EJEAFChe

Electronic Journal of Environmental, Agricultural and Food Chemistry

ISSN: 1579-4377

$C_2H_2F_4$-SCFE-OLEORESINS OF BLACK PEPPER (*PIPER NIGRUM L.*) AND GINGER (*ZINGIBER OFFICINALE (L.) ROSC.*) FROM VIETNAM: ANTIMICROBIAL TESTINGS, GAS CHROMATOGRAPHIC ANALYSIS AND OLFACTORIC EVALUATION

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KEYWORDS

$C_2H_2F_4$-SCFE-Oleoresins, pepper, ginger, antimicrobial testing, compositions, GC-FID, GC-MS, olfactoric evaluation.

ABSTRACT

$C_2H_2F_4$-SCFE-oleoresins of black pepper (dried fruits) and ginger (dried roots) from Vietnam were tested for their antimicrobial activities against some different strains of yeast, Gram-(+)- and Gram-(-)-bacteria by agar diffusion and agar dilution method, respectively. Both oleoresins showed a significant high antimicrobial activity against the Gram-(+)-bacteria *Staphylococcus aureus* and *Enterococcus faecalis*. In addition, strong effects of the *Zingiber officinale* oleoresin against the Gram-(-)-bacteria *Klebsiella pneumoniae* and the yeast *Candida albicans* were observed.

For qualitative and quantitaive investigations of the compositions of the SCF-extracts, gas chromatographic methods (GC-FID and GC-MS with columns of different polarities) were used and the results correlated with that of the antimicrobial testings. As main compounds of the $C_2H_2F_4$-SCFE-oleoresins of pepper β-caryophyllene (56.8%), limonene (18.9%), terpinolene (3.9%) and *p*-cymene (3.1%) as well as of ginger α-zingiberene (42.1%), β-sesquiphellandrene (14.2%), β-bisabolene (11.3%), *cis*-menth-2-en-1-ol (9.8%), α-curcumene (8.5%) and (*E,E*)-α-farnesene (5.9%) were found.

INTRODUCTION

In continuation of our research work on the field of combined data interpretation of antimicrobial testings (agar diffusion and agar dilution methods) and gas chromatographic analysis (GC-FID and GC-MS, using fused silica columns of different polarity) of aroma samples [1-7], in a systematic investigation of aroma compounds and odorous samples (e.g. essential oils and extracts) of a running, international project, the supercritical fluide extraction-oleoresins ($C_2H_2F_4$-SCFE-method [16,17,18]) of black pepper (*Piper nigrum* L., Piperaceae) and ginger (*Zingiber officinale* (L.) Rosc., Zingiberaceae) from Vietnam were investigated. The obtained antimicrobial and analytical data of these oleoresins were compared with that ones of CO_2-SCFE-oleoresins of the identical plant material [1] to get informations about influences on the use of different SCF-extraction methods. Although the medical, pharmaceutical, perfumistic, cosmetic and food flavouring properties as well as the compositions of different samples (essential oils, extracts, etc.) of pepper and ginger have already been discussed by many researchers [e.g. 8-15], no detailed informations about the composition, olfactorical properties and antimicrobial activities of CO_2-SCF-extracts (oleoresins) of *Piper nigrum* and *Zingiber officinalis* from Vietnam were published until now. In addition, data of the influence of single main compounds of the black pepper and ginger oleoresin on the antimicrobial effects were not available.

Therefore, the objectives of this research was to test these $C_2H_2F_4$-SCFE-oleoresins of pepper and ginger by means of usual agar diffusion and agar dilution method against these properties of various strains of microorganisms [19-37], after improvement of some parameters [4-7]. Furthermore, gas chromatograpic (GC-FID and GC-MS) analyses and olfactoric evaluations were used to get informations about the composition of each sample. As result of the combined data interpretation the above mentioned influence of the main compounds on the antimicrobial effects of the pepper and ginger oleoresins should be ascertained and -as mentioned above- these data sets compared with that ones of CO_2-SCFE-oleoresins of the identical plant material [1].

