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Artigo

CHEMICAL COMPOSITION, CIRCADIAN RHYTHM AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF *Piper divaricatum*: A NEW SOURCE OF SAFROLE

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The essential oils from leaves, stems and fruits of *Piper divaricatum* were analyzed by GC-MS. The tissues showed high safrole content: leaves (98%), fruits (87%) and stems (83%), with yields of 2.0, 4.8 and 1.7%, respectively. This is a new alternative source of safrole, a compound widely used as a flavoring agent and insecticide. The leaf´s oil showed antibacterial activity against gram-negative bacteria while safrole was active against *Salmonella Typhimurium* and *Pseudomonas aeruginosa.* In addition, the study of circadian rhythm of the safrole concentration in the essential oils of leaves showed a negligible variation of 92 to 98%.

Keywords: *Piper divaricatum*; Piperaceae; safrole.

INTRODUCTION

Safrole, the main component of sassafras oil (*Ocotea odorífera)*, is a natural compound, widely found in the plant kingdom at a broad range of concentrations in species of the Aristolochiceae, Lauraceae and Piperaceae families.[1]

Safrole is classified as a weak hepatocarcinogen in rodents and possibly in humans.[2] It is an important raw material for the chemical industry, being used as a fragrance, flavoring agent and in the synthesis of piperonyl butoxide (PBO), a crucial ingredient in pyrethroid insecticides.[3] Natural pyrethrum insecticide is not sufficiently effective for profitable marketing without the addition of PBO as a synergist, therefore its commercialization depends on continued availability of PBO.[4,5] Japan, Italy and the United States are the most important markets for sassafras oil, with total annual demand estimated to be around 2,000 tonnes. The demand for sassafras oil depends on the market for PBO and heliotropin, a compound commonly used in flagrances and flavoring agents and obtained by oxidation of safrole/isosafrole. Global consumption of heliotropin is increasing, particularly in Eastern Europe and Asia, and sassafras oil is the preferred raw material for its production.[5] In Brazil, *Piper hispidinervum*, a safrole-rich shrub endemic in the state of Acre, is cultivated as an alternative source for the obtention of safrole.[6]

In a systematic study of Piperaceae phytochemistry, we identified *Piper divaricatum* as containing high levels of safrole in the essential oil extracted from its tissues. *P. divaricatum* is an aromatic plant found mainly in Atlantic Forest in Brazil. It is used in folk medicine to treat rheumatism and cramps and also as an insecticide.[7] Due to the medicinal proprieties of plant, we tested the antibacterial activity of essential oils from *P. divaricatum* tissues. In addition, the circadian rhythm of the essential oil composition from leaves of this plant was investigated.

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EXPERIMENTAL

Plant material

Fresh leaves, fruits and stems of *P. divaricatum* ($N = 3$) were collected in the city of Itabuna, Bahia state, Brazil, in January 2011. For the circadian rhythm study, leaves were collected on January 4, 2011 at 3 a.m., 6 a.m., 9 a.m., 12 a.m., 3 p.m., 6 p.m., 9 p.m. and 12 p.m. The botanical material was identified by Dr. E. F. Guimarães (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Brazil) and a voucher specimen (Kato-1063) was deposited at the Herbarium of the Instituto de Botânica, São Paulo, Brazil.

Obtention and analysis of the essential oils

The essential oils from fresh leaves, fruits and stems were obtained by hydrodistillation using a Clevenger-type apparatus. A 100 g amount of fresh plant tissue and 250 mL of H_2O were used and the distillation was carried out for 2 h after the mixture reached boiling point. Traces of water present in the essential oils were removed by treatment with Na_2SO_4. The samples were kept at -20 °C in a freezer until further analysis. Yields were calculated based on the weight of the fresh material.

The essential oils were analyzed by GC-MS (60-240 ºC at 3 ºC min rate) on a Varian 431-GC device coupled to a Varian 220-MS instrument using a fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm) coated with DB-5. MS spectra were obtained using electron impact at 70 eV, a scan interval of 0.5 s and fragments from 40 to 550 Da (all samples were analyzed in triplicate).

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Perkin-Elmer instrument, under the same conditions and with the same column as reported for GC–MS.

