Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: https://www.tandfonline.com/loi/iphb20

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To cite this article: Noel R. Monks, Alexandre Ferraz, Sergio Bordignon, Katia R. Machado, Martha F.S. Lima, Adriana B. Rocha & Gilberto Schwartsmann (2002) In vitro Cytotoxicity of Extracts from Brazilian Asteraceae, Pharmaceutical Biology, 40:7, 494-500, DOI: 10.1076/phbi.40.7.494.14681

To link to this article: https://doi.org/10.1076/phbi.40.7.494.14681

Pharmaceutical Biology
2002, Vol. 40, No. 7, pp. 494–500

1388-0209/02/4007-494$16.00

In vitro Cytotoxicity of Extracts from Brazilian Asteraceae

Noel R. Monks[1], Alexandre Ferraz[2], Sergio Bordignon[1], Katia R. Machado[1], Martha F.S. Lima[1], Adriana B. da Rocha[1,2] and Gilberto Schwartsmann[1,2]

[1]Centro Integrado do Câncer (CINCAN), Universidade Luterana do Brasil (ULBRA), Canoas, RS, Brazil; [2]South American Office for Anticancer Drug Development (SOAD), Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

Abstract

Aqueous and organic extracts of Asteraceae (Compositae) collected from the State of Rio Grande do Sul, Brazil, have been tested *in vitro* for cytotoxic activity against human solid tumour cell lines. Twenty-five species, 125 extracts in total, were screened against HT29 human colon adenocarcinoma cells and NCI-H460 human non-small cell lung cancer cells. Twenty-five extracts from 11 species demonstrated cytotoxicity at 100 µg/ml against one or both of the cell lines tested. Further analysis was performed on the active extracts using three cell lines HT29, NCI-H460, and U373 human glioblastoma cells, to determine the IC_{50} and the degree of tumour cell line selectivity. Extracts from *Baccharis coridifolia, Baccharis ochracea*, *Eupatorium macrocephalum*, *Eupatorium pedunculosum* and *Stenachaenium riedelii* all produced IC_{50} values below 5 µg/ml. Comparison of the IC_{50} results between cell lines identified that *Baccharis coridifolia, Baccharis ochracea, Eupatorium laevigatum* and *Pluchea sagittalis* extracts produced differential sensitivity across the panel of three cell lines. These species are currently under further investigation with the ultimate objective of isolation and identification of the active principles responsible for the anti-proliferative activity.

Keywords: *In vitro* screening, cytotoxic activity, Asteraceae, Rio Grande do Sul, Brazil.

Introduction

Combinatorial chemistry, rational drug design and high throughput screening of synthetic chemical libraries are all techniques currently utilised by the pharmaceutical industry in the search for new, more potent, less toxic anti-cancer agents. In parallel, pharmaceutical companies are also looking to nature as a source of new lead compounds. To date, a significant proportion of the cytotoxic agents used for the treatment of human malignancies are derived from the screening of natural products, the most successful example of which are the vinca alkaloids, anthracyclines, taxoids and camptothecin derivatives (Mans et al., 2000; Schwartsmann, 2000). To satisfy the demand for new anti-cancer drugs, scientists are scouring the world for new species of plants, animals, marine and micro-organisms which demonstrate potent anti-cancer properties. Rainforests, deep sea thermal vents and coral reefs are examples of ecosystems which are currently being explored for novel sources of anti-neoplastic molecules (Cragg et al., 1997; Cragg & Newman, 1999; Schwartsmann et al., 2001). It has been estimated that only 5–15% of the 250,000 higher plants species have been tested both phytochemically and pharmacologically, thus leaving a huge potential to encounter novel anti-cancer molecules (Balandrin et al., 1993).

As a part of this international effort, we have established a permanent *in vitro* anti-cancer screening programme for the testing of Brazilian flora and marine species against a panel of human tumour cell lines (Mans et al., 2000). Plant species are selected using a number of methods, these include ethnobotanical information, i.e., plants used in popular medicines and chemotaxonomy were plants are selected due to the presence of a important chemical compound in a congeneric species. Along side rational plant selection, we are also running a programme of random testing. This approach may be considered less productive, but in the past, yielded important successes, most notably taxol (Farnsworth, 1994).

