Selective tumor cell growth inhibition by lignans and a seco-triterpenoid from Combretum mellifluum

Patrick da Silva Mirowski\textsuperscript{a}, Mariáh Ojeda\textsuperscript{b}, Luiz Gustavo Kollet\textsuperscript{a}, Talita Vilalva Freire\textsuperscript{a}, Arnildo Pott\textsuperscript{c}, Walmir Silva Garcez\textsuperscript{a}, Renata Trentin Perdomo\textsuperscript{b} and Fernanda Rodrigues Garcez\textsuperscript{a}\textsuperscript{b}

\textsuperscript{a}Bioactive Natural Products Research Laboratory, Institute of Chemistry, Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil; \textsuperscript{b}Laboratory of Molecular Biology and Cell Culture, School of Pharmaceutical Sciences, Food Technology, and Nutrition, Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil; \textsuperscript{c}Institute of Biosciences, Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil

\textbf{ABSTRACT}

Two new tetrahydrofuran lignans 1-2, along with 2,3-seco-lup-20(29)-en-2,3-dioic acid (3), (-)-larreatricin (4), and 15 additional compounds were isolated from \textit{Combretum mellifluum} (Combretaceae). Their structures were determined by 1D- and 2D-NMR spectroscopic data and HRESIMS. Another 15 compounds were identified after HPLC-DAD-MS/MS analysis. Tested against HT-29 (colon) neoplastic cells, lignan 1 showed marked cytotoxicity (\textit{GI}_{50} = 3.9 \mu M) and high selectivity (SI > 227), compared with non-neoplastic NIH/3T3 cells, while 2 proved less cytotoxic, despite exhibiting SI > 75. Seco-triterpene 3 was strongly cytotoxic to 786-0 (kidney) and HT-29 cells (\textit{GI}_{50} = 0.5 and 2.9 \mu M, respectively), proving roughly 107 and 18 times more selective for these cell lines, respectively, than for NIH/3T3 cells. After 48 h of incubation, 1-3 exhibited potent cytostatic activity against HT-29 cells at all concentrations tested, while 3 had a cytocidal effect on 786-0 cells at 25 \mu g.mL\textsuperscript{-1}.

\textbf{1. Introduction}

\textit{Combretum mellifluum} Eichler (Combretaceae), a shrub native to the Cerrado biome, is found in North, Northeast, Midwest, and Southeast Brazil, as well as Argentina.
Paraguay, Bolivia, and Peru (Pott and Pott 1994; Grandtner and Chevrette 2013; Combretaceae in Flora do Brasil 2020). The Combretaceae, a family of predominantly pantropical distribution, comprises approximately 20 genera, of which *Combretum*, the largest, with roughly 250 species, is distributed throughout tropical and subtropical regions (Hutchings et al. 1996). *Combretum* species, particularly those from the African continent, have drawn considerable attention for the several in vitro and in vivo biological activities reported for their extracts and isolated compounds, including notable anticancer properties (De Morais Lima et al. 2012). However, a number of *Combretum* species—including *C. mellifluum*—have not yet been investigated as potential sources of bioactive compounds. According to Flora do Brasil (Combretaceae in Flora do Brasil 2020), *C. mellifluum* Eichler is the valid name of the now synonym *C. discolor* Taub. As part of our ongoing focus on the search for potentially antineoplastic agents from plants native to the Cerrado biome of Midwest Brazil, the present study led to the isolation of two new tetrahydrofuran lignans of the 7,9'-epoxy type (1 and 2), as well as of the previously known seco-triterpenoid 2,3-seco-lup-20(29)-en-2,3-dioic acid (3) and the lignan (∼)-larreatricin (4), in addition to 15 compounds (5–19) comprising ellagic acid derivatives and pentacyclic triterpenoids, among other substances, from the stems, roots, and leaves of *C. mellifluum* (Figure 1). Another 15 compounds were fully or tentatively identified after HPLC-DAD-MS/MS analysis of EtOH extracts.

Compounds 1–4 were further evaluated for cytotoxicity against human neoplastic HT-29, 786-0, MCF-7, and PC-03 cell lines, and the non-neoplastic NIH/3T3 murine line.