EXPERIMENTAL

Samples

The air-dried fruits of black pepper and roots of ginger from Vietnam (the crops were harvested in 2004) were ground separately in an attrition mill to a size of 0.15-0.25 mm and the oleoresins pepper A and ginger A obtained by a high-pressure-CO_2-extractor [16,] equipped with a 5 dm^3 volume extractor-vessel as well as the oleorseins pepper B and ginger B by a $C_2H_2F_4$-laboratory-extractor [17] under following conditions (continuos flow, draining every 60 min.):

Material	*Temperature (°C)*	*Pressure (bar)*	*Time (min.)*	*Yield (%)/Colour*
Pepper A	28-32	6.8-7.2	180	2.2/yellow
Pepper B	19-21	5.0	20	1.6/yellow
Ginger A	12-14	4.6-5.0	120	3.7/yellow
Ginger B	13-14	3.4	180	1.1/yellow

Using an azeotropic distillation of the raw materials (moisture) for quality control, yields of 6.8%/7.2% (pepper A/B) and 7.0%/6.5% (ginger A/B) of extracts were found.

Eugenol and tetracycline hydrochloride (= achromycine hydrochloride – 25g) are products from Sigma-Aldrich with numbers W24,670-0 and T3383-25G, Ciproxin^R 500mg-tablettes = 582mg ciproflaxoxacine hydrochloride/water) from Bayer Austria Co., Vienna and Lidaprim^R-infusion-bottle (250mg containing 0.8g sulfmetrol and 0.16g trimethoprim) from Nycomed Austria Co., Vienna.

Antimicrobial testings

As test microorganisms (colony-forming-units = cfu/cm^3), Gram-(+)-bacteria *Staphylococcus aureus* (ATCC 6538P, $1x10^{13}$) and *Enterococcus faecalis* (clinically isolated, $1x10^{13}$), Gram-(-)-bacteria *Escherichia coli* (ATCC 8739, $2x10^{12}$), *Proteus vulgaris* (clinically isolated, $3x10^{13}$), *Pseudomonas aeruginosa* (G 28, $1.2x10^{9}$), *Samonella sp.* (clinically isolated, $3x10^{12}$) and *Klebsiella pneumoniae* (clinically isolated, $1x10^{13}$) as well as the yeast *Candida albicans* (ATCC 10231, $3.6x10^{11}$) – all products from the National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria – were used.

The antimicrobial activity was studied by two methods: Agar diffusion disc method using Whatman No. 1 filter paper discs (6mm) and quantities of 6µl of the sample, each. After cultivation of the bacteria and the yeast at 37°C for 24^h the diameter of the inhibition zone (IZ) was measured [4-7] as well as agar serial tube dilution method with results as minimum inhibitory concentration (MIC) in accordance to [4-6] as follows: The essential oils and reference compounds were added to brine, containing 1.0% (v/v) Tween 80 at the appropriate volumes to produce final concentrations of the samples in the range of 100-1000ppm; the Petri dishes were inoculated by pipetting $0.1cm^3$ of the desired culture and 6.0µL of the samples as well as the reference compounds (the tablettes of Ciproxin^R were added as solution in saline at a quantity of 300µg) on paper discs (6mm) and then incubated at 37°C for 24h.

Olfactoric evaluations

All investigated samples were olfactorically evaluated by professional perfumers and/or olfactorically trained chemists and the aroma described as pleasant spicy-peppery with fresh top- and woody-herbal base-notes (black pepper) and as pleasant spicy, camphoraceous-minty top- and warm-ginger-like base-notes (ginger). In general, a high olfactoric quality for both samples were certificated.

GC/FID

A GC-14A with FID and integrator C-R6A-Chromatopac (Shimadzu Co.) resp. a GC-3700 with FID (Varian Co.) and integrator C-R1B-Chromatopac (Shimadzu Co.) were used; carrier gas: hydrogen; injector-temp.: 250°C; detector-temp.: 300°C; temp.-progr.: 40°C/5 min. to 280°C/5 min. with a heating-rate of 6°C/min.; columns: 30m x 0.32mm bonded FSOT-RSL-200 (OV-5-type) fused silica (film thickness: 0.25 micron; Biorad Co.) and 50m x 0.32mm bonded Stabilwax (film thickness: 0.50micron; Restek Co.); quantification by %-peak-area-calculation. Retention indices of the constituents were compared with published data [38-44] or own data.

GC/MS

A GC-17A with QP5000 (Shimadzu Co.) and data-system Compaq-ProLinea (Class5k-software, Shimadzu Co.), a GC-17A with QP5050 (Shimadzu Co.) with data-system PentiumII (Class5k-software, Shimadzu Co.), a GC-HP5890 with HP5970-MSD (Hewlett-Packard Co.) and Pentium-PC (Böhm Co., ChemStation-software) resp. a GCQ (Finnigan-Spectronex Co.) and Gateway-2000-PS75 (Siemens-Nixdorf Co.; GCQ-software) were used; carrier gas: helium; injector-temp.: 250°C; interface-heating: 300°C; ion-source-heating: 200°C; EI-mode, 70 eV; scan-range: 41-550 amu; other parameters see GC/FID-part. Mass spectra correlations with Wiley-, NBS-, NIST- and private library spectra on-line as well as published ones [38,41,43].

