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Article

Antibacterial evaluation of *Styrax pohlii* and isolated compounds

Camila Spereta Bertanha[1], Susane Hellen Utrera[1], Valéria Maria Melleiro Gimenez[2], Milton Groppo[3], Márcio Luis Andrade e Silva[1], Wilson Roberto Cunha[1], Carlos Henrique Gomes Martins[1], Ana Helena Januário[1], Patrícia Mendonça Pauletti[1,*]

[1]Center for Research in Exact and Technological Sciences, University of Franca, [2]Claretiano University Center, [3]Faculty of Philosophy, Sciences and Literature of Ribeirão Preto, University of São Paulo

The antibacterial activity of the compounds egonol (1) and homoegonol (2), of the crude ethanolic extract of *Styrax pohlii* (Styracaceae) aerial parts (EE), and of its *n*-hexane (HF), EtOAc (EF), *n*-BuOH (BF), and hydromethanolic (HMF) fractions was evaluated against the following microorganisms: *Streptococcus pneumoniae* (ATCC 6305), *S. pyogenes* (ATCC 19615), *Haemophilus influenzae* (ATCC 10211), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 10031). The broth microdilution method was used for determination of the minimum inhibitory concentration (MIC) during preliminary evaluation of antibacterial activity. The EE yielded MIC values of 400 µg/mL for *S. pneumoniae* and *P. aeruginosa* and 300 µg/mL for *H. influenzae*. The HF and EF fractions exhibited enhanced antibacterial activity, with MIC values of 200 µg/mL against *S. pneumoniae*, but only EF displayed activity against *H. influenzae* (MIC 200 µg/mL). The best MIC value with compounds 1 and 2 (400 µg/mL) was obtained for (1) against *S. pneumoniae* and *P. aeruginosa*. Therefore, 1 exhibited weak antibacterial activity against these standard strains.

Uniterms: *Styrax pohlii/pharmacognosy.* Styracaceae/*pharmacognosy. Styrax pohlii*/ethanolic extract/ antibacterial activity. Egonol/antibacterial activity. Homoegonol/antibacterial activity. Natural products/ evaluation.

As atividades antimicrobianas das substâncias egonol (1) e homoegonol (2), do extrato etanólico das partes aéreas de *Styrax pohlii* (Styracaceae) (EE), bem como das frações *n*-hexano (HF), AcOEt (EF), *n*-BuOH (BF) e hidrometanólica (HMF) foram avaliadas frente aos seguintes microorganismos: *Streptococcus pneumoniae* (ATCC 6305), *S. pyogenes* (ATCC 19615), *Haemophilus influenzae* (ATCC 10211), *Pseudomonas aeruginosa* (ATCC 27853) e *Klebsiella pneumoniae* (ATCC 10031). O método de microdiluição em caldo foi utilizado para a determinação da concentração inibitória mínima (CIM) na avaliação preliminar da atividade antimicrobiana. EE mostrou valores de CIM de 400 µg/mL para *S. pneumoniae* e *P. aeruginosa*, e 300 µg/mL para *H. influenzae*. As frações HF e EF apresentaram melhora na atividade antimicrobiana, com valores de CIM de 200 µg/mL frente *S. pneumoniae*, mas apenas EF apresentou ação contra *H. influenzae* (200 µg/mL). Em relação às substâncias 1 e 2, o melhor valor de CIM (400 µg/mL) foi obtido por 1 frente a *S. pneumoniae* e *P. aeruginosa*, que exibiu fraca atividade antimicrobiana contra estas cepas padrões.

Unitermos: *Styrax pohlii*/farmacognosia Styracaceae/farmacognosia. *Styrax pohlii*/extrato etanólico/ atividade antimicrobiana. Egonol/atividade antimicrobiana. Homoegonol/atividade antimicrobiana. Produtos naturais/avaliação.

INTRODUCTION

A major concern in the public health field is the ease of development of antibiotic resistance by bacteria, since infectious diseases caused by resistant microorganisms are responsible for increased health costs as well as high morbidity and mortality, particularly in developing countries (Nickerson *et al.*, 2009a; 2009b; Gangoué-Piéboji *et al.*, 2009). The rapid emergence of bacterial resistance is attributable to such diverse factors as the complex genetics of the microorganism; the increasing transport of humans, animals, and goods between countries; the widespread use of antibiotics; and the lack of precise therapeutic choices for high-risk patients (Stefani, 2009).

