Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 160165, 5 pages
http://dx.doi.org/10.1155/2013/160165

Research Article

Antifungal Constituents from the Roots of *Piper dilatatum* Rich.

Ruilan Alves dos Santos,[1] Clécio Souza Ramos,[2] Maria Cláudia M. Young,[3] Thamilles Gonçalves Pinheiro,[1] André M. Amorim,[4] Massuo J. Kato,[5] and Ronan Batista[6]

[1] *Departamento de Estudos Básicos e Instrumentais, Universidade Estadual do Sudoeste da Bahia, BR 415, Km 03, s/n, 45700-000 Itapetinga, BA, Brazil*
[2] *Departamento de Ciências Moleculares, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros, s/n, 52171-030 Recife, PE, Brazil*
[3] *Seção de Fisiologia e Bioquímica de Plantas, Instituto de Botânica, CP 3005, 01061-970 São Paulo, SP, Brazil*
[4] *Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna, Km 16, 45662-900 Ilhéus, BA, Brazil*
[5] *Instituto de Química, Universidade de São Paulo, CP 26077, 05513-970 São Paulo, SP, Brazil*
[6] *Instituto de Química, Universidade Federal da Bahia, Rua Barão de Geremoabo, s/n, Ondina, 40170-290 Salvador, BA, Brazil*

Correspondence should be addressed to Ronan Batista; ronbatis@ufba.br

Received 8 May 2013; Revised 10 June 2013; Accepted 11 June 2013

Academic Editor: Joaquin Campos

The compounds (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain (**1**), flavokavain B (**2**), β-sitosterol (**3**), and stigmasterol (**4**) are reported here as chemical constituents of *Piper dilatatum* Rich. (Piperaceae). Their structures were determined on the basis of their spectroscopic data (^{1}H and ^{13}C NMR, MS, and IR). The antifungal activities of pyrone **1** (1 μg) and chalcone **2** (100 μg) were determined by means of direct bioautography against *Cladosporium cladosporioides* and *C. sphaerospermum*. Results indicate *P. dilatatum* as a candidate for the development of novel antifungal phytotherapic products as well as point out pyrone **1** as a promising hit compound in the quest for novel antifungal agents.

1. Introduction

An alarming and remarkable increase in the incidence of deep-seated disseminated mycoses has been observed in the last decades, and it may be credited to the advent of aggressive cancer chemotherapy, highly effective immunosuppressants for organ transplantation, long-term use of corticoids, widespread use of powerful broad spectrum antibacterial agents, and the explosion in the number of cases of human immunodeficiency virus (HIV) infection [1]. Taking into account the increasing emergence of resistance to current antimycotic agents, new efforts have been devoted to the discovery of new antifungal lead compounds with different mechanisms of action [2]. Within this context, natural products have been proven to be an excellent source of novel chemical entities in drug discovery [3, 4].

Piper is one of the most diverse genera among the basal lineages of angiosperms in tropical wet forest around the world. The diversification centers of *Piper* species include Southeast Asia, Southeast Mexico, Andes, Colombian (Chocó department) and Brazilian Amazon, and Atlantic forest in Brazil [5]. The Brazilian forests harbor 283 *Piper* species [6], and approximately 10% of them have already been chemically investigated [7].

Piper dilatatum Rich. is a shrub, 1.5–2 m tall, usually found in gaps and clearings with white spicate inflorescences consisting of several thousand flowers [8]. The chemical investigation on its leaves revealed the presence of six prenylated benzoic acid derivatives and three chalcones [9]. The essential oil from the leaves has been found to contain α-phellandrene, Δ-3-carene, and bicyclogermacrene as major

constituents [10]. Despite these investigations, no previous phytochemical studies have been conducted on *P. dilatatum* growing in Brazil.

This study describes the isolation and characterization of (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain (**1**) and flavokavain B (**2**) in the roots of *P. dilatatum*, along with β-sitosterol (**3**) and stigmasterol (**4**) in its leaves. In addition, the antifungal activity of compounds **1** and **2** were determined by direct bioautography against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum*.