In parallel, extracts which demonstrate *in vitro* cytotoxicity, in our cell line panel are sent for further testing at the US National Cancer Institute (NCI) *in vitro* screening program,

Accepted: April 30, 2002

Address correspondence to: Dr. Noel R. Monks, Dana-Farber Cancer Institute, Smith Building – Rm 934, 44 Binney Street, Boston – 02115, MA, USA. Tel.: +1-617-632-4172; Fax: +1-617-632-4680; E-mail: Noel_Monks@dfci.harvard.edu

which includes 60 phenotypically and genotypically characterised cell lines of human origin (Shoemaker et al., 1988; Monks et al., 1997).

The Asteraceae (Compositae) family is made up of 1100 genera (~25,000 species), of which 191 genera (~1900 species) have been identified in Brazil (Barroso et al., 1991). A high proportion of this family, approximately 600 species, are found in the State of Rio Grande do Sul (personal communication, C. Mondin, Universidade Federal do Rio Grande do Sul, 2001). Asteraceae are commonly used in folklore medicine across the world, with greater that 1000 species being registered in ethnomedical databases, such as EthnobotDB (http://ars-genome.cornell.edu/Botany/aboutethnobotdb.html) and NAPRALERT (Farnsworth, 1994). Approximately 291 species of Asteraceae have been reported to have ethnomedical uses for the treatment of cancer (Hartwell, 1968; Graham et al., 2000). Of these, 43 species are cited as used in South America with 12 species in Brazil.

Numerous reports have described experimental studies which demonstrate Asteraceae species to have anti-tumour activity, frequently due to the presence of sesquiterpene lactones. Recent examples include *Emilia sonchifolia* (L.) DC. (Shylesh & Padikkala, 2000), *Alomia myriadenia* Schultz-Bip. ex Baker (Zani et al., 2000), *Inula britannica* L. var *chinensis* (Rupr.) Reg (Park & Kim, 1998) and *Mikania minima* (Baker) Robinson (Barrero et al., 2000).

In this report we describe the *in vitro* screening of extracts from Asteraceae species collected in the State of Rio Gande do Sul which is located in southern Brazil, the majority of which have no prior reports of anti-tumour activity (denoted by an asterisk in Table 1).

Methodology

Collection and extraction of plant materials

Plant material was collected throughout the state Rio Grande do Sul, southern Brazil. Botanical identification of the plant material was made by Dr. Sergio Bordignon and exsiccates are deposited at the herbarium of the Universidade Luterana do Brasil (HERULBRA). The species tested in this study, including common names, popular uses (Brazil and Argentina) and the parts tested (organic and aqueous), are detailed in Table 1.

Plant materials were dried in the dark at ambient temperature, powdered and extracted by maceration for 48 h in both water and ethanol. Extracts were subsequently filtered and concentrated by either rotary evaporation (organic) or lyophilisation (aqueous) and stored at −20 °C. Immediately prior to testing, the organic extracts were dissolved in DMSO, and diluted in culture medium to give a final, *in vitro*, DMSO concentration of 0.25% v/v, at which no growth inhibitory activity was observed in each of the cell lines tested. Aqueous extracts were dissolved in culture medium.

Cell culture maintenance

The HT29 human colon adenocarcinoma (ATCC No HTB-38), NCI-H460 human large cell lung carcinoma (ATCC No. HTB-177) and U373 human glioblastoma astrocytoma (ECACC No. 89081403) cell lines were maintained as exponentially growing cultures in RPMI 1640 culture medium, supplemented with 10% foetal bovine serum, pH 7.4. All cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in air (100% humidity).

Cytotoxicity screening

As part of our general screening programme, all plant extracts were primarily tested at a concentration of 100 µg/ml against HT29 and NCI-H460 cells to eliminate extracts which did not produce the required level of activity (SRB absorbance below the time zero control).