***Correspondence:** P. M. Pauletti. Universidade de Franca. Av. Dr. Armando Salles de Oliveira, 201, 14404-600 – Franca – SP, Brasil. E-mail: pmpauletti@unifran.br

Natural medicinal products have been used for millennia to treat various ailments. Although many have been superseded by conventional pharmaceutical approaches, there is a resurgence of interest in natural products by physicians (Boukraa, 2008; Shimizu *et al.*, 2001). In addition, the existence of resistant bacterial strains reinforces the need for development of novel safe and effective antibacterials that can combat infectious diseases caused by bacteria (Gangoué-Piéboji *et al.*, 2009; Coutinho *et al.,* 2009).

The literature describing traditional Brazilian medicine contains references to the use of plant species belonging to the genus *Styrax* (Styracaceae), which have been mainly employed in the treatment of gastrointestinal diseases and fevers. *Styrax pohlii* A. DC., known in Brazil as "pindaíba", "pindaúba", "benjoeiro", "estoraqueiro", and "árvore-de-bálsamo", is used as a folk medicine to relieve fever (Lorenzi, 1998; Rodrigues, Carvalho, 2008). It grows in the States of São Paulo, Minas Gerais, Goiás, and Mato Grosso do Sul, mainly in the Cerrado region, a biome characterized by rich biodiversity but threatened by agriculture (Lorenzi, 1998; Ratter *et al*., 1997). Phytochemical investigations of *Styrax* species have revealed a predominance of shikimate derivatives, such as lignan derivatives of 3,7-dioxabicyclo[3.3.0]octane, butanolide, and tetrahydrofuran; neolignan derivatives of dihydrobenzofuran; *nor*-neolignan derivatives of benzofuran, phenylpropanoids, and phenolic acids; and pentacyclic saponins and triterpenes (Pauletti *et al*., 2006). Previous studies have shown that some lignans, neolignans and *nor*-neolignans from *Styrax* and their derivatives display significant antimicrobial and cytotoxic activities (Pauletti *et al.*, 2000; Teles *et al.*, 2005; Hirano *et al.*, 1994; Öztürk *et al*., 2008).

Therefore, as part of our ongoing biological studies into the antibacterial activity of natural compounds (Pauletti *et al.*, 2000; Scalon Cunha *et al.*, 2007; Cunha *et al.*, 2010; Silva *et al.*, 2009), we now provide a preliminary report on the antibacterial activities of compounds 1-2, the crude ethanolic extract (EE), and the *n*-hexane (HF), EtOAc (EF), *n*-BuOH (BF), and hydromethanolic (HMF) fractions of *Styrax pohlii* against the microorganisms *Streptococcus pneumoniae*, *S. pyogenes*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, which have not yet been described.

MATERIAL AND METHODS

Collection and identification

S. pohlii A. DC. was collected in the municipality of Luis Antonio, state of São Paulo, Brazil, in October 2008, and identified by Prof. V. M. M. Gimenez and Prof. M. Groppo. A voucher specimen (SPFR12168) was deposited in the Herbarium of the Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo.

Extraction and isolation

The air-dried, powdered stems and leaves (2.43 kg) of *S. pohlii* were extracted with EtOH by maceration at room temperature. After filtration, the solvents were removed under reduced pressure to yield 87.2 g of extract. The EE (20.3 g) was then dissolved in MeOH/H_2O (1:4, v/v) and successively partitioned with *n*-hexane, EtOAc, and *n*-BuOH. After solvent removal using a rotary evaporator, each partition phase yielded 1.39, 2.97, and 3.80 g of extract respectively. Additionally, the residual hydromethanolic phase furnished 4.12 g. In a previous study, our research group had fractionated the *n*-hexane fraction and obtained compounds 1 (27.3 mg) and 2 (22.0 mg) (Bertanha *et al*, 2012).

