, 752, 696, 642, 593; ^1H NMR spectral data ($CDCl_3$): δ 3.71 (2H, *s*, H-7), 7.24–7.42 (5H, *m*, Ar-H); ^{13}C NMR spectral data ($CDCl_3$): δ 23.4 ($\underline{C}H_2$), 117.8 ($\underline{C}N$), 127.8 (C-3, C-5), 127.9 (C-4), 129.0 (C-2, C-6), 129.8 (C-1); EIMS, 70 eV, *m*/*z* (rel. int.): 117 (100), 118 (9), 116 (42), 91 (9), 90 (47), 89 (29), 63 (12), 51 (13), 50 (9), 39 (9).

3.7.2. 2-(2-Methoxyphenyl)acetonitrile (10)

Yellow liquid; 310 mg (67%), R_t (HP-5MS) 13.82 min, RI 1350; FTIR (ATR) cm^{-1}: 2939, 2837, 2249 (C≡N), 1600 (ar. C=C), 1591, 1495, 1463, 1412, 1291, 1247 (ar. C–O–al. C), 1163, 1108, 1026 (ar. C–O–al. C), 751, 567; ^1H NMR spectral data ($CDCl_3$): δ 3.68 (2H, *s*, H-7), 3.86 (3H, *s*, CH_3), 6.79–7.00 (2H, *m*, H-3, H-5), 7.22–7.37 (2H, *m*, H-4, H-6); ^{13}C NMR spectral data ($CDCl_3$): δ 18.6 ($\underline{C}H_2$), 55.4 ($\underline{C}H_3O$), 110.3 (C-3), 118.0 ($\underline{C}N$), 118.5 (C-1), 120.7 (C-5), 129.1 (C-4), 129.4 (C-6), 156.6 (C-2); EIMS, 70 eV, *m*/*z* (rel. int.): 147 (100), 132 (60), 116 (19), 107 (56), 104 (27), 91 (23), 89 (14), 78 (14), 77 (47), 51 (19).

3.7.3. 2-(3-Methoxyphenyl)acetonitrile (11)

Yellow liquid; 277.5 mg (69%), R_t (HP-5MS) 14.43 min, RI 1373; FTIR (ATR) cm^{-1}: 2940, 2837, 2354, 2251 (C≡N), 1683, 1601 (ar. C=C), 1489, 1455, 1414, 1284, 1259 (ar. C–O–al. C), 1148, 1108, 1039 (ar. C–O–al. C), 879, 778, 742, 690, 544; ^1H NMR spectral data ($CDCl_3$): δ 3.71 (2H, *s*, H-7), 3.81 (3H, *s*, CH_3), 6.73–6.91 (3H, *overlapping signals*, H-2, H-4, H-6), 7.28 (1H, *dd*, $J_{4,5}$ = 8 Hz, $J_{5,6}$ = 7 Hz, H-5); ^{13}C NMR spectral data ($CDCl_3$): δ 23.5 ($\underline{C}H_2$), 55.2 ($\underline{C}H_3O$), 113.4 (C-4),[1] 113.5 (C-2),[1] 117.7 ($\underline{C}N$), 120.0 (C-6), 128.3 (C-5), 130.1 (C-1), 160.0 (C-3); EIMS, 70 eV, *m*/*z* (rel. int.): 147 (100), 146 (19), 132 (27), 117 (22), 116 (24), 104 (20), 90 (17), 77 (66), 63 (17), 51 (17).