RESULTS AND DISCUSSION

Eugenol as a volatile phenolic compound with well-known antimicrobial activity against many strains of microorganisms was tested using it as a standard substance. This flavor component shows excellent effects against all strains using both methods, agar diffusion and agar dilution. Therefore, its antimicrobial data (IZ=inhibition zones and MIC=minimum inhibition concentrations) were taken as natural reference compound for further testings. Additionally, the antibiotics Ciproxin^R, Lidaprim^R and tetracycline hydrochloride wer used as synthetic references. Surprisingly, Lidaprim^R was not effective against the Gram-(-)-bacteria *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (see **Table 1**).

Both $C_2H_2F_4$-SCFE-oleoresins (samples B) were found to show a high activity against the Gram-(+)-bacteria *Staphylococcus aureus* (pepper: IZ=20 and MIC=60; ginger: IZ=19 and MIC=60) and

Enterococcus faecalis (pepper: IZ=9 and MIC=600; ginger: IZ=7 and MIC=600). In addition, the *Z. officinalis* oleoresin is strongly effective against *Klebsiella pneumoniae* (IZ=8 and MIC=600) and against the yeast *Candida albicans* (IZ=8 and MIC=600); see **Table 1**).

Table 1. Antimicrobial activities of pepper and ginger oleoresins from Vietnam as well as of the reference compounds

Compounds and Essential Oils	**Inhibition Zones (IZ) in mm and Minimum Inhibition Concentrations (MIC) in ppm in accordance to test-microorganisms and methods**									
	Staphylococcus aureus/ Enterococcus faecalis		***Escherichia coli/ Proteus vulgaris***		***Pseudomonas aeruginosa/ Salmonella sp.***		***Klebsiella pneumoniae***		***Candida albicans***	
	IZ	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**
Pepper oleoresin A	20/10	600/600	-/-	-/-	-/-	-/-	10	6	15	600
Pepper oleoresin B	20/9	60/600	-/-	-/-	-/-	-/-	-	-	-	-
Ginger oleoresin A	12/18	60/60	-/-	-/-	-/-	-/-	-	-	10	600
Ginger oleoresin B	19/7	60/600	-/-	-/-	-/-	-/-	8	600	8	600
Eugenol	30/30	600/600	28/30	600/600	25/28	600/600	28	600	32	600
Ciproxin[R]	35/33	600/600	22/25	600/600	32/10	600/600	25	600	-	-
Lidaprim[R]	27/27	600/600	11/23	60/600	-/8	-/60	-	-	-	-
Tetracycline hydrochloride	15/22	600/600	11/13	600/600	15/10	600/600	20	600	-	-
- no inhibition observed										

The correlation with antimicrobial data of the previous investigated CO_2-SCFE-oleoresins (samples A) of the identical pepper and ginger plant material showed differences in the activity of pepper oleoresin A, with effects against *Klebsiella pneumoniae and Candida albicans*, which were not found by use of pepper oleoresin B. In contrast to ginger oleoresin A (no effects), oleoresin B was found to be highly antimicrobial active against *Klebsiella pneumonia* (see **Table 1**).

Using gas chromatography (GC-FID and GC-MS with 2 columns of different polarity) in combination with olfactoric evaluations, the purity and composition of the $C_2H_2F_4$-SCF-extracts were investigated and following results found:

The black pepper sample B from Vietnam shows more than 3% (calculated as %-peak area of GC-FD analysis using an apolar column) of the sesquiterpene hydrocarbons β-caryophyllene (56.8%), limonene (18.9%), terpinolene (3.9%) and *p*-cymene (3.1%), while for oleoresin A the following published [1] main compounds were found: β-caryophyllene (43.9%) and *allo*-aromadendrene (4.4%) as well as of the monoterpene hydrocarbons limonene (17.1%), δ-3-carene (11.8), α-phellandrene (4.4%), sabinene (3.1%) and myrcene (3.1%; see **Table 2**).

