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Artigo

Cytotoxicity of *Wedelia paludosa* D.C. extracts and constituents

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RESUMO: "Citotoxidade de extratos e constituintes de *Wedelia paludosa* D.C." *Wedelia paludosa* D.C. (Asteraceae) é uma planta ornamental facilmente encontrada em várias regiões do Brasil, principalmente nos estados de Santa Catarina, São Paulo, Minas Gerais, Bahia e Pernambuco. Objetivando descobrir novas substâncias citotóxicas a partir desta espécie, o extrato hidrometanólico de *W. paludosa* (HME) e as frações diclorometânica (FD) e aquosa (FA) resultantes de sua partição em CH_2Cl_2-H_2O foram avaliados utilizando-se o bioensaio em *Artemia salina*. A fração diclorometânica (FD) apresentou a maior atividade citotóxica (CL_{50} = 140,6 μg/mL), e sua análise por cromatografia líquida de alta eficiência empregando-se fase reversa (FR-CLAE) revelou os ácidos caurenóico (**1**, 6,22 ± 0,23%) e grandiflorênico (**2**, 3,22 ± 0,31%) como constituintes majoritários. As amostras HME (CL_{50} = 980 μg/mL), FD (CLC_{50} = 140,6 μg/mL), **1** (CL_{50} = 15,9 μg/mL) e **2** (CL_{50} = 29,8 μg/mL) foram citotóxicas contra *A. salina*, enquanto que a fração aquosa (FA, CL_{50} >> 1000 μg/mL) mostrou-se inativa. Conclui-se que a citotoxidade observada para HME e FD pode ser atribuída à presença dos ácidos caurenóico (**1**) e grandiflorênico (**2**) nestes extratos.

Unitermos: *Wedelia paludosa,* Asteraceae, atividade citotóxica, toxidade em *Artemia salina*, ácido caurenóico, ácido grandiflorênico, CLAE.

ABSTRACT: *Wedelia paludosa* D.C. (Asteraceae) is an ornamental species occurring in many regions of Brazil. Aiming to find new cytotoxic compounds, the hydromethanol extract of *W. paludosa* (HME), as well as the dichloromethane (DF) and water (WF) fractions resulting from its partition, were submitted to the brine shrimp lethality bioassay (BSLB) in order to evaluate their cytotoxicity. Dichloromethane fraction (DF) was shown to be the most cytotoxic fraction (LC_{50} = 140.6 μg/mL), and its analysis by reversed phase high performance liquid chromatography (RP-HPLC) revealed *ent*-kaurenoic (**1**, 6.22 ± 0.23%) and grandiflorenic (**2**, 3.22 ± 0.31%) acids as important constituents. HME (LC_{50} = 980 μg/mL), DF (LC_{50} = 140.6 μg/mL), **1** (LC_{50} = 15.9 μg/mL) and **2** (LC_{50} = 29.8 μg/mL) were found to be cytotoxic, while the water fraction (WF, LC_{50} >> 1000 μg/mL) was inactive. As conclusion, the cytotoxicity observed for HME and DF is mainly due to the presence of **1** and **2** in their constitution.

Keywords: *Wedelia paludosa,* Asteraceae, cytotoxic activity, brine shrimp lethality bioassay, kaurenoic acid, grandiflorenic acid, HPLC.

INTRODUCTION

W. paludosa D.C. (Asteraceae) is an ornamental species easily found in many regions of Brazil, especially in the states of Pernambuco, Bahia, Minas Gerais, São Paulo and Santa Catarina, where it is known as "pseudo-arnica", "pingo-de-ouro" or "margaridão" (Bresciani et al., 2000). Apart from its ornamental uses, this plant is often employed in folk medicine to treat various ailments, including cough, infectious and dolorous conditions (Roque et al., 1987). Several biological effects have been described for the ethanol extract of *W. paludosa* D.C. aerial parts including antinociceptive (Block et al., 1998a), trypanosomicidal (Chiari et al., 1996), hypoglycemic (Block et al., 1998b) and antifungal (Sartori et al., 2003) activities, among others. Previous phytochemical studies on this plant afforded the kaurane diterpenes *ent*-kaur-16-en-19-oic acid (**1**, kaurenoic acid) and *ent*-kaur-9(11),16-dien-19-oic acid (**2**, grandiflorenic acid) as major compounds, besides other related diterpenes, triterpenes and eudesmanolide lactones as minor constituents (Roque et al., 1987; Ferreira et al., 1994; Block et al., 1998a; Block et al., 1998b; Batista et al., 1999; Carvalho et al., 2001; Batista et al., 2005).