2. Materials and Methods

2.1. General Procedures. IR spectra were measured in KBr pellets on a Perkin-Elmer infrared spectrometer model 1750. Circular dichroism (CD) spectrum was measured in CH_3OH with a JASCOORD/UV-6 spectropolarimeter and optical rotation on a Perkin-Elmer 241 polarimeter. HREIMS spectra were obtained on a Bruker Daltonics MicrOTOF mass spectrometer. LREIMS spectra were measured at 70 eV on a VG Platform II spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, on a Bruker AC200 apparatus. $CDCl_3$ (Aldrich) was used as solvent and TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (J) in Hz. Silica gel (Merck, 230–400 mesh) was used for column chromatographic separations, while silica gel 60 PF254 (Merck) was used for analytical (0.25 mm) TLC chromatography. HPLC analyses of extracts and pure compounds were performed on a Shimadzu instrument using a C18 column (250 × 4.6 mm, 5 μm) from Supelco eluted in a gradient mode starting with $CH_3CN : H_2O$ (3 : 7) for 8 min, raising to 100% of CH_3CN in 37 min, with detection at 254 nm. GC-FID analyses were carried out on a Shimadzu 17A instrument equipped with an HP DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, and cross-linked 5% phenylmethyl silicone). Temperature gradient: from 100° to 280° at 3° min^{-1} and then held at 300° during 15 min. The flow rate of carrier gas (He) was 1.6 mL min^{-1}.

2.2. Plant Material. Roots and leaves of *P. dilatatum* were collected in Ilhéus, Bahia, Brazil, in March 2008. The species was identified by Dr. André Márcio Amorim (Universidade Estadual de Santa Cruz, Brazil), and a voucher specimen (Piper 001) was housed at the Herbarium of CEPEC/CEPLAC in Ilhéus, Bahia, Brazil.

2.3. Extraction and Isolation of Constituents. The dried and powdered roots of *P. dilatatum* (203 g) were extracted with CH_2Cl_2 (3 × 500 mL) by maceration for two days. The resulting solution was filtered and concentrated under vacuum to afford the crude dichloromethanic extract of the roots (**DER**, 2.99 g), which was partially suspended (2.90 g) in hexanes to yield a yellow precipitate, which was filtered and dried (1.32 g). The hexanic solution was then concentrated under vacuum to give the hexanic fraction residue (1.68 g). Part of the precipitate (641 mg) was submitted to column chromatography over silica gel (100 g) yielding 70 fractions of 20 mL each by means of a gradient elution with hexanes (200 mL, fractions 1–10), hexanes-ethyl acetate 9 : 1 (200 mL, fractions 11–21), hexanes-ethyl acetate 8 : 2 (400 mL, fractions 22–42), hexanes-ethyl acetate 6 : 4 (400 mL, fractions 43–63), and ethyl acetate (150 mL, fractions 64–70) to yield the pyrone **1** (fractions 18–23, 553 mg) as pure compound. The hexane fraction (1.68 g) was subjected to vacuum liquid chromatography over silica gel, employing hexanes (100 mL, fraction 1), hexanes-ethyl acetate 9 : 1 (500 mL, fractions 2–6), hexanes-ethyl acetate 8 : 2 (500 mL, fractions 7–11), and hexanes-ethyl acetate 1 : 1 (300 mL, fractions 12–14) to give 14 fractions of 100 mL each. The fraction 4 afforded the pure chalcone **2** (46 mg).

The leaves of *P. dilatatum* were dried and pulverized, and the corresponding plant material (119 g) was extracted with CH_2Cl_2 (3 × 800 mL) by maceration, to give, after concentration under reduced pressure, the corresponding crude dichloromethanic extract of the leaves (**DEL**, 4.24 g). Part of this extract (4.06 g) was chromatographed over silica gel using mixtures of hexanes and ethyl acetate with increasing polarities to give 15 fractions of 250 mL each. Fraction 6 (365 mg) was rechromatographed over silica gel to give 20 fractions, 30 mL per fraction, using a gradient elution with dichloromethane (100 mL, fractions 1–4), dichloromethane-methanol 99 : 1 (200 mL, fractions 5–11), dichloromethane-methanol 95 : 5 (200 mL, fractions 12–17), and dichloromethane-methanol 9 : 1 (100 mL, fractions 18–20) to afford the mixture of compounds **3** and **4** (Fractions 7-8, 32 mg).