The main compounds of the Vietnamese ginger oleoresin B are α-zingiberene (42.1%), β-sesquiphellandrene (14.1%), β-bisabolene (11.3%), *cis*-menth-2-en-1-ol (9.8%), α-curcumene (8.5%) and (*E,E*)-α-farnesene (5.9%), whereas for the CO_2-SCFE-oleoresin A of ginger α-zingiberene (36.9%), β-sesquiphellandrene (15.3%), β-bisabolene (8.8%), (*E,E*)-α-farnesene (7.0%) and α-curcumene (6.6%) as well as the monoterpene hydrocarbon camphene (3.2%; see **Table 2**) were found.

Table 2. Compositions of pepper and ginger $C_2H_2F_4$-SCFE-oleoresins B in correlation to previous published data of CO2-SCFE-oleoresins [1] A (%-peak area of GC using an apolar fused silica OV-5-type column)

Compound	RI	PepperA/B	GingerA/B	Odor in accordance to [8, 10, 47 - 49]
cis-3-Hexenol	859	tr[1]/tr	tr/tr	green, grassy, weak fatty
α-Thujene	930	0.1/0.4	0.7/tr	camphoraceous, herbal
α-Pinene	939	0.2/tr	0.3/0.1	pine-like, warm, weak herbal
Camphene	955	0.1/tr	3.2/0.6	camphoraceous, fresh, clean
Heptanol	966	tr/nd	1.7/tr	oily, weak herbal
Sabinene	975	3.1/1.2	tr/tr	warm, oily-peppery, woody, spicy, weak herbal
β-Pinene	979	2.2/tr	0.1/nd	resinous-piney, dry-woody
1-Octen-3-ol	981	nd[2]/tr	tr/nd	mushroom-like, moody, earthy
Myrcene	991	3.1/0.2	0.6/tr	weak citrus- and lime-like
2-Octanol	995	tr/nd	2.0/tr	fatty, oily, weak earthy
α-Phellandrene	1003	4.4/0.9	tr/nd	citrus-like, weak peppery
1,4-Cineole	1015	nd/0.2	tr/nd	fresh, eucalyptus-like, camphoraceous
α-Terpinene	1017	tr/0.5	nd/tr	weak lemon- and citrus-like
p-Cymene	1025	0.9/3.1	0.1/0.6	weak citrus-, lemon- and bergamot-like
Limonene	1029	17.1/18.9	1.2/0.9	lemon-like
β-Phellandrene	1031	tr/0.4	0.2/tr	peppery, minty, weak citrus-like
δ-3-Carene	1033	11.8/1.1	tr/tr	sweet, limonene-like
1,8-Cineole	1035	0.1/0.2	0.1/0.6	fresh, eucalyptus-like
γ-Terpinene	1060	tr/1.3	nd/tr	citrus-like, herbal
cis-Sabinene hydrate	1071	tr/0.2	nd/nd	warm, spicy, peppery, weak woody
Terpinolene	1089	0.2/3.9	0.2/nd	pleasant, sweet-piney
trans-Sabinene hydrate	1095	tr/0.1	nd/nd	warm, herbal, spicy, woody
Linalool	1097	0.8/1.6	0.3/0.5	fresh, floral
cis-Menth-2-en-1-ol	1122	nd/0.1	7.1/9.8	fresh, minty
trans-Menth-2-en-1-ol	1141	nd/nd	1.8/2.1	fresh, minty
cis-β-Terpineol	1144	tr/tr	0.1/tr	woody-earthy
Camphor	1146	tr/nd	0.1/tr	fresh, camphoraceous
Isoborneol	1161	nd/nd	tr/tr	camphoraceous, weak piney
Borneol	1169	nd/tr	1.3/0.2	camphoraceous, peppery, earthy
Terpinen-4-ol	1177	tr/0.1	tr/0.2	warm, peppery, woody, weak liliac-like
α-Terpineol	1189	0.9/0.2	0.5/tr	sweet, floral, liliac-note
Safranal	1195	tr/nd	nd/nd	herbal, saffron-like
Neral	1236	nd/nd	0.3/nd	floral, fruity, sweet
Geranial	1265	nd/nd	0.7/tr	floral, sweet, weak fruity
Bornyl acetate	1289	nd/nd	tr/nd	piney, sweet-balsamic, herbal
Isobornyl acetate	1286	nd/nd	tr/nd	mild piney, oily-camphoraceous, balsamic
δ-Elemene	1335	0.9/tr	nd/nd	herbal, spicy
α-Cubebene	1349	1.5/1.8	nd/tr	woody, spicy
α-Copaene	1377	tr/1.3	tr/tr	weak woody, herbal
β-Elemene	1389	2.2/0.9	tr/nd	herbal, spicy, woody
β-Caryophyllene	1419	43.9/56.8	tr/0.8	spicy, woody, terpene-like
γ-Elemene	1432	0.2/0.7	nd/nd	herbal, spicy
Aromadendrene	1441	tr/tr	nd/tr	woody, spicy
(*Z*)-β-Farnesene	1444	tr/tr	nd/tr	herbal, spicy
α-Humulene	1455	2.3/1.2	tr/0.1	woody, spicy
allo-Aromadendrene	1460	3.3/0.8	nd/nd	spicy, herbal, woody
Germacrene B	1462	tr/tr	nd/nd	spicy, dry-woody