Antibacterial assay

The following microorganisms were used for evaluation of antibacterial activity: *Streptococcus pneumoniae* (ATCC 6305), *S. pyogenes* (ATCC 19615), *Haemophilus influenzae* (ATCC 10211), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 10031). Minimum inhibitory concentration (MIC) values for each sample were determined in triplicate by using the broth microdilution method (CLSI, 2009). The samples were dissolved in DMSO (Merck) at 0.5 mg/mL and then diluted in Brain Heart Infusion broth or, for *H. influenzae* only, BD Haemophilus Test Medium Agar (Difco Labs), to concentrations ranging from 400 to 20 μg/mL. The final DMSO concentration in the culture medium was 5% (v/v), which was used as a negative control. The inoculum was adjusted to each organism to yield a cell concentration of 5×10^5 colony forming units (CFU/mL). One inoculated well was included to control the adequacy of the broth for organism growth. One non-inoculated well, free of antibacterial agent, was also included to ensure medium sterility. Chloramphenicol and vancomycin were used as controls, dissolved in DMSO (Merck) at 0.1 and 10 mg/mL respectively, and then diluted in Brain Heart Infusion broth or BD Haemophilus Test Medium Agar to 0.02 mg/mL. The controls were evaluated at final concentrations ranging from 0.01 to 5.9 μg/mL. The microplates (96-well) were incubated at 37 °C for 24 h. Then, 30 μL of resazurin 0.01% aqueous

solution (Sigma-Aldrich) was added to determine microorganism viability (Sarker *et al.*, 2007). The MIC was determined as the lowest concentration of the sample capable of inhibiting microorganism growth.

RESULTS AND DISCUSSION

A previous study on the CH_2Cl_2-CH_3OH (2:1, v/v) crude extract from the leaves of *S. ferrugineus* demonstrated antimicrobial activity, as detected by inhibition of *Cladosporium sphaerospermum*, *Candida albicans*, and *Staphylococcus aureus* (Pauletti *et al*., 2000). This finding has encouraged us to conduct biological and chemical investigations of metabolites isolated from other species of this genus.

Regarding the antibacterial assay, the crude EtOH extract (EE), as well as the *n*-hexane (HF), EtOAc (EF), *n*-BuOH (BF), and hydromethanolic (HMF) fractions of *S. pohlii*, were screened for their antibacterial activity against five different bacterial strains, three of which were Gram-positive and two were Gram-negative. The preliminary antibacterial activities, expressed as the MIC, are shown in Table I.

The EtOH extract (EE) yielded MIC values of 300 µg/mL against *H. influenzae* and 400 µg/mL against *S. pneumoniae* and *P. aeruginosa*. EE did not inhibit the growth of the two other tested strains. Therefore, EE exhibits moderate activity against *H. influenzae*, *S. pneumoniae* and *P. aeruginosa*, as described by Holetz *et al.* (2002).

Compared with EE, the *n*-hexane fraction (HF) provided superior antibacterial activity against the Gram-positive *S. pneumoniae*, with a MIC value of 200 µg/mL. However, unlike EE**,** HF did not significantly reduce the growth of *H. influenzae*. In addition, HF displayed an MIC of 400 µg/mL against *P. aeruginosa*, but was considered ineffective against *S. pyogenes*, *H. influenzae*, and *K. pneumoniae*. According to these results, the HF fraction exhibits promising activity against *S. pneumoniae*.

The best MIC value obtained for the EtOAc fraction (EF) was 200 µg/mL, against the Gram-negative *H. influenzae* and the Gram-positive *S. pneumoniae*. This result is superior to those obtained with EE against the same strains (MIC = 300 and 400 µg/mL, respectively). In addition, EF displayed an MIC of 400 µg/mL against *P. aeruginosa* and *S. pyogenes*. Thus, EF was the only assayed sample that exhibited moderate antibacterial activity against *S. pyogenes*, as compared with the data obtained by Holetz *et al.* (2002).

The *n*-BuOH fraction (BF) exhibited moderate antibacterial activity against *P. aeruginosa*, *S. pneumoniae*, and *H. influenzae* (MIC = 400 µg/mL), and did not significantly inhibit the growth of the two other tested strains, just as found for EE. The hydromethanolic fraction (HMF) displayed moderate antibacterial activity against *P. aeruginosa*. Thus, the BF and HMF fractions are not promising antibacterials against the strains tested in this work when compared with the HF and EF fractions.