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Considering that prior studies on the hydromethanol extract of *Wedelia asperrima* Benth. have identified wedeloside (**3**) and other correlated diterpene aminoglycosides as main toxic and potential antitumor constituents (Eichholzer et al., 1981; MacLeod et al., 1990), we decided to investigate the cytotoxicity of the hydromethanol extract of *W. paludosa* and fractions, using the brine shrimp lethality bioassay (BSLB) as a general bioassay tool in order to find new cytotoxic compounds.

The brine shrimp lethality bioassay (BSLB) has been used as an efficient, rapid and inexpensive front-line test for the detection of bioactive compounds (Meyer et al., 1982; Ghisalberti, 1997; Lhullier et al., 2006; Stefanello et al., 2006; Silva et al., 2007; Nunes et al., 2008; Shoeb et al., 2008; Subhan et al., 2008). This bioassay requires small amount of sample (Solis et al., 1993), and it generally correlates well with cytotoxic (McLaughlin et al., 1998) and trypanosomicidal (Zani et al., 1995) activities.

MATERIAL AND METHODS

Reference substances

Kaurenoic (**1**) and grandiflorenic (**2**) acids were previously isolated from the aerial parts of *W. paludosa* D.C. (Batista et al., 1999). Purity of the samples was checked by HPLC and NMR analysis (Batista et al., 2005).

Plant material

Aerial parts of *Wedelia paludosa* D.C. (Asteraceae) were collected in Belo Horizonte, Brazil, in April 1998, and authenticated by Drª. Telma M. S. Grandi, Department of Botany, Universidade Federal de Minas Gerais - UFMG, at which herbarium a voucher specimen was deposited under the code BHCB 19033. The plant material was dried at 40 °C during 72 h, powdered and stored at low temperatures (-15 ºC) until use.

Hydromethanol extraction (HME)

The aerial parts of *W. paludosa* D.C. (20 g) were extracted once with 60 mL of methanol-water (1:1), at 50 ºC, under shaking, for 2 hours. After being filtered, the hydromethanol extract (HME) was concentrated in a rotavapor evaporator (50 °C) up to dry residue (3.76 g), yielding about 18.8% of the dried plant material (DPM).

Partition of HME

The HME residue (3.0 g) was suspended in water (100 mL) under sonication and extracted with dichloromethane (2 x 100 mL). The water and dichloromethane solution were concentrated in a rotavapor evaporator (60 °C and 40 ºC, respectively), affording the respective water (WF, 2.60 g, 16.3% of the DPM) and dichloromethane (DF, 0.13 g, 0.8% of the DPM) fraction residues.

Artemia salina assay

Brine shrimp eggs were hatched in artificial sea water (40 g/L, sea salt). After 24 hours incubation at warm room temperature (27-30 ºC) under artificial light, nauplii were collected with a Pasteur pipette and kept for an additional 24 hours under the same conditions, in order to reach the metanauplii stage. Residue and reference substance samples were prepared by dissolving separately 50 mg of HME, WF and DF residues or 1 mg of each reference substance, respectively, in 1 mL of dimethylsulfoxide (DMSO). In triplicate, aliquots of 12.5, 25.0, 50.0 and 100.0 µL from these samples were diluted to 5 mL of seawater containing 10-20 metanauplii. Both a negative control, containing 100 µL of DMSO, and a positive control, containing lapachol in DMSO, were included in each experiment. Twenty-four hours later, the number of survivors was counted, and the lethal concentration 50% (LC_{50}) was calculated using Probit analysis with 95% confidence intervals (Finney, 1971).

RP-HPLC analyses

Analyses were carried out on a Merck-Hitachi apparatus (Germany) composed of pump L-6200A, automatic injector AS-2000A, UV-VIS detector L-4250 and integrator D-2500. An ODS column (150 x 4.0 mm I.D., 5 µm) was employed (Merck, Germany) at a temperature of 35 °C, flow rate of 1.0 mL/min and wavelength of 220 nm. Isocratic elution of 60% CH_3CN in water was employed. Solvents used were of HPLC grade (Merck, Darmstadt, Germany) and were degassed by sonication before use. Analyses were performed in triplicate and each sample was injected onto the HPLC apparatus in duplicate.

The HME, WF and DF residues (20 mg) were separately dissolved in CH_3CN (2 mL), filtered on Adsorbex RP-18 cartridges (Merck, Germany), previously conditioned with CH_3CN (2 mL), and then centrifuged at 10,000 rpm during 5 min, prior to injection onto the HPLC apparatus.