Identification of safrole in the essential oils was performed by comparing its mass spectrum and t_r (retention time) with a known authentic compound as well as by computed matching of the acquired mass spectra to those stored in the NIST21 and NIST107 mass

Table 1. Chemical constituents of the essential oils from tissues of *P. divaricatum*

Chemical constituents	Leaves				Stems				Fruits			
	AIE[a])	AIL[b])	t_r	%	AIE	AIL	t_r	%	AIE	AIL	t_r	%
Safrole	**1287**	**1285**	**18.9**	**98.0±0.1**	**1287**	**1285**	**18.9**	**83.0±0.2**	**1287**	**1285**	**18.9**	**87.0±0.1**
Daucene	1377	1380	22.9	0.4±0.6	-	-	-	-	-	-	-	-
Cadina-1(6),4-diene <*cis*>	1458	1461	26.7	1.1±0.3	1458	1461	26.7	5.0±0.4	1458	1461	26.7	3.2±0.3
γ-Gurjunene	-	-	-	-	-	-	-	-	1475	1475	27.3	1.7±0.1
δ-Amorphene	-	-	-	-	1507	1511	28.9	1.1±0.3	1508	1511	28.8	4.5±0.5

AIE [a]) Experimentally determined. AIL [b]) Literature values ref. 9

spectral library of the GC/MS data system. Retention indices (RI) for the safrole were determined according to Vandendool and Kratz,[8] as described elsewhere.[9] Quantification of safrole in the essential oils was performed on the basis of their GC peak areas compared against the GC peak area of the authentic compound, obtained from safrole solutions diluted in methanol (0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL). Standard safrole was purchased from Sigma.

Antibacterial activity

The minimum inhibitory concentration (MIC) of the oils and safrole were determined through the broth dilution method.[10] The following bacterial species were used in the assays: gram-negative – *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella enterica* Ser. *Typhimurium*; gram-positive – *Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus.* An aliquot of 0.1 mL of each test bacterium (10^8 cells/mL) was placed into tubes containing Mueller Hinton broth plus safrole or essential oils at concentrations ranging from 4.8 to 5,000 μg/mL. The MIC was defined as the lowest oil concentration that caused visible inhibition of growth after 24 h of incubation at 37 °C. An aliquot 0.1 mL was drawn from the tubes and added to Petri dishes containing Mueller Hinton agar to determine the minimum bactericidal concentration (MBC). MBC was defined as the lowest concentration resulting in no growth after incubation of 24 h at 37 ºC. All assays were performed in duplicate. Also, when the MIC values could not be determined because of the turbidity produced by the safrole or essential oils, the MBC was evaluated for the whole set of tubes.

RESULTS AND DISCUSSION

The essential oil yields from hydrodistillation of fresh leaves, fruits and stems of *P. divaricatum* were 2.0, 4.8 and 1.7% (w/w), respectively. Qualitative and quantitative analysis of the essential oils from the three plant parts by GC-FID and GC-MS (Figure 1) showed phenylpropanoid safrole as the predominant constituent, at 98, 87 and 83%, respectively (Table 1).

In addition, the circadian variation of safrole content in the essential oil from fresh leaves of *P. divaricatum* was investigated by GC-FID and GC-MS. The results indicated no significant change in the safrole content (Table 2).

The chemical composition previously reported for essential oil from leaves of *P. divaricatum* did not reveal the presence of safrole, but identified other phenylpropanoids including methyleugenol (63.8%) and eugenol (23.6%) in specimens collected from Marajo Island, Pará state, Brazil.[11] In addition, terpenes such as linalool (29%), β-pinene (25%) and α-pinene (18.8%) were found in specimens collected in Guaramiranga, Ceará state, Brazil.[7] Safrole has been identified in the essential oil of several *Piper* species, at concentrations ranging from 0.2 to 90%, with *P. hispidinervum* showing the highest reported content (Table 3).

The antibacterial activity of the safrole and essential oils from *P. divaricatum* is shown in Table 4. The leaf oil was broadly inhibitory

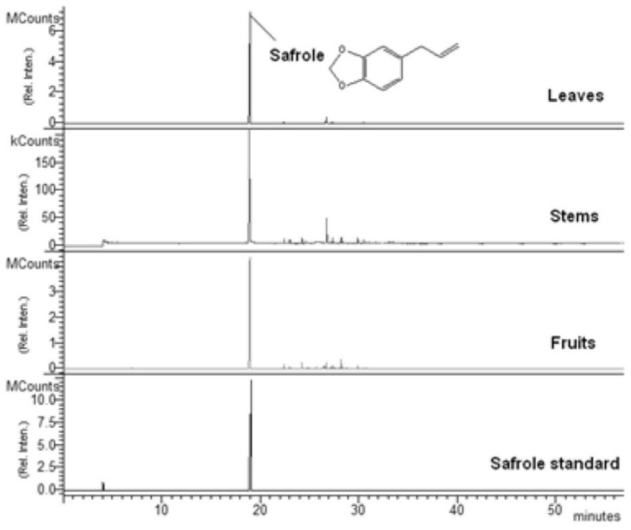

Figure 1. *Chromatogram (GC) of essential oils from leaves, stems and fruits of P. divaricatum and standard safrole*