In conclusion, differences in antibacterial effect were more evident for the HF fraction when assayed against the tested Gram-positive bacteria. HF was not effective against Gram-negative bacteria, perhaps due to the presence of the outer membrane in the latter microorganisms (Horiuchi *et* al., 2007). The HF fraction was thus selected for a further purification process as described previously (Bertanha *et al.*, 2012), which yielded egonol (**1**) and homoegonol (**2**) (Figure 1).

TABLE I - Minimum inhibitory concentration values (MIC) obtained for the crude ethanolic extract of *S. pohlii*, fractions and isolated compounds

Sample	MIC [µg/mL]				
	K. pneumoniae	*P. aeruginosa*	*S. pyogenes*	*S. pneumoniae*	*H. influenzae*
EE	> 400	400	> 400	400	300
HF	> 400	400	> 400	200	> 400
EF	> 400	400	400	200	200
BF	> 400	400	> 400	400	400
HMF	> 400	400	> 400	> 400	> 400
Egonol (1)	> 400	400	> 400	400	> 400
Homoegonol (2)	> 400	> 400	> 400	> 400	> 400
Positive Controls[a]	0.046 (c)	5.9 (c)	0.37 (v)	0.37 (v)	0.046 (c)

[a]Positive Controls: (c)- Chloranphenicol (v)- Vancomycin

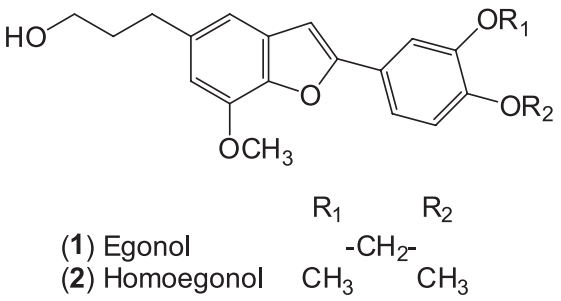

FIGURE 1 - Chemical structures of the compounds isolated.

Regarding the antibacterial activity of the isolated compounds (Table I), egonol (1) displayed weak activity against *P. aeruginosa* and *S. pneumoniae* (MIC = 400 µg/mL), according to Ríos and Recio (2005). As HF displayed the same activity as **1** against *P. aeruginosa*, it might be suggested that 1 is the compound responsible for the activity initially observed for the HF fraction, at least against *P. aeruginosa*. However, data for *S. pneumoniae* showed that **1** displayed half of the activity found for HF against this same strain. Similar findings are frequently observed in the area of natural product research after fractionation processes (Harvey, 2009; Yeh *et al.*, 2012). Homoegonol (2) was considered inactive, at least in the present assay conditions.

Compounds 1 and 2 are quite similar, differing mainly in the presence of a methylenedioxyl group or two methoxyl groups at the phenyl ring connected to C-2 of the 5-propylbenzofuran core, respectively. Therefore, considering their antibacterial activity, it can be suggested that the presence of a methylenedioxyl group in compound 1 may improve its activity, since it was more active than compound 2. The mechanism whereby benzofuran derivatives exert their *in vitro* antibacterial effect is unclear. However, 1, 2, and some semi-synthetic egonol derivatives have been shown to possess antifungal and antibacterial activities against several species, including *Staphylococcus aureus*, *Candida albicans, Cladosporium sphaerospermum*, *Bacillus subtilis*, and *Escherichia coli* (Pauletti *et al.*, 2000; Öztürk *et al*., 2008). Nevertheless, further studies are still required to improve the antibacterial activity of these natural products and obtain a better understanding of the mode of action of benzofuran derivatives.

CONCLUSION

The EtOH extract (EE) of *S. pohlii* showed moderate activity against the Gram-negative bacterial species *H. influenzae*. Chemical analysis of the EE yielded four fractions. The *n*-hexane fraction exhibits promising activity against the Gram-positive *S. pneumoniae* bacterium and was thus selected for further purification, which yielded egonol (1) and homoegonol (2). Furthermore, biological assay results suggested that compound 1 inhibits the growth of *P. aeruginosa* and *S. pneumoniae*. Further studies are ongoing to assess other potentially important biological effects of this plant species.