*(+)-(7S,8R)-Epoxy-5,6-didehydrokavain (**1**).* White crystalline solid; IR ν_{max} (KBr): 2986, 1768, 1655, 1263, 1034, 948, and 740 cm^{-1}; $[\alpha]_D^{25}$ = +140.3 (c 0.5, $CHCl_3$); CD: λ_{max} (MeOH) 285 (+), 240 (+); LREIMS m/z (rel. int.) 244 [M] (10), 187 (20), 138 (93), 95 (78), and 69 (100); HREIMS m/z = 244.0800 (calcd. for $C_{14}H_{12}O_4$: 244.0736); ^{1}H NMR (200 MHz, $CDCl_3$, ppm, J in Hz): δ 5.49 (1H, d, J = 2.2, H-3), 6.09 (1H, d, J = 2.2, H-5), 3.60 (1H, d, J = 1.3, H-7), 4.11 (1H, d, J = 1.3, H-8), 7.36–7.26 (5H, m, H-10 to H-14), and 3.79 (3H, s, $OC\underline{H}_3$); ^{13}C NMR: δ 163.3 (C-2), 89.1 (C-3), 170.5 (C-4), 100.3 (C-5), 159.0 (C-6), 57.9 (C-7), 60.0 (C-8), 134.9 (C-9), 125.5 (C-10), 128.5 (C-11), 128.8 (C-12), 128.5 (C-13), 125.5 (C-14), and 55.9 ($O\underline{C}H_3$). All data are in agreement with those reported in the literature for (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain [11].

*Flavokavain B (**2**).* Yellow crystalline solid; LREIMS m/z (rel. int.) 284 [M$^+$] (56), 207 (100), 180 (76), 135 (47), 152 (43), and 77 (48); HREIMS m/z = 284.1043 (calcd. for $C_{17}H_{16}O_4$: 284.1048); ^{1}H NMR (200 MHz, $CDCl_3$, ppm, J in Hz): δ 7.61–7.59 (2H, m, H-2/H-6), 7.42–7.38 (3H, m, H-3/H-4/H-5), 7.78 (1H, d, J = 12.3, H-α), 7.92 (1H, d, J = 12.3, H-β), 6.11 (1H, d, J = 2.6, H-3′), 5.97 (1H, d, J = 2.2, H-5′), 3.84 (3H, s, 4′-$OC\underline{H}_3$), and 3.92 (3H, s, 6′-$OC\underline{H}_3$). ^{13}C NMR: δ 135.54 (C-1), 128.34 (C-2/C-6), 128.85 (C-3/C-5), 127.51 (C-4), 130.05 (C-α), 142.33 (C-β), 192.64 (C=O), 106.31 (C-1′), 162.51 (C-2′), 93.75 (C-3′), 168.39 (C-4′), 91.25 (C-5′), 166.23 (C-6′), 55.82

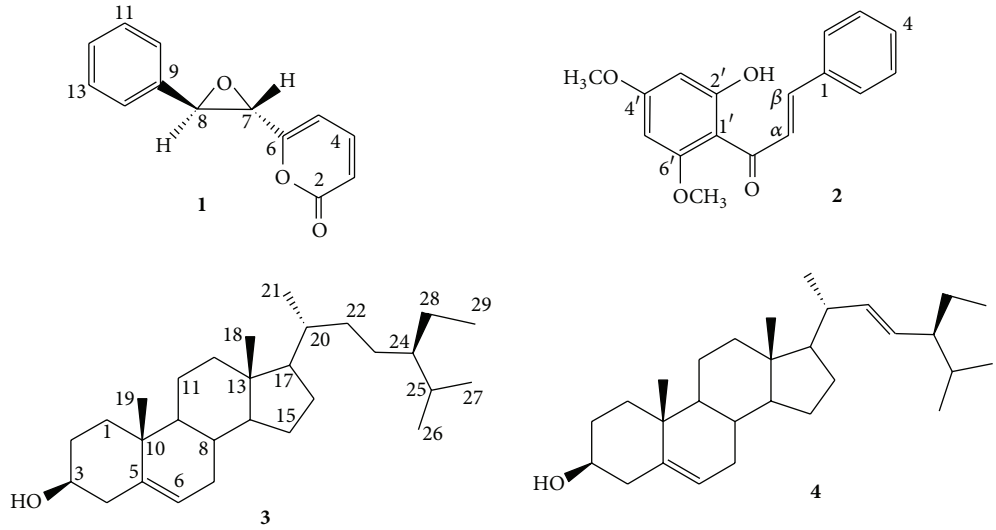

Figure 1: Structures of the secondary metabolites **1**–**4** isolated from *P. dilatatum*.