Table 2. Percentage content of safrole found in the essential oil from leaves of *P. divaricatum* and yields of oil during circadian rhythm analysis

Harvest time (h)	Safrole content (%)	Oil yields (%)
3	98.1 ±0.1	1.0 ±0.1
6	92.0 ±0.2	0.8 ±0.1
9	92.4 ±0.1	0.8 ±0.1
12	95.0 ±0.1	1.0 ±0.2
15	92.5 ±0.2	1.0 ±0.1
18	92.6 ±0.3	1.5 ±0.2
21	95.3 ±0.1	2.0 ±0.2
24	96.1 ±0.2	0.8 ±0.3

Table 3. Relative percentage of safrole in essential oil from *Piper* species previously reported

Piper species	Safrole	Ref.
P. hispidinervum	90	5
P. auritum	87	12
P. betel	52	13
P. betle	46	14
Piper obliquum	45	15
Piper betel	40	16
P. Silvestre	32	17
P. betel	27	18
P. callosum	22	19
P. affinis hispidinervum	18	4
P. carpunya	15	20
P. guineense	2	21
P. marginatum	0.2 – 64	22

Table 4. Antibacterial activity of safrole and essential oils from *Piper divaricatum*

Strains	Essential oils							
	Safrole		Leaf		Stem		Fruit	
Gram-negative	MIC[a])	MBC[b])	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	625	2500	625	1250	> 625[c])	-	> 625	-
Salmonella enterica Ser. Typhimurium	312.5	625	625	625	312.5	-	> 625	1250
Pseudomonas aeruginosa	312.5	-	625	1250	> 625	-	> 625	-
Gram-positive	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	> 625	-	625	-	> 625	-	> 625	-
Bacillus cereus	625	-	625	-	> 625	-	> 625	-
Listeria monocytogenes	156.2	-	156.2	-	156.2	-	78	-

MIC [a]) Minimum inhibitory concentration. MBC[b]) Minimum bactericidal concentration (μg/mL). [c]) MIC was not determinate due to turbidity produced by compounds at concentrations higher than 625 μg/mL.

against all strains and proved bactericidal against gram-negative bacteria. Safrole was especially active against *S. typhimurium* and *P. aeruginosa,* often involved in gastroenteritis episodes and nosocomial human infections, respectively.[23] Conversely, the oils from the stems and fruits were frequently not active at concentrations lower than 625 μg/mL and not bactericidal. At high concentrations, these oils increased turbidity in the tubes precluding determination of MIC (Table 4). Nevertheless, the fruit oil was highly active at 78 μg/mL against *L. monocytogenes,* which has been associated with human meningoencephalitis.[24] Accordingly, the antibacterial activity of the essential oils of *P. divaricatum* was at least partially attributed to the presence of safrole, found in 98% of the leaf oil and was also present at high concentrations in the fruit and stem oils. Phenylpropanoids and terpenoid compounds with antimicrobial properties have been widely reported in plant essential oils.[25] However, the synergism among different chemical compounds can be crucial for antibacterial activity, which also depends on the bacterial strain.[26]

CONCLUSIONS

In summary, the leaf essential oil showed higher safrole content than essential oils obtained from *O. odorífera* (80-90% of raw oil depending on source)[27] and *P. hispidinervum* (80-90%),[5] both known natural sources of safrole. Furthermore, the high content of safrole found in both fruits and stems means the whole plant can be used for extraction of safrole. This study revealed that *P. divaricatum* is a potential safrole source that can be exploited economically in a sustainable and environmentally responsible way from preserved forest areas. The study also revealed antibacterial activity of leaf oil against gram-negative bacteria.