Extraction and isolation procedures and cytotoxicity assay data, as well as HRESIMS, 1D- and 2D- NMR, and LC-DAD-MS data are available in the supplementary material.
2. Results and discussion

Compound 1 was obtained as an amorphous solid and assigned the molecular formula C_{18}H_{20}O_{3} based on HRESIMS data (m/z 285.1491 [M+H]^{+}, calcd for C_{18}H_{21}O_{3} 285.1490) indicating nine indices of hydrogen deficiency. {^{1}}H and {^{13}}C NMR data (Table S1) revealed the compound to be a tetrahydrofuran lignan of the 7,9\’-epoxy type (El Gamal et al. 1997; Li et al. 2011), in which both aryl groups were para-hydroxylated. In the {^{1}}H NMR spectrum, an oxymethine doublet at δ_{H} 4.23 (1H, J = 9.5 Hz) and a pair of broad triplets of oxymethylene protons at δ_{H} 3.75 (1H, J = 8.3) and δ_{H} 3.90 (1H, J = 8.3) showed correlations with HSQC signals at δ_{C} 90.3 and 74.1, respectively, being thus assigned to H-7/C-7 and H-9\’a, H-9\’b/C-9\’. Signals for a secondary methyl group at δ_{H} 0.92 (d, J = 6.5 Hz) and a pair of diastereotopic oxymethylene protons at δ_{H} 2.53 (dd, J = 13.7 and 5.5 Hz) and 2.83 (dd, J = 13.7 and 5.5 Hz), also apparent in the {^{1}}H NMR spectrum, showed one-bond correlations with HSQC carbon signals at δ_{C} 14.7 and 38.4, respectively, being therefore ascribed to methyl-9 and the methylene group linked to C-7\’. Typical signals for the AA’BB’ protons of the two para-hydroxylated aromatic rings were observed as four doublets at δ_{H} 6.74 and 7.14, as well as at δ_{H} 6.72 and 7.00 (8H, d, J = 8.5 Hz), which showed one-bond correlations in the HSQC spectrum with carbon signals at δ_{C} 116.2, 129.0, 116.1 and 130.6, respectively. Long-range HMBC connectivities of H-7 (δ_{H} 4.23) with C-2,6 (δ_{C} 129.0) and C-9 (δ_{C} 14.7), of H-7a/H-7\’b (δ_{C} 6.74/6.75) with C-1\’ (δ_{C} 132.4), C-2,6\’ (δ_{C} 130.6) and C-8 (δ_{C} 49.5), of H-3 (δ_{H} 6.74) and H-7 (δ_{H} 4.23) with C-1 (δ_{C} 132.2), of H-3\’ (δ_{H} 6.72) with C-1\’ (δ_{C} 132.4), of methyl-9 (δ_{H} 0.92) with C-8 (δ_{C} 49.5), and of H-9a/H-9b (δ_{H} 3.75/3.90) with C-7 (δ_{C} 90.3) confirmed these assignments, together with additional {^{1}}H-{^{1}}H correlations provided by the COSY spectrum. According to the literature, the typical chemical shifts of the oxymethine proton H-7 and methyl-9 (δ_{H} 4.23 and 0.92, respectively), as well as the C-7 resonance at δ_{C} 90.3, in the NMR spectra of 1 defined the trans-relationship of the methyl group at C-8 and the aryl group linked to C-7 (Rimando et al. 1994). Key NOE effects discernible from a NOESY experiment between methyl-9 and H-8\’ (δ_{H} 2.21), methyl-9 and H-7, and H-7 and H-8\’ revealed a cofacial relationship between these hydrogens, and therefore a trans-orientation of H-8 to both H-7 and H-8\’, thereby substantiating the relative stereochemistry of 1 as described above. The proton and carbon resonances of the tetrahydrofuran ring and methyl-9, as well as their corresponding {^{1}}H-{^{1}}H constants, showed remarkable resemblance with those of structurally related lignans bearing a trans-orientation of H-7 and H-8 and also of H-8 and H-8\’ (Liang et al. 2013; Yamauchi et al. 2013). The positive optical rotation of 1 ([α]_{D}^{20} + 52.5 (c 0.06, CHCl_{3}) was also similar to the foregoing (7R,8R,8\’R)-lignans, which differed from 1 only in the substitution pattern of the oxygenated groups on the aromatic rings (Liang et al. 2013; Yamauchi et al. 2013)—features that substantiated establishing the novel compound 1 as shown (Figure 1), which was named mellifluin A.