HT29 cells were plated into 96-well cell culture plates, at 3.5×10^3 cells per well in 100 µl and NCI-H460 cells at 1×10^3 per well. After 24 h, 100 µl of growth medium containing the extracts was added to the wells in triplicate giving a final concentration of 100 µg/ml. Medium and DMSO (0.25% v/v) controls were used. Following addition of the extracts, plates were incubated for 72 h, after which cellular growth was determined using the sulforhodamine B colorimetric protein assay (Skehan et al., 1990). Colorimetric readings where made at 540 nm (using a Labsystems Multiscan EX plate reader and Genesis-lite software). Those extracts which produce an absorbance lower than that of the time-zero value in one or both of the two cell lines (approximately 10% and 5% of the control growth for the HT29 and NCI-H460, respectively) were considered to be cytotoxic and subsequently submitted for further investigation. The time-zero control was generated by cellular fixation, using 50% TCA, prior to the addition of the extracts.

Growth inhibition studies

The IC_{50} (concentration which inhibits cellular growth by 50%) was determined against HT29, NCI-H460 and U373 (2.5×10^3 cells per well) cells using the methods described above. Cells were treated in triplicate with a log 10 concentration range (0.1, 1, 10 and 100 µg/ml) of each extract for 72 h (in the case of the organic stems extract of *Pluchea sagittalis*, a maximum concentration of 500 µg/ml was used). The IC_{50} values were estimated from a semi-log plot of extract concentration against SRB absorbance as a percentage of vehicle control growth (0.25% DMSO).

Statistical analysis

The values reported are the mean ± SD of three replicate experiments. The raw data were analysed by one way analysis of variance (ANOVA – Tukey's test) to determine the statistical difference (Instat software, Version 2.0 –

Table 1. Asteraceae species screened for cytotoxic activity.

Botanical Name[a]	Common name	Popular use	Plant parts tested[b]	References
Baccharis coridifolia DC.	Mio-mio, Vassourinha, Alecrim	Poison, Digestive, Diaphoretic	L + S	1, 3, 5
**Baccharis ochracea* Spreng.	Erva-santa, Carqueja		L + S	3
**Baccharis spicata* (Lam.) Baill.			L, S	
**Chaptalia nutans* (L.) Polak.	Língua-de-vaca	Amenorrhea, Sore, Swelling, Fumitory, Bechic, Asthma, Genital infections, Diuretic, Fever, Ophthalmic, Respiratory infections, Anti-inflammatory	L + F + S, R	1, 3, 5, 7
**Dasyphyllum brasiliense* (Spreng.) Cabr.	Guaiapá-parreira, Cipó-agulha		L, S	10
**Dasyphyllum spinescens* (Less.) Cabr.	Açucará, Sucará, Espinho-de-agulha, Espinho-de-santo-antônio, Não-me-toque		L, S, F	10
**Eupatorium casarettoi* (Robinson) Steyrmark	Eupatório-de-casaretto, Vassoura- do-campo, Vassoura-bichada		L, S, F	11
**Eupatorium inulaefolium* H.B.K.	Cambará	Antifertility, Cold, Collyrium, Cough, Ophthalmic, Sore, Aborticide, Menstrual cycle regulation.	L, S	1, 5, 6
**Eupatorium laevigatum* Lam.	Cambará, Cambará-falso	Injuries of bad character, Analgesic, Purgative, Menstrual cycle regulation, Aborticide, Bechic, Digestive.	L, S, R	3, 4, 5, 6, 11
**Eupatorium macrocephalum* Less.	Charrúa-grande	Bechic, Aborticide, Digestive.	T, L + F	3, 5, 6
**Eupatorium pedunculosum* Hook. et Arn.	Eupatório		L, S	11
**Eupatorium tremulum* Hook. et Arn.	Eupatório, Vassourão-do-brejo.		L, S	11
**Gochnatia polymorpha* (Less.) Cabr.	Cambará-de-folha-grande, Cambará-do-mato, Tatané-moroti.	Catarrh and Respiratory infections, Base for diverse medicines.	L, S	3, 7
**Mikania dentata* Spreng.			L + S	
Mikania hirsutissima DC.	Cipó-Cabeludo, Guaco-cabeludo,Cipó-almecega-Cabeludo, Erva-dutra, Herva Dutra.	Albuminuria, Cystitis, Diuretic, Anti-albuminuric, Molluscicidal, Nephritis, Urethitis, Back pain, Chronic Diarrhea, Paralysis, Rheumatism.	L, S, F	2, 3
**Piptocarpha sellowii* (Sch. Bip.) Baker	Braço-forte		L, S	
**Pluchea sagittalis* (Lam.) Cabr.	Lucera, Erva-lucera, Lucero, Quitoco, Tabacarana, Madrecravo	Anti-flatulence, Stomach and Liver infections, Genital infections, Wound healing, Digestive, Diuretic, Stimulant, Fever, Respiratory infections, Anti- inflammatory, Sedative, Headache, Anti-hysterical.	L, S, F, R	1, 4, 5, 6, 8
**Senecio brasiliensis* (Spreng.) Cabr.	Flor-das-almas, Catião, Craveiro-do-campo, Erva-lanceta, Malmequer-amarelo, Maria-mole.	Cure for injuries.	L, S, R	3