3.7.4. 2-(4-Methoxyphenyl)acetonitrile (12)

Yellow liquid; 450 mg (58%), R_t (HP-5MS) 14.91 min, RI 1392; FTIR (ATR) cm^{-1}: 3002, 2936, 2837, 2250 (C≡N), 1612 (ar. C=C), 1510, 1463, 1416, 1303, 1245 (ar. C–O–al. C), 1178, 1109, 1029 (ar. C–O–al. C), 906, 810, 755, 705, 580; ^1H NMR spectral data ($CDCl_3$): δ 3.65 (2H, *s*, H-7), 3.80 (3H, *s*, CH_3), 6.89 (2H, *overlapping signals*, H-3, H-5), 7.20 (2H, *overlapping signals*, H-2, H-6); ^{13}C NMR spectral data ($CDCl_3$): δ 22.6 ($\underline{C}H_2$), 55.2 ($\underline{C}H_3O$), 114.5 (C-3, C-5), 118.4 ($\underline{C}N$), 122.0 (C-1), 129.1 (C-2, C-6), 159.2 (C-4); EIMS, 70 eV, *m*/*z* (rel. int.): 147 (100), 146 (44), 132 (36), 116 (21), 107 (21), 104 (16), 91 (11), 78 (10), 77 (38), 51 (12).

3.8. In vitro antimicrobial activity

3.8.1. Test microorganisms

The synthesized compounds **1–5**, **7–9** were tested against a panel of microorganisms (isolates), including Gram-positive *Staphylococcus aureus* (two strains isolated from human material sample), *Bacillus cereus* (food isolate), *S. lutea* (food isolate), Gram-negative *Salmonella enteritidis* (food isolate), *Klebsiella pneumoniae* (human sample isolate), *Escherichia coli* (human sample), *Shigella* sp. (human sample), yeast *Candida albicans* (human sample isolate) and mould *A. fumigatus* (house dust isolate). Cultures of the bacterial species were maintained on Nutrient agar while the fungal cultures on Sabouraud dextrose agar at the appropriate optimal temperature (37 and 30 °C, respectively).

3.8.2. Screening of antimicrobial activity

Antimicrobial activity was evaluated using a broth microdilution method (NCCLS, 2003). The minimum inhibitory concentrations (MIC) determination was performed by a serial dilution method in 96-well microtiter plates. Microorganisms were cultured at 37 °C in Mueller Hinton agar (MHA) for bacteria and Sabouraud dextrose agar (SDA) for fungi (30 °C). After 18 h of cultivation, bacterial suspensions were made in Mueller Hinton broth and their turbidity was standardized to 0.5 McFarland. Absorbance of every suspension was adjusted to a standard one using a McFarland Densitometer (DEN-1B, Biosan). The final density of the bacterial and yeast's inoculums was 5×10^5. Suspension of the mould was made in Sabouraud dextrose broth and its turbidity was confirmed by viable counting in Thoma chamber with 1×10^4 as the final size of the inoculums. Stock solutions of the compounds were prepared in 0.05% aq. Tween 80 (Hammer et al., 1999) in the concentration range from 0.0005 to 20.00 mg/ml. The highest concentration, 0.025% (v/v) in any well, of Tween 80 had no effect of the growth of the microorganisms. After making the dilutions of the test substances, the inoculum was added to all wells and the plates were incubated at 37 °C during 24 h (bacteria) or at 30 °C for 48 h (fungal strains). Tetracycline and nystatin served as positive controls, while the solvent (aqueous 0.05% Tween 80, v/v), showing no visible effect, was used as the negative one. One non-inoculated well, free of the antimicrobial agents, was also included to ensure medium sterility. The bacterial growth was determined by adding 20 μl of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) aq. solution (Radulović et al., 2010). MIC was defined as the lowest concentration of the sample that inhibited visible growth (red colored pellet on the bottom of the wells after the addition of TTC). To determine the minimal bactericidal/fungicidal concentrations (MBC/MFC), broth was taken from each well without visible growth and inoculated in MHA for 24 h at 37 °C for bacteria or in SDA for 48 h at 28 °C (mould) and 30 °C (yeast). The MBC/MFC was defined as the lowest concentration of a tested sample at which the inoculated microorganisms were 99.9% killed. All experiments were done in duplicate and repeated three times.

3.9. Statistical analysis

To determine whether there is a statistically significant difference among the obtained results for antifungal and antibacterial activity assays, variance analyses were carried out using the SPSS 10.0 software package. Values of $p < 0.05$ were considered to be significantly different.

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