An ion peak at m/z 337.1419 [M+Na]^{+} observed in the HRESIMS of compound 2 was consistent with the molecular formula C_{19}H_{22}O_{4} (calcd for C_{19}H_{22}NaO_{4} 337.1416), indicative of nine degrees of unsaturation. NMR data revealed the structure of 2 to differ from that of 1 by the presence of a hydroxy substituent at C-8\’ and a methoxy group at C-4\’ (δ_{H} 3.78/δ_{C} 55.6) instead of a hydroxy functionality (Table S1). This
assumption was supported by the presence of a tetrasubstituted carbon at δC 82.3 in the spectrum of 2, with no signal for the methine carbon at C-8’, as well as the appearance of methyl-9 protons as a doublet at δH 0.88 (J = 6.8 Hz), along with signals attributable to the deshielded diastereotopic protons H-9’a and H-9’b (δH 3.63 and 4.13, respectively), whose geminal couplings, J = 9.2 Hz, were confirmed by correlations shown by the COSY spectrum. Accordingly, long-range HMBC correlations were observed from H-9, H-9’a, H-7’a, and H-7 to the hydroxylated C-8’. Likewise, the location of the methoxy group was proposed at C-4’, as implied by the cross-correlations between the methoxy protons and C-4’, reinforced by long-range connectivities of H-2’,6’ with C-4’ and C-7’. As with lignan 1, the chemical shifts of H-7, methyl-9, and C-7 led to assigning a trans-orientation of the aryl and methyl groups. The foregoing evidence, combined with the positive optical rotation value of 2 ([α]D 20 + 24.0 (c 0.1, MeOH)) and NOESY correlations between H-7 and H-9, as well as between H-7’a and H-8, H-7’b and H-9, and H-9’b and H-8, supported the proposal that 2 had the same stereochemistry as 1, in addition to a β-orientation of the hydroxy group at C-8’. Therefore, the structure of the novel lignan 2, named mellifluin B, was established as shown (Figure 1).

The molecular formula of 3 was deduced as C30H48O4 based on HRESIMS (m/z 473.3621 [M + H]+) data. The 1H NMR spectrum exhibited signals for two olefinic methylene protons at δH 4.68 (d, J = 2.3 Hz) and 4.56 (dd, J = 2.3 and 1.3 Hz), a vinyl methyl group at δH 1.68, and six tertiary methyl groups at δH 0.78, 0.92, 0.94, 1.02, 1.17, and 1.25. This information, together with 30 carbon signals observed in the 13C NMR spectrum, particularly those of a trisubstituted double bond at δC 109.6 (CH2) and 151.1 (C), were characteristic of a lup-20(29)-ene-type triterpenoid (Mahato and Kundu 1994) (Table S2). The absence of characteristic C-2 methylene and C-3 carbinol carbon resonances and the presence, instead, of two carboxylic carbon signals at δC 178.2 and 187.3 suggested that triterpene 3 has a tetracyclic core whose two carboxyl groups originated from the oxidative cleavage of ring A. This assumption was further confirmed by an isolated AB set of proton doublets in the NMR spectrum at δH 2.59 and 2.46 (J = 19.7 Hz), which were ascribed to the methylene hydrogens at C-1. One of the carboxyl carbons was assigned to the C-2 position, given its long-range correlation with H-1a (δH 2.46), which in turn showed a two-bond correlation with C-10. Further HMBC correlations from H3-23 (δH 1.25) and H3-24 (δH 1.17) to the carboxyl carbon at δC 187.3 placed the second carboxyl group at the other end (C-3) of the opened ring A. These and other 1D- and 2D- NMR data were in full agreement with those reported for 2,3-seco-lup-20(29)-en-2,3-dioic acid (Figure 1) (Gao et al. 2010). This A-seco lupane derivative, first isolated from Salacia hainanensis (Hippocrateaceae) (Gao et al. 2010) and further described for only two other species—Fagus hayatae (Fagaceae) (Lai et al. 2012) and Juglans hopeiensis (Juglandaceae) (Peng et al. 2020)—is being reported for the first time for the Combretaceae.