Table 1. Continued

Botanical Name[a]	Common name	Popular use	Plant parts tested[b]	References
**Solidago chilensis* Meyen	Erva-lanceta, Arnica, Lanceta.	Gastrointestinal disturbances, Wound healing, Analgesic, Vermifuge, Diuretic, Post-partum, Headache, Calming baths.	L, S, R	3, 5, 6, 9
**Stenachaenium riedelii* Baker		Stomachic.	L + F, S, R	6
Tagetes minuta L.	Chinchilla, Cravo-de-defunto, Rabo-de-rojão, Rabo-de-foquete, Cravo-de-mato, Voadeira	Aperient, Bronchodilator, Diaphoretic, Diuretic, Dyspepsia, Emmenagogueue, Gastritis, Hypertension, Anti-Hysterical, Anti-Inflammatory, Piles, Poison, Purgative, Spasmolytic, Stimulant, Stomachic, Vermifuge, Viricide, Anti-rheumatic, Genital infections, Insect repellent, Anti-flatulence, Calmative, Hepatic infections.	L + F, R	1, 4, 5, 7
**Vernonia balansae* Hieron.	Tatatai		L, L + S	
**Vernonia muricata* DC.	Cambarazinho		L + F, S	
**Vernonia nudiflora* Less.	Alecrim-do-campo		L, S	
**Vernonia tweediana* Baker	Mata-pasto, Assapeixe, Chamarita, Erva-de-laguna, Lingua-de-vaca, Orelha-de-mula		L, S, F	3

References for popular names and use (1) EthnobotDB – (http://ars-genome.cornell.edu/Botany/aboutethnobotdb.html); (2) Rain-tree.com – (http://www.rain-tree.com); (3) Corrêa, 1984; (4) Lorenzi, 2000; (5) Amat, 1983; (6) Zardini, 1983a; (7) Zardini, 1983b; (8) Burkart et al., 1974; (9) Simões et al., 1995; (10) Cabrera and Klein, 1973; (11) Cabrera and Klein, 1989.

[a] The asterisk denotes those species which have no prior reference to anti-tumour activity, both ethnomedical and scientific.

[b] L – leaves, S – stems, F – flowers, R – roots, "+" denotes a mixture of plant parts.

Graphpad Software, Inc.). Differences above the 95% confidence interval ($P < 0.05$) were considered as statistically significant.