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REFERENCES

BERTANHA, C.S.; BRAGUINE, C.G.; MORAES, A.C.; GIMENEZ, V.M.; GROPPO, M.; SILVA, M.L.; CUNHA, W.R.; JANUÁRIO, A.H.; PAULETTI, P.M. Cyclooxygenase inhibitory properties of *nor*-neolignans from *Styrax pohlii*. *Nat. Prod. Res.*, v.26, n.24, p.2323-2329, 2012.

BOUKRAA, L. Additive activity of royal jelly and honey against *Pseudomonas aeruginosa*. *Altern. Med. Rev.*, v.13, n.4, p.330-333, 2008.

CLINICAL AND LABORATORY STANDARDS INSTITUTE. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically* M7-A8. Wayne: CLSI, 2009. 10 p.

COUTINHO, H.D.; VASCONCELLOS, A.; LIMA, M.A.; ALMEIDA-FILHO, G.G.; ALVES, R.R. Termite usage associated with antibiotic therapy: enhancement of aminoglycoside antibiotic activity by natural products of *Nasutitermes corniger* (Motschulsky 1855). *BMC Complem. Altern. Med.*, v.9, n.35, p.1-4, 2009.

CUNHA, W.R.; DE MATOS, G.X.; SOUZA, M.G.; TOZATTI, M.G.; ANDRADE E SILVA, M.L.; MARTINS, C.H.; DA SILVA, R.; DA SILVA FILHO, A.A. Evaluation of the antibacterial activity of the methylene chloride extract of *Miconia ligustroides*, isolated triterpene acids, and ursolic acid derivatives. *Pharm. Biol.*, v.48, n.2, p.166-169, 2010.

GANGOUÉ-PIÉBOJI, J.; EZE, N.; NGONGANG DJINTCHUI, A.; NGAMENI, B.; TSABANG, N.; PEGNYEMB, D.E.; BIYITI, L.; NGASSAM, P.; KOULLA-SHIRO, S.; GALLENI, M. The *in-vitro* antimicrobial activity of some medicinal plants against beta-lactam-resistant bacteria. *J. Infect. Dev. Ctries.*, v.3, n.9, p.671-680, 2009.

HARVEY, A.L. The continuing value of natural products for drug discovery. *Rev. Salud Anim.*, v.31, n.1, p.8-12, 2009.

HIRANO, T.; GOTOH, M.; OKA, K. Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. *Life Sci.*, v.55, n.13, 1061-1069, 1994.

HOLETZ, F.B.; PESSINI, G.L.; SANCHES, N.R.; CORTEZ, D.A.G.; NAKAMURA, C.V.; DIAS FILHO, B.P. Screening of some plants used in the brazilian folk medicine for the treatment of infectious diseases. *Mem. Inst. Oswaldo Cruz.*, v.97, n.7, p.1027-1031, 2002.

HORIUCHI, K.; SHIOTA, S.; HATANO, T.; YOSHIDA, T.; KURODA, T.; TSUCHIYA, T. Antimicrobial activity of oleanolic acid from Salvia officinalis and related compounds on vancomycin-resistant enterococci (VRE). *Biol. Pharm. Bull.*, v.30, n.6, p.1147-1149, 2007.

LORENZI, H. *Árvores brasileiras I*. 2.ed. Nova Odessa: Plantarum, 1998. 368 p.

NICKERSON, E.K.; HONGSUWAN, M.; LIMMATHUROTSAKUL, D.; WUTHIEKANUN, V.; SHAH, K.R.; SRISOMANG, P.; MAHAVANAKUL, W.; WACHARAPRECHASGUL, T.; FOWLER, V.G.; WEST, T.E.; TEERAWATANASUK, N.; BECHER, H.; WHITE, N.J.; CHIERAKUL, W.; DAY, N.P.; PEACOCK, S.J. *Staphylococcus aureus* bacteraemia in a tropical setting: patient outcome and impact of antibiotic resistance. *PLoS One*, v.4, n.1, p.e4308, 2009a.