The identities of the previously known lignans (-)-larreatricin (4) and anolignan C (5), and the triterpenoids combregenin (6), arjunegenin (7), arjunolic, betulinic, oleanolic, and ursolic acids (8-11, respectively), arjunglucoside I (12), lupeol (13), and lupe-none (14), in addition to the ellagic acid derivatives 3,3’,4-tri-O-trimethylflavellagic acid (15) and ellagic acid 4-O-α-L-rhamnoside (16, also termed eichwellenol C), the flavonol
quercetin (17), para-hydroxyacetophenone (18), and α-tocopherol (19), were verified by comparing NMR spectroscopic data with those reported elsewhere (Rimando et al. 1994; Konno et al. 1990; Bisoli et al., 2008; Gossan et al. 2016; Santos et al. 2018; Balde et al. 2019; Serafin et al. 2007; Yang et al. 1998; Awad et al. 2017) (Tables S3-S7) and/or with authentic samples. (-)-Larreatricin $\{[\alpha]_D^{20} = 11.7 (c 0.1, MeOH)\}$ (4), which occurs in both enantiomeric forms, particularly in the genera Larrea (Zygophyllaceae) and Krameria (Krameriaceae) (Konno et al. 1990; Achenbach et al. 1991), lacks in previous records for the Combretaceae, while a single record of the occurrence of anolignan C (5) was found for the Combretaceae—namely, in Anogeissus acuminata (Rimando et al. 1994)—and this lignan is therefore being described for the first time in the genus Combretum. The occurrence of 18 is being reported for the first time for the Combretaceae, as is compound 15 for the genus Combretum.

A subsequent LC-DAD-MS, negative-ion-mode analysis of the EtOH extracts of stems, roots, and leaves yielded another 15 compounds (20-34)—gallic and ellagic acid derivatives, quercetin-O-hexosides, rutin, and isomers of arjunolic acid and arjunogenin, among other constituents—identified or tentatively characterized based on retention time, online UV, and HRESIMS data, including mass-spectrometric fragmentation patterns. These data were subsequently compared with published information, authentic standards, or both (Table S8).

Following isolation, compounds 1–4 were evaluated for in vitro growth-inhibiting activity against MCF-7 (breast), HT-29 (colon), 786-0 (kidney), and PC-03 (prostate) human cancer cell lines, as well as against non-neoplastic NIH/3T3 (murine fibroblast) cells, based on the SRB assay (Monks et al. 1991) (Table S9). Although devoid of anti-proliferative activity against 786-0 and PC-03 cells, compound 1 exhibited not only significant potency against HT-29 cells (GI$_{50}$ = 3.9 μM), but also a high selectivity index (SI > 227). By contrast, lignan 2 proved less cytotoxic than 1 to HT-29 cells (GI$_{50}$ = 10.5 μM), but exhibited a significant SI (>75) and weak cytotoxicity against the other cell lines. SI values of compounds 1 and 2 for HT-29 cells were notable not only for their magnitude (>227 and >75, respectively), but also for being at least 454 and 151 times higher, respectively, than the SI of doxorubicin. Compound 4, the other tetrahydrofuran lignan tested, proved mildly cytotoxic to HT-29 cells (GI$_{50}$ = 17.9 μM), but 48 times more selective for this cell line than for NIH/3T3 non-tumor cells. The A-seco-triterpene 3 strongly suppressed proliferation of 786-0 and HT-29 cells, as demonstrated by GI$_{50}$ values of 0.5 and 2.9 μM, respectively, similar to values obtained for doxorubicin (0.3 and 1.5 μM, respectively). It also proved roughly 107 and 18 times more selective for these carcinoma cell lines, respectively, than for non-neoplastic NIH/3T3 cells. Also noteworthy is that these significant SI values obtained for 786-0 and HT-29 cells were roughly 214 and 35 times higher, respectively, than those observed for doxorubicin.

Although 3 and 4 had previously been isolated from other plant species, this study is the first to yield information on the antitumor potential of these compounds.