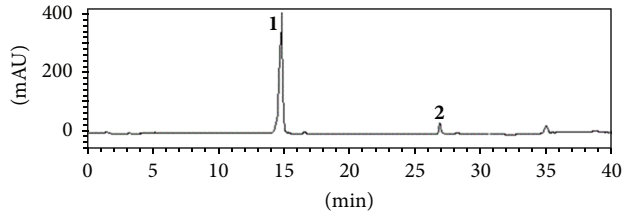

Figure 2: Chromatographic profile (HPLC) of the crude CH_2Cl_2 extract from *P. dilatatum* roots. **1**, (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain; **2**, flavokavain B. For chromatographic conditions, see Section 2.

($4'$-O$\underline{C}H_3$), and 55.57 ($6'$-O$\underline{C}H_3$). All data are very similar to those reported in the literature for flavokavain B [12–14].

*Mixture of β-Sitosterol (**3**) and Stigmasterol (**4**).* Amorphous white powder: IR,[1]H, and [13]C NMR data are in agreement with those reported in the literature for *β*-sitosterol and stigmasterol [15–17].

2.4. Antifungal Bioassay. The microorganisms used in the antifungal assays *C. cladosporioides* (Fresen.) de Vries SPG 140 and *C. sphaerospermum* (Penzig) SPC 491 have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. Assays were performed in triplicate using the direct bioautography method in agreement with the literature procedure [17–20]. Ten microliters of the solutions was prepared, in different concentrations, corresponding to 20, 10, 5, and 1 μg for pure compounds and 400, 200, and 100 μg for the crude extracts. The samples were applied to TLC plates, with these being eluted with hexanes-EtOAc (4 : 1) followed by complete removal of the solvent at room temperature. The chromatograms were then sprayed with a spore suspension of fungi in glucose and salt solution and incubated for 72 h in the darkness in a moistened chamber at 25°C. Clear inhibition zones appeared against a dark background, indicating the minimal amount of compound required (Table 1). Nystatin was used as the positive control (detection limit 1 μg), whereas ampicillin and chloramphenicol were used as negative controls.

Table 1: Antifungal activity of extracts and compounds from *P. dilatatum* against *Cladosporium cladosporioides* and *C. sphaerospermum*.

Sample	Antifungal activity (μg)[a]	
	C. cladosporioides	*C. sphaerospermum*
DER	200 μg	200 μg
DEL	—	—
1	1 μg	1 μg
2	100 μg	100 μg
Nystatin	1 μg	1 μg

[a]Minimum amount required for the inhibition of fungal growth on a thin-layer chromatographic plate (TLC).

3. Results and Discussion

Extracts **DER** and **DEL** were assessed for their antifungal activity against *Cladosporium* fungi, and the results are shown in Table 1. As can be seen, only the root extract was active, inhibiting growth of both fungal strains at the 200 μg treatment.

The crude extracts from roots and leaves were fractionated using a silica gel chromatography and afforded compounds **1**–**4** (Figure 1). Their structures were determined on the basis of their IR, MS, [1]H, and [13]C NMR data as well as confirmed by comparison with the literature data.

The crude CH_2Cl_2 extract from the roots of *P. dilatatum* afforded (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain (**1**) and flavokavain B (**2**). Compound **1** was found to compose approximately 40% of the extract or 0.6% of the dry weight. In addition, HPLC analysis (Figure 2) revealed **1** as the major

component, representing 87% of **1** and 5% of **2**. ^{1}H and ^{13}C NMR spectral data as well as CD curve for **1** were identical to those reported for (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain, which was previously isolated from *Piper rusbyi* leaves [11]. The literature search reveals that this is the first report on the occurrence of the pyrone **1** from *P. dilatatum* and represents the first report of the occurrence of this class of compounds in a plant species native to Brazilian and perhaps also to American forests.