NICKERSON, E.K.; WEST, T.E.; DAY, N.P.; PEACOCK, S.J. *Staphylococcus aureus* disease and drug resistance in resource-limited countries in south and east Asia. *Lancet Infect. Dis.*, v.9, n.2, p.130-135, 2009b.

OZTÜRK, S.E.; AKGÜL, Y.; ANIL, H. Synthesis and antibacterial activity of egonol derivatives. *Bioorg. Med. Chem.,* v.16, n.8, p.4431-4437, 2008.

PAULETTI, P.M.; ARAÚJO, A.R.; YOUNG, M.C.; GIESBRECHT, A.M.; BOLZANI, V.D. *Nor*-Lignans from the leaves of *Styrax ferrugineus* (Styracaceae) with antibacterial and antifungal activity. *Phytochemistry*, v.55, n.6, p.597-601, 2000.

PAULETTI, P.M.; TELES, H.L.; SILVA, D.H.S.; ARAÚJO, A.R.; BOLZANI, V.S. The Styracaceae. *Rev. Bras. Farmacogn.*, v.16, n.4, p.576-590, 2006.

RATTER, J.A.; RIBEIRO, J.F.; BRIDGEWATER, S. The Brazilian cerrado vegetation and threats to its biodiversity. *Ann. Bot.*, v.80, n.3, p.223-230, 1997.

RÍOS, J.L.; RECIO, M.C. Medicinal plants and antimicrobial activity. *J. Ethnopharmacol.*, v.100, n.1-2, p.80-84, 2005.

RODRIGUES, V.E.G.; CARVALHO, D.A. Florística de plantas medicinais nativas de remanescentes de floresta estacional semidecidual na região do Alto Rio Grande – Minas Gerais. *Cerne*, v.14, n.2, p.93-112, 2008.

SARKER, S.D.; NAHAR, L.; KUMARASAMY, Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods*, v.42, n.4, p.321-324, 2007.

SCALON CUNHA, L.C.; ANDRADE E SILVA, M.L.; CARDOSO FURTADO, N.A.; VINHÓLIS, A.H.; GOMES MARTINS, C.H.; DA SILVA FILHO, A.A.; CUNHA, W.R. Antibacterial activity of triterpene acids and semi-synthetic derivatives against oral pathogens. *Z. Naturforsch. C,* v.62, n.9-10, p.668-722, 2007.

SHIMIZU, M.; SHIOTA, S.; MIZUSHIMA, T.; ITO, H.; HATANO, T.; YOSHIDA, T.; TSUCHIYA, T. Marked potentiation of activity of beta-lactams against methicillin-resistant *Staphylococcus aureus* by corilagin. *Antimicrob. Agents Chemother.,* v.45, n.11, p.3198-3201, 2001.

SILVA, M.L.; MARTINS, C.H.; LUCARINI, R.; SATO, D.N.; PAVANB, F.R.; FREITAS, N.H.; ANDRADE, L.N.; PEREIRA, A.C.; BIANCO, T.N.; VINHOLIS, A.H.; CUNHA, W.R.; BASTOS, J.K.; SILVA, R.; DA SILVA FILHO, A.A. Antimycobacterial activity of natural and semi-synthetic lignans. *Z. Naturforsch. C*, v.64, n.11-12, p.779-784, 2009.

STEFANI, S. Evolution in the antibiotic susceptibility and resistance. *Infez. Med.*, v.17, suppl.3, p.5-12, 2009.

TELES, H.L.; HEMERLY, J.P.; PAULETTI, P.M.; PANDOLFI, J.R.; ARAUJO, A.R.; VALENTINI, S.R.; YOUNG, M.C.; BOLZANI, V.S.; SILVA, D.H. Cytotoxic lignans from the stems of *Styrax camporum* (Styracaceae). *Nat. Prod. Res.*, v.19, n.4, p. 319-323, 2005.

YEH, J.-C.; GARRARD, I.J.; CHO, C.-W.C.; BLIGH, S.W.A.; LU, G.; FAN, T.-P.; FISHER, D. Bioactivity-guided fractionation of the volatile oil of *Angelica sinensis* radix designed to preserve the synergistic effects of the mixture followed by identification of the active principles. *J. Chromatogr. A.,* v.1236, n.4, p.132-138, 2012.

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