The strong cytotoxicity of compounds 1 and 2 against HT-29 cells and of 3 against HT-29 and 786-0 cells, together with their high selectivity indexes, led us to investigate the effects of 1-3 on the viability of these human tumor cells and murine non-neoplastic cells. As shown by the curves for HT-29 cells after 48 h of incubation,
compounds 1 and 2 (Figures S1A and S1B) exhibited potent cytostatic activity (positive y values in the graphs) at all concentrations tested (0.25 to 250 \( \mu \text{g.mL}^{-1} \)), while proving inactive against non-neoplastic NIH/3T3 cells (100% cell viability) at all concentrations tested. Triterpene 3 promoted a marked reduction in the viability of HT-29 and 786-0 cells even at 0.25 \( \mu \text{g.mL}^{-1} \), the lowest concentration tested, while no effect on NIH/3T3 cells was observed at up to 2.5 \( \mu \text{g.mL}^{-1} \) (Figure S2). Although 3 exhibited similar antiproliferative (cytostatic) effects against HT-29 cells when employed at 0.25, 2.5, and 25 \( \mu \text{g.mL}^{-1} \) (points above zero in the curve), it had a cytocidal effect on 786-0 cells at 25 \( \mu \text{g.mL}^{-1} \) and higher concentrations, as revealed by negative y values on the curve.

3. Conclusion
In addition to information on the hitherto unreported chemical composition of C. melilifluum, the present study also revealed the species to be a promising source of compounds with potential for development of anticarcinogenic drugs. The \( \text{GI}_{50} \) and SI values obtained by testing seco-lupane-type triterpene 3 on human HT-29 colon cancer cells and, particularly, 786-0 kidney carcinoma cells, as well as by testing the new lignans 1 and 2 on HT-29 colon cancer cells, call for detailed investigation of the mechanisms of action of these three compounds, as well as studies on their synergistic potential when associated with current antineoplastic drugs.

Supplementary material
Supplementary data related to this article are available online, alongside Tables S1-S9, and Figures S1–S81.

Acknowledgments
This investigation was supported by the Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT-MS, grants 020793, 025220), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, finance code 001) and the Conselho Nacional de Ciência e Tecnologia (CNPq, grants awarded to P.S.M. and T.V.F.), and CPq-PROPP-UFMS (process 26/2021 CPQ-PROPP). Thanks are also extended to Maria de Fátima Vanderlei de Souza (Universidade Federal da Paraíba, João Pessoa, Brazil) for the 500 MHz NMR spectra of compound 3.

Disclosure statement
No potential conflict of interest was reported by the authors.