Results and discussion

Table 1 details the species and plants parts (aqueous and organic extracts) which were initially included in the study. In total, 25 species (125 extracts) were screened (data not presented) of which 11 species (25 extracts) demonstrated cytotoxic activity at 100 µg/ml in one or both of the HT29 and NCI-H460 cell lines (Table 2). All six of the *Eupatorium* species tested were found to be cytotoxic *in vitro*. A number of literature reports have demonstrated the *Eupatorium* genus to have cytotoxic activity and from these a number of molecules have been isolated, primarily sesquiterpene lactones (Table 3). This is the first report demonstrating anti-proliferative activity against human tumour cell lines by these six *Eupatorium* species.

Tagetes minuta, a plant which has previously been reported to have marginal activity *in vivo* (Lewis lung carcinoma) by the NCI (Ickes et al., 1973), was found to be inactive in our screening model. The genus *Vernonia* contains a number of species with ethnomedical references for use against cancer, such as *V. anthelmintica* (L.) Willd., *V. cinera* Lees. *and V. fasciculata* Michx. (Hartwell, 1968). A number of cytotoxic sesquiterpene lactones have also be isolated from the *Vernonia* species *V. amygdalina* Del. and *V. hymenolepis* A. Rich (Kupchan et al., 1969a,b). Notably, all four of the *Vernonia* species tested in this study did not demonstrate *in vitro* cytotoxic activity.

Single concentration screening provides relatively little information regarding the true activity of the extracts and the cytotoxic component(s) within. Instead, it facilities the rapid analysis of a large number of extracts and the identification of those extracts with cytotoxic activity and the elimination of those which are inactive. This methodology is similar to that currently employed by the NCI's natural products group as a pre-screen to eliminate extracts which are not consid-

Table 2. *In vitro* growth inhibitory activity of extracts from Brazilian Asteraceae plants species.

Species	Extract[a]	Cell lines[b] (IC_{50} µg/ml) HT29	U373	NCI-H460	ANOVA[c]
Baccharis coridifolia	Leaves + Stems (O)	0.3 ± 0	0.32 ± 0.02	0.23 ± 0.01	***
	Leaves + Stems (A)	3.9 ± 0.1	4.3 ± 0.3	3.0 ± 0	***
Baccharis ochracea	Leaves + Stems (O)	4.2 ± 0.5	3.3 ± 0.4	2.0 ± 0.6	**
	Leaves + Stems (A)	27 ± 0	28 ± 3	4.3 ± 0.7	***
Eupatorium casarettoi	Leaves (O)	28 ± 3	23 ± 3	26 ± 4	ns
	Flowers (O)	17 ± 8	11 ± 9	3.9 ± 0.5	ns
	Flowers (A)	24 ± 4	18 ± 3	27 ± 6	ns
	Stems (O)	28 ± 8	32 ± 6	34 ± 4	ns
Eupatorium inulifolium	Leaves (O)	35 ± 0	36 ± 12	33 ± 3	ns
Eupatorium laevigatum	Stems (O)	83 ± 17	33 ± 6	37 ± 7	***
	Leaves (O)	36 ± 5	27 ± 5	34 ± 5	ns
Eupatorium macrocephalum	Stems (O)	35 ± 5	32 ± 6	30 ± 0	ns
	Leaves + Flowers (O)	1.6 ± 0.8	5.4 ± 3.6	5 ± 2	ns
	Leaves + Flowers (A)	30 ± 9.5	30 ± 8.7	32 ± 4	ns
Eupatorium pedunculosum	Leaves (O)	3.6 ± 0.1	2.9 ± 1	3 ± 0	ns
Eupatorium tremulum	Leaves (O)	34 ± 3.5	35 ± 5	30 ± 0.6	ns
	Stems (O)	27 ± 6.5	27 ± 6	31 ± 1	ns
Piptocarpha sellownii	Leaves (O)	31 ± 1	29 ± 2	30 ± 2	ns
Pluchea sagittalis	Leaves (O)	31 ± 1	11 ± 3	12 ± 6	**
	Stems (O)	126 ± 21	49 ± 4	32 ± 1	***
	Flowers (O)	83 ± 15	34 ± 3	31 ± 0	***
Stenachaenium riedelii	Leaves + Flowers (O)	2.8 ± 0.4	2.2 ± 0.3	1.9 ± 0.5	ns
	Leaves + Flowers (A)	44 ± 4	44 ± 11	29 ± 3	ns
	Stems (O)	4.1 ± 1	2.7 ± 1	2.9 ± 0.1	ns
	Roots(O)	25 ± 5	14 ± 7	11 ± 5	ns