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REFERENCES

1. Costa, P. R. R.; *Quim. Nova* **2000**, *23*, 357.
2. Ueng, Y. F.; Hsieh, C. H.; Don, M. J.; Chi, C. W.; Ho, L. K.; *Chem. Res. Toxicol.* **2004**, *17*, 1151.
3. Braga, N. P.; Cremasco, M. A.; Valle, R. C. C. R.; *Braz. J. Chem. Eng*. **2005**, *22*, 257.
4. Bizzo, H. R.; Lopes, D.; Abdala, R. V.; Pimentel, F. A.; De Souza, J. A.; Pereira, M. V. G.; Bergter, L.; Guimarães, E. F.; *Flavour Fragr. J.* **2001**, *16*, 113.
5. Maia, J. G.; Goeldi, M. P. E.; Green, C. L.; Milchard, M. J.; *Perfumer & Flavorist* **1993**, *18*, 19.
6. Maia, J. G. S.; Andrade, E. H. A.; *Quim. Nova* **2009**, *32*, 595.
7. Almeida, J. G. L.; Silveira, E. R.; Pessoa, O. D. L.; Nunes, E. P.; *J. Essent. Oil Res.* **2009**, *21*, 228.
8. Van Den Dool, H.; Kratz, P. D. J.; *J. Chromatogr.* **1963**, *11*, 463.
9. Adams, R. P.; *Identification of essential oil components by Gas Chromatography Mass Spectroscopy*, 4th ed., Allured Publishing Corporation: Illinois, 2007.
10. Koneman, E. W.; Allen, S. D.; Janda, W. M.; Schreckenberger, M. P.; Winn Jr, W. C.; *Diagnóstico Microbiológico – Texto e Atlas Colorido*, 5ª ed.; MEDSI: São Paulo, 2001.
11. Da Silva, J. K. R.; Andrade, E. H. A.; Guimarães, E. F.; Maia, J. G. S.; *Nat. Prod. Commun.* **2010**, *5*, 477.
12. Monzote, L.; Garcia, M.; Montalvo, A. M.; Scull, R.; Miranda, M.; *Mem. Inst. Oswaldo Cruz* **2010**, *105*,168.
13. Pino, J. A.; Rosado, A.; Rodriguez, M.; Garcia, D.; *J. Essent. Oil Res*. **1998**, *10*, 333.
14. Mohottalage, S.; Tabacchi, R.; Guerin, P. M.; *Flavour Fragr. J.* **2007**, *22*, 130.
15. Misra, P.; Kumar, A.; Khare, P.; Gupta, S.; Kumar, N.; Dube, A.; *J. Med. Microbiol*. **2009**, *58,* 1058.
16. Guerrini, A.; Sacchetti, G.; Rossi, D.; Paganetto, G.; Muzzoli, M.; Andreotti, E.; Tognolini, M.; Maldonado, M. E.; Bruni, R.; *Environ. Toxicol. Pharmacol.* **2009**, *27*, 39.
17. Jirovetz, L.; Puschmann, C.; Buchbauer, G.; *Fleischhacker,* W.; Shafi, P. M.; Arif, M. M.; *Scientia Pharmaceutica* **1999**, *67*, 305.
18. Gurib-Fakim, A.; *Planta Med.* **1994**, *60*, 376.
19. Hwang, L. S.; Wang, C. K.; Sheu, M. J.; Kao, L. S.; *ACS Symposium Series. 506 (Phenolic Compd. Food Their Eff. Health I)* 1992, p. 200.
20. van Genderen, M. H. P.; Leclercq, P. A.; Delgado, H. S.; Kanjilal, P. B.; Singh, R. S.; *Spectroscopy* **1999**, *14*, 51.
21. Vargas, L.; Velasco-Negueruela, A.; Perez-Alonso, M. J.; Pala-Paul, J.; Vallejo, M. C. G.; *J. Essent. Oil Res*. **2004**, *16*, 122.
22. Andrade, E. H. A.; Carreira, L. M. M.; Silva, M. H. L.; Silva, J. D.; Bastos, C. N.; Sousa, P. J. C.; Guimarães, E. F.; Maia, J. G. S.; *Chem. Biodivers.* **2008**, *5*, 197.
23. Vincent, J. L.; Bihari, D. J.; Suter, P. M.; Bruining, H. A.; White, J.; Wolff, M.; Spencer, R. C.; Hemmer, M.; *JAMA* **1995**, *274*, 639.
24. Chau, T. T.; Campbell, J. I.; Schultsz, C.; Chau, N. V.; Diep, T. S.; Baker, S.; Chinh, N. T.; Farrar, J. J.; van Doorn, H. R.; *PLoS Med* **2010**, *7*, 1.
25. Silva, A. B.; Silva, T.; Franco, E. S.; Rabelo, S. A.; Lima, E. R.; Mota, R. A.; da Câmara, C. A. G.; Pontes-Filho, N. T.; Lima-Filho, J. V.; *Braz. J. Microbiol.* 2010, *41*, 158.
26. Sonboli, A.; Babakhani, B.; Mehrabian, A. R.; *Z. Naturforsch* **2006**, *61*, 160.
27. Miglierini, G.; *Chemistry Today* **2008**, *26*, 59.