The structure of compound **2**, a yellow crystalline solid of molecular formula $C_{17}H_{16}O$, was identified as the chalcone flavokavain B, and its spectroscopic data were identical to those reported in the literature [12–14]. Flavokavain B has been isolated from several *Piper* species [21], including *P. dilatatum* leaves [9], but it is the first report from the roots of *P. dilatatum*.

β-Sitosterol (**3**) and stigmasterol (**4**) have been also reported here as chemical constituents on *P. dilatatum* leaves. *β*-Sitosterol (**3**) is often isolated from *Piper* species, while stigmasterol (**4**) is rarely found in this genus [21].

Given the interesting *in vitro* and *in vivo* biological activities already described for **1** and **2** [11, 22], and bearing in mind that these compounds were found as the main chemical constituents of the roots of *P. dilatatum*, it was assumed that both substances **1** and **2** would be responsible for the crude extracts antifungal activity (Table 1). Thus, these secondary metabolites were assayed against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum*, and the results are shown in Table 1. Both pyrone **1** (1 *μ*g) and chalcone **2** (100 *μ*g) exhibited antifungal activity, with pyrone **1** as the most potent substance (1 *μ*g). Considering that pyrone **1** is the most abundant constituent on the roots of *P. dilatatum*, the present study identifies this plant species as a candidate for the development of novel antifungal phytotherapic products. Furthermore, the potent antifungal activity of pyrone **1** provides a new and promising hit for the pursuit of more active and selective antifungal agents.

4. Conclusions

This study describes the first report of the occurrence of (+)-(7*S*,8*R*)-epoxy-5,6-didehydrokavain (**1**) in *Piper dilatatum*. The potent antifungal activity observed for **1** identifies this plant species as a promising candidate for the development of novel antifungal phytotherapic products. Moreover, the antifungal activity of pyrone **1** provides a new hit for the development of new antifungal derivatives.

Acknowledgments

The authors thank FAPESB and CNPq for grants and financial support. MJK is grateful to CNPq and FAPESP for funding. Dr. Pablo A. García (University of Salamanca, Spain) and Dr. Christopher S. Jeffrey (University of Nevada, USA) are also acknowledged for their nomenclature assistance and English revision, respectively.

References

[1] R. Di Santo, "Natural products as antifungal agents against clinically relevant pathogens," *Natural Product Reports*, vol. 27, no. 7, pp. 1084–1098, 2010.

[2] R. Musiol and W. Kowalczyk, "Azole antimycotics—a highway to new drugs or a dead end?" *Current Medicinal Chemistry*, vol. 19, no. 9, pp. 1378–1388, 2012.

[3] F. E. Koehn and G. T. Carter, "The evolving role of natural products in drug discovery," *Nature Reviews Drug Discovery*, vol. 4, no. 3, pp. 206–220, 2005.

[4] D. J. Newman and G. M. Cragg, "Natural products as sources of new drugs over the 30 years from 1981 to 2010," *Journal of Natural Products*, vol. 75, no. 3, pp. 311–335, 2012.

[5] M. A. Jaramillo and R. Callejas, "Current perspectives on the classification and phylogenetics of the genus *Piper* L," in *Piper: A Model Genus for Studies of Phytochemistry, Ecology, and Evolution*, L. A. Dyer and A. D. N. Palmer, Eds., p. 219, Academic/Plenum Publishers, New York, NY, USA, 2004.

[6] R. A. de Figueiredo and M. Sazima, "Pollination biology of Piperaceae species in southeastern Brazil," *Annals of Botany*, vol. 85, no. 4, pp. 455–460, 2000.

[7] M. J. Kato and M. Furlan, "Chemistry and evolution of the Piperaceae," *Pure and Applied Chemistry*, vol. 79, no. 4, pp. 529–538, 2007.