ORCID
Fernanda Rodrigues Garcez http://orcid.org/0000-0002-3995-4472
References

Achenbach H, Utz W, Usubillaga A, Rodriguez HA. 1991. Lignans from Krameria ixina. Phytochemistry. 30(11):3753–3757.
Awad BM, Habib ES, Ibrahim AK, Wanas AS, Radwan MM, Helal MA, Elsohly MA, Ahmed SA. 2017. Cytotoxic activity evaluation and molecular docking study of phenolic derivatives from Achillea fragrantissima (Forssk.) growing in Egypt. Med Chem Res. 26(9):2065–2073.
Balde ES, Camara AK, Traoré MS, Baldé NM, Megalizzi V, Pieters L, Balde AM. 2019. The hypoglycemic and cytotoxic activity of the leaves extract of Combretum glutinosum Perr ex DC. J Pharmacogn Phytochem. 8:2230–2237.
Bisoli E, Garcez WS, Hamerski L, Tieppo C, Garcez FR. 2008. Bioactive pentacyclic triterpenes from the stems of Combretum laxum. Molecules. 13(11):2717–2728.
Combretaceae in Flora do Brasil. 2020. Jardim Botânico do Rio de Janeiro; [accessed 2021 Mar 30]. http://reflora.jbrj.gov.br/reflora/floradobrasil/FB6907.
De Morais Lima GR, de Sales IRP, Caldas Filho MRD, de Jesus NZT, Falcão HDS, Barbosa Filho JM, Cabral AGS, Souto AL, Tavares JF, Batista LM. 2012. Bioactivities of the genus Combretum (Combretaceae): a review. Molecules. 17(8):9142–9206.
El Gamal AA, Takeya K, Itokawa H, Halim AF, Amer MM, Saad H-EA. 1997. Lignan bis-glucosides from Galium sinaicum. Phytochemistry. 45(3):597–600.
Gao H-Y, Guo Z-H, Cheng P, Xu X-M, Wu L-J. 2010. New triterpenes from Salacia hainanensis Chun et How with α-glucosidase inhibitory activity. J Asian Nat Prod Res. 12(10):834–842.
Gossan DPA, Magid AA, Yao-Kouassi PA, Josse J, Gangloff SC, Morjani H, Voutquenne-Nazabadioko L. 2016. Antibacterial and Cytotoxic triterpenoids from the roots of Combretum racemosum. Fitoterapia. 110:89–95.
Grandtner MM, Chevrette J. 2013. Dictionary of trees, volume 2: South America. 1st ed. Amsterdam: Academic Press.
Hutchings A, Scott AH, Lewis G, Cunningham AB. 1996. Zulu medicinal plants – an inventory. Pietermaritzburg: University of KwaZulu-Natal Press.
Konno C, Lu Z-Z, Xue H-Z, Erdelmeier CAJ, Meksuriyen D, Che C-T, Cordell GA, Soejarto DD, Waller DP, Fong HHS. 1990. Furanoid lignans from Larrea tridentata. J Nat Prod. 53(2):396–406.
Lai Y-C, Chen C-K, Tsai S-F, Lee S-S. 2012. Triterpenes as α-glucosidase inhibitors from Fagus hayatae. Phytochemistry. 74:206–211.
Li Y, Cheng W, Zhu C, Yao C, Xiong L, Tian Y, Wang S, Lin S, Hu J, Yang Y, et al. 2011. Bioactive neolignans and lignans from the bark of Machilus robusta. J Nat Prod. 74(6):1444–1452.
Liang C-Q, Hu J, Luo R-H, Shi Y-M, Shang S-Z, Gao Z-H, Wang R-R, Zheng Y-T, Xiong W-Y, Zhang H-B, et al. 2013. Six new lignans from the leaves and stems of Schisandra sphenanthera. Fitoterapia. 86:171–177.
Mahato SB, Kundu AP. 1994. 13C NMR spectra of pentacyclic triterpenoids—a compilation and some salient features. Phytochemistry. 37(6):1517–1575.
Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolf A. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst. 83(11):757–766.
Peng X, Lin Y, Liang J, Zhou M, Zhou J, Rua H. 2020. Triterpenoids from the barks of Juglans hopeiensis. Phytochemistry. 170:112201.
Pott A, Pott VJ. 1994. Plantas do Pantanal. Brasília: Empresa Brasileira de Pesquisa Agropecuária.
Rimando AM, Pezzuto JM, Farnsworth NR, Santisuk T, Neutrakul V, Kawanishi K. 1994. New lignans from Anogeissus acuminata with HIV-1 reverse transcriptase inhibitory activity. J Nat Prod. 57(7):896–904.
Santos H, Jr Lopes K, Alves D, Carvalho MG, Oliveira D. 2018. Ursolic acid and cis-tiliroside produced by Merremia tomentosa affect oviposition of Leucoptera coffeella on coffee plants. Quim Nova. 41:302–309.
Serafin C, Nart V, Malheiros A, Souza MM, Fischer L, Delle Monache J, Delle Monache F, Cechinel Filho V. 2007. Bioactive phenolic compounds from aerial parts of *Plinia glomerata*. Z Naturforsch C J Biosci. 62(3–4):196–200.

Yamauchi S, Kumamoto M, Ochi Y, Nishiwaki H, Shuto Y. 2013. Structure-plant growth inhibitory activity relationship of lariciresinol. J Agric Food Chem. 61(50):12297–12306.

Yang S-W, Zhou B-N, Wisse JH, Evans R, Van der Werff H, Miller JS, Kingston DGI. 1998. Three new ellagic acid derivatives from the bark of *Eschweilera coriacea* from the Suriname rainforest. J Nat Prod. 61(7):901–906.