Calibration graphs

Five point calibration curves were obtained from CH_3CN standard solutions of kaurenoic (1.0 mg/mL) and grandiflorenic (0.5 mg/mL) acids, injected in the range of 5-40 µL. The solutions were analyzed and the corresponding peak areas were compared against the mass of kauranes injected. Each point of the graph

Table 1. Cytotoxicity of HME, WF, DF, kaurenoic and grandiflorenic acids.

Sample	LC_{50} (μg/mL)	95% confidence interval (μg/mL)
HME	980.1	(707.3 - 1800.1)
WF	>> 1000 [(a)]	- - -
DF	140.6	(127.9 - 154.6)
Kaurenoic acid (**1**)	15.9	(13.5 - 20.6)
Grandiflorenic acid (**2**)	29.8	(27.2 - 32.4)
Lapachol	68.1	(57.2 - 79.1)

[(a)] 100% of survivors at maximum assayed WF concentration (1000 μg/mL).

was the mean of five measurements. Linear regression equations were obtained by computer analysis, employing SigmaPlot software (Jandell Co., USA). Identification of the diterpenoid peaks in the extracts was accomplished by comparison with the retention time of standard solutions injected in the same conditions.

RESULTS AND DISCUSSION

The cytotoxic effects of HME, WF, DF, kaurenoic (**1**) and grandiflorenic (**2**) acids are depicted in Table 1. According to the literature parameters (Meyer et al., 1982; Solis et al., 1993), hydromethanolic extract (HME), dichloromethane fraction (DF), kaurenoic (**1**) and grandiflorenic (**2**) acids were cytotoxic to brine shrimp nauplii (LC_{50} < 1000 μg/mL), being diterpene **1** (LC_{50} = 15.9 μg/mL) more toxic than **2** (LC_{50} = 29.8 μg/mL), while the water fraction (WF) was inactive (LC_{50} >> 1000 μg/mL). The LC_{50} value obtained in the present work for kaurenoic acid (**1**, LC_{50} = 15.9 μg/mL) is in agreement with literature data (LC_{50} = 16 μg/mL) (Fatope et al., 1996). At the best of our knowledge, the cytotoxic activities observed for grandiflorenic acid (**2**, LC_{50} = 29.8 μg/mL) and *W. paludosa* extracts (HME, LC_{50} = 980.1 μg/mL; DF, LC_{50} = 140.6 μg/mL) are, for the first time, reported in this work.

Dichloromethane fraction (DF) was shown to be more cytotoxic (LC_{50} = 140.6 μg/mL) than hydromethanol extract (HME, LC_{50} = 980.1 μg/mL) and water fraction (WF, LC_{50} >> 1000 μg/mL). These results agree with the higher cytotoxicity observed for chloroform than methanol extract of *Wedelia calendulacea*, suggesting that kaurenoic acid (**1**) might be the principal cytotoxic constituent of this species (Mottakin et al., 2004).

Thus, we were motivated to quantify both kaurenoic (**1**) and grandiflorenic (**2**) acids in dry dichloromethane fraction (DF) of *W. paludosa*, employing the RP-HPLC quantitation method which was previously developed and validated for these diterpenes in the aerial parts of *W. paludosa* D.C. (Batista et al., 2005).

Good response linearity was obtained for both diterpenes with r^2 values of 0.9998 (**1**) and 0.9999 (**2**) (peak area vs. mass). The linear equations obtained for **1** and **2** were y = 32208,5x + 2432,8 and y =108421,3x + 25392,8 respectively. The quantitation limit of the method was established as 1.25 μg for **1** (RSD = 2.07%) and 0.63 μg for **2** (RSD = 2.67%).

An example of typical chromatograms obtained from HME, WF and DF analysis is shown in Figure 1. The contents of kaurenoic (**1**) and grandiflorenic (**2**) acids in the dry DF residue were determined as 6.22 ± 0.23% and 3.22 ± 0.31%, respectively. These diterpenes were not detected by the HPLC apparatus in HME and WF residues.

Several biological effects have been described for kaurenoic (**1**) and grandiflorenic (**2**) acids (Ghisalberti, 1997). These diterpenes (**1** and **2**) caused total lysis of trypomastigotes of the protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease (American trypanosomiasis), at a concentration of 0.68 mg/mL (Batista et al., 1999). In addition, **1** and **2** were shown to be potent stimulators of uterine contraction, and their presence in *Aspilia mossambicensis* may explain why female chimpanzees consume such plant more frequently than males do (Page et al., 1992). Among more recently reported activities for **1**, we can stand out the antimicrobial (Zgoda-Pols et al., 2002), anti-platelet aggregation (Yang et al., 2002), analgesic (Block et al., 1998a), antifungal (Sartori et al., 2003), smooth muscle relaxant (Cunha et al., 2003), hypoglycemic (Bresciani et al., 2004), cytotoxic and embryotoxic (Costa-Lotufo et al., 2002) effects, among others (García et al., 2007).