[8] D. W. Kikuchi, E. Lasso, J. W. Dalling, and N. Nur, "Pollinators and pollen dispersal of *Piper dilatatum* (Piperaceae) on Barro Colorado Island, Panama," *Journal of Tropical Ecology*, vol. 23, no. 5, pp. 603–606, 2007.

[9] C. Terreaux, M. P. Gupta, and K. Hostettmann, "Antifungal benzoic acid derivatives from *Piper dilatatum* in honour of Professor G. H. Neil Towers 75th birthday," *Phytochemistry*, vol. 49, no. 2, pp. 461–464, 1998.

[10] J. B. Cysne, K. M. Canuto, O. D. L. Pessoa, E. P. Nunes, and E. R. Silveiraa, "Leaf essential oils of four *Piper* species from the state of Ceará—northeast of Brazil," *Journal of the Brazilian Chemical Society*, vol. 16, no. 6B, pp. 1378–1381, 2005.

[11] N. Flores, G. Cabrera, I. A. Jiménez et al., "Leishmanicidal constituents from the leaves of *Piper rusbyi*," *Planta Medica*, vol. 73, pp. 2006–2011, 2007.

[12] P. Boeck, C. A. Bandeira Falcão, P. C. Leal et al., "Synthesis of chalcone analogues with increased antileishmanial activity," *Bioorganic and Medicinal Chemistry*, vol. 14, no. 5, pp. 1538–1545, 2006.

[13] P. Boeck, P. C. Leal, R. A. Yunes et al., "Antifungal activity and studies on mode of action of novel xanthoxyline-derived chalcones," *Archiv der Pharmazie*, vol. 338, no. 2-3, pp. 87–95, 2005.

[14] H. R. W. Dharmaratne, N. P. D. Nanayakkara, and I. A. Khan, "Kavalactones from *Piper methysticum*, and their ^{13}C NMR spectroscopic analyses," *Phytochemistry*, vol. 59, no. 4, pp. 429–433, 2002.

[15] J.-Y. Cai, L. Zhao, and D.-Z. Zhang, "Chemical constituents from *Bletilla ochracea* Schltr," *Chemical Research in Chinese Universities*, vol. 23, no. 6, pp. 705–707, 2007.

[16] D. Kongduang, J. Wungsintaweekul, and W. de-Eknamkul, "Biosynthesis of *β*-sitosterol and stigmasterol proceeds exclusively via the mevalonate pathway in cell suspension cultures of *Croton stellatopilosus*," *Tetrahedron Letters*, vol. 49, no. 25, pp. 4067–4072, 2008.

[17] R. B. Zanon, D. F. Pereira, T. K. Boschetti, M. dos Santos, and M. L. Athayde, "Fitoconstituintes isolados da fração em

diclorometano das folhas de *Vernonia tweediana* Baker," *Revista Brasileira de Farmacognosia*, vol. 18, pp. 226–229, 2008.

[18] L. Rahalison, M. Hamburger, M. Monod, E. Frenk, and K. Hostettmann, "Antifungal tests in phytochemical investigations: comparison of bioautographic methods using phytopathogenic and human pathogenic fungi," *Planta Medica*, vol. 60, no. 1, pp. 41–44, 1994.

[19] M. N. Lopes, A. C. de Oliveira, M. C. M. Young, and V. D. S. Bolzani, "Flavonoids from *Chiococca braquiata* (Rubiaceae)," *Journal of the Brazilian Chemical Society*, vol. 15, no. 4, pp. 468–471, 2004.

[20] J. V. Marques, R. O. S. Kitamura, J. H. G. Lago, M. C. M. Young, E. F. Guimarães, and M. J. Kato, "Antifungal amides from *Piper scutifolium* and *Piper hoffmanseggianum*," *Journal of Natural Products*, vol. 70, no. 12, pp. 2036–2039, 2007.

[21] V. S. Parmar, S. C. Jain, K. S. Bisht et al., "Phytochemistry of the genus *Piper*," *Phytochemistry*, vol. 46, no. 4, pp. 597–673, 1997.

[22] J. N. Tabudravu and M. Jarspars, "Anticancer activities of constituents of kava (*Piper methysticum*)," *The South Pacific Journal of Natural Science*, vol. 23, pp. 26–29, 2005.