α-Curcumene	1481	nd/0.1	6.6/8.5	spicy, herbal
Germacrene D	1485	0.2/0.4	1.1/0.9	dry-spicy, weak woody
α-Zingiberene	1495	nd/nd	36.9/42.1	warm-spicy, weak woody
(*E*,*E*)-α-Farnesene	1504	tr/0.2	7.0/5.9	warm-floral, herbal
β-Bisabolene	1506	0.1/tr	8.8/11.3	warm, spicy, sweet-balsamic
Germacrene A	1508	tr/tr	nd/nd	dry-spicy, weak woody
γ-Cadinene	1514	tr/tr	1.3/tr	dry-woody, mild
β-Sesquiphellandrene	1522	nd/nd	15.3/14.2	spicy, mild
δ-Cadinene	1525	tr/0.3	nd/nd	warm-spicy, woody
Caryophyllene oxide	1583	0.1/1.1	tr/0.5	warm-spicy, woody

[1]trace compound (less than 0.1%)

[2]not detected

In general, it can be seen that the $C_2H_2F_4$-SCFE-oleoresins of pepper and ginger in comparison with the CO_2-SCF-extracts have less compounds, however the main components in higher concentrations.

The composition of the black pepper and ginger extract volatiles are characteristic for samples of these species, presented in many papers (e.g. [11, 13-15, 39, 42]).

In addition, the gas chromatographic data of the $C_2H_2F_4$-SCFE-oleoresins are in accordance to that one of olfactoric evaluations (using correlations with odor attributes published elsewhere [8,10,47-51]) and therefore, both results prove also the high quality of these samples.

Summarizing this combined and comparative investigation of antimicrobial activities and composition of *Piper nigrum* and *Zingiber officinale* $C_2H_2F_4$- and CO_2-SCF-extracts from Vietnam, we can state:

1.) Eugenol as natural reference substance was found to possess the highest antimicrobial potential of all samples tested. This result proves that both, agar diffusion and agar dilution methods furnish reproducible antimicrobial data in testing aroma samples, while surprisingly the synthetic antibioticum Lidaprim[R] was not effective against the Gram-(-)-bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (contrary to the also used synthetic antibiotics Ciproxin[R] and tetracycline hydrochloride).

2.) Significant antimicrobial effects of all four oleoresins were found only against the Gram-(+)-bacteria *Staphylococcus aureus* and *Enterococcus faecalis*. Against the yeast *Candida albicans* both ginger, but only the CO_2-SCFE-oleoresin of pepper were strongly effective. In addition, the pepper oleoresin A and the ginger oleoresin B show medium to high antimicrobial activity against the Gram-(-)-bacterium *Klebsiella pneumoniae*.

3.) By means of GC-FID and GC-MS analyses characteristic compositions of volatiles for such black pepper and ginger samples were found, with wellknown dominating mono- and sesquiterpene hydrocarbons in both cases. In general, for the $C_2H_2F_4$-SCFE-oleoresins, in comparison with the CO_2-SCFE-oleoresins, less constituents were identified, but the main compounds in higher concentrations.

4.) Although some papers report that mono- and sesquiterpene hydrocarbons do not show any or only very weak antimicrobial effects against bacteria, fungi and yeasts (e.g. [25,36], we found a high activity of these group of compounds [4, 7].

Further investigations, in combination with antimicrobial testings and GC (and olfactometry), should be done to get more data for a satisfying answer of the question, how effective pure aroma chemicals in complex aroma samples (e.g. essential oils and extracts) really are.

ACKNOWLEDGEMENT

The authors are grateful to *Univ.-Prof.Dr. Heinz Schilcher*, professor emer. of „Freie Universität Berlin", for the valuable informations and discussions on the field of antimicrobial testing methods for natural products.

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