CONCLUSION

Considering the cytotoxic activity showed by kaurenoic (**1**) and grandiflorenic (**2**) acids, by dichloromethane fraction (DF) and hydromethanol extract (HME) of *W. paludosa*, and the absence of this effect from water fraction (WF), along with the contents of diterpenes **1** (6.22 ± 0.23%) and **2** (3.22 ± 0.31%) determined in dichloromethane fraction (DF) by RP-HPLC, we can conclude that *W. paludosa* hydromethanol extract (HME) and dichloromethane fraction (DF) are cytotoxic mainly due to the presence of kaurenoic and grandiflorenic acids in their constitution.

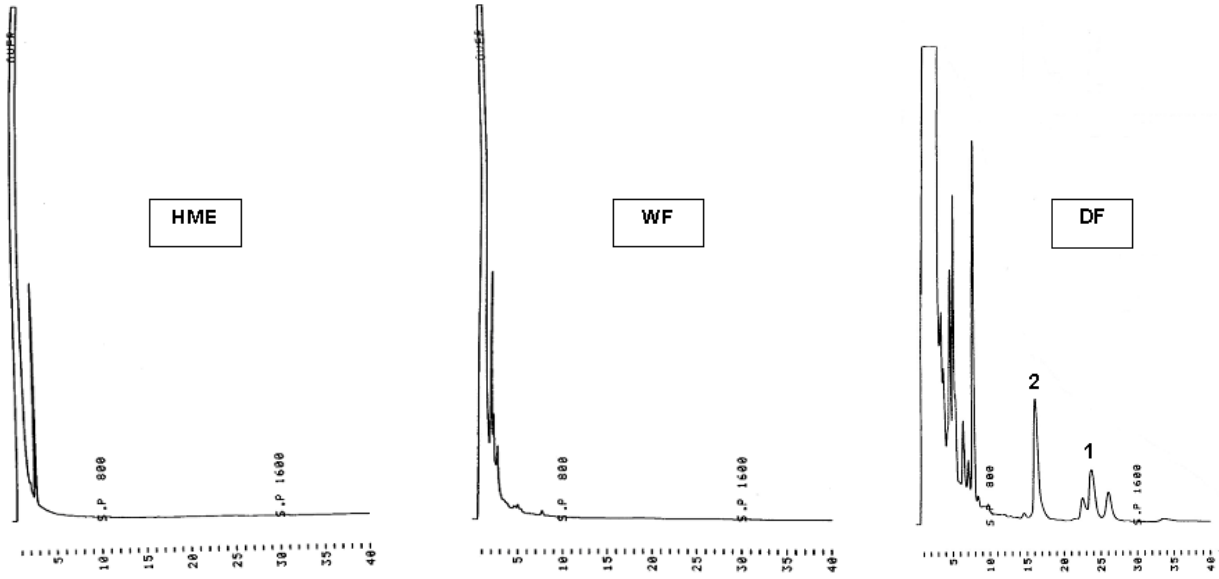
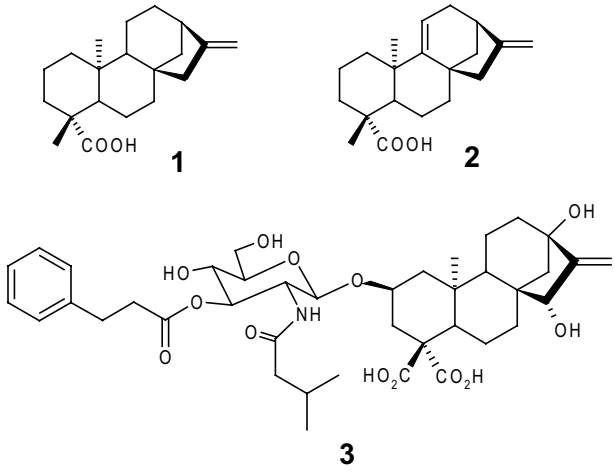


Figure 1. RP-HPLC chromatograms obtained from the analysis of the hydromethanol extract (HME), water fraction (WF) and dichloromethane fraction (DF). **1**, kaurenoic acid; **2**, grandiflorenic acid. Chromatographic conditions: see material and methods section.



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