[a] O – Organic, A – Aqueous.
[b] HT29 – Human colon adenocarcinoma, U373 – Human glioblastoma astrocytoma, NCI-H460 – Human large cell lung carcinoma.
[c] ANOVA – One way analysis of variance, ns – Not significant, * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$.

Table 3. Literature reports of *Eupatorium* species and the compounds isolated which have previously demonstrated anti-tumour activity.

Species	Name	Chemical class	Reference
Eupatorium altissimum L.	Eupatorin	Flavone	(Dobberstein et al., 1977)
Eupatorium cannabinum L.	Eupatoriopicrin	Sesquiterpene lactone	(Hladon & Chodera, 1975)
Eupatorium cuneifolium (Tourn.) L.	Eupacunin	Sesquiterpene lactone	(Kupchan et al., 1971)
Eupatorium formosanum HAY.	Eupatolide	Sesquiterpene lactone	(Lee et al., 1972)
Eupatorium hecatanthum (DC) Bak.			(Mongelli et al., 2000)
Eupatorium rotundifolium L.	Euparotin, Eupachlorin, Eupatundin	Sesquiterpene lactones	(Kupchan et al., 1969c)
Eupatorium rotundifolium L.	Eupachlorin acetate	Sesquiterpene lactone	(Kupchan et al., 1968)
Eupatorium rotundifolium L.	Euparotin acetate	Sesquiterpene lactone	(Kupchan et al., 1967)
Eupatorium semiserratum DC	Eupaserrin and Deacetyleupaserrin	Sesquiterpene lactones	(Kupchan et al., 1973)
Eupatorium semiserratum DC	Eupatorin	Flavone	(Kupchan et al., 1965)

ered active enough to be tested against the 60 cell line panel (Sausville & Feigal, 1999).

Baccharis coridifolia was found to be the most potent species tested. Both the organic and aqueous extracts from leaves and stems resulted in IC_{50} concentrations below 5 µg/ml. This result can be expected as *Baccharis coridifolia* is well known in both southern Brazil and Argentina, as the cause of livestock poisoning (Habermehl et al., 1985; Jarvis

et al., 1996). This toxicity has been attributed to the presence of highly toxic macrocyclic trichothecene antibiotics (Jarvis et al., 1988). A dichloromethane extract from *Baccharis coridifolia* has previously demonstrated potent activity against KB cells (ED_{50} = 4.2 µg/ml) (Mongelli et al., 1997) and detailed phytochemical studies have identified schottenol glucoside as one of the active components (Arisawa et al., 1985). A number of other species also demonstrated potent *in vitro* anti-tumour activity (below 5 µg/ml), including *Baccharis ochracea* (leaves and stems – organic), *Eupatorium macrocephalum* (leaves and flowers – organic), *Eupatorium pedunculosum* (leaves – organic) and two extracts from *Stenachaenium riedelii* (leaves and flowers – organic and stems – organic).

Using a one way analysis of variance (ANOVA), extracts which demonstrated statistically different sensitivities between the cell lines were identified; these results are displayed in Table 2. The majority of the extracts did not result in a detectable difference in the activity between the three cell lines. In fact, a number of the potent extracts such as those from *Eupatorium macrocephalum*, *Eupatorium pedunculosum* and *Stenachaenium riedelii* did not demonstrate any statistical variance. Of the species tested, *Pluchea sagittalis* was found to be the most interesting. A statistical difference was observed in all three of the extracts tested (leaves, stems and flowers). In each case, the HT29 cell line was found to be significantly less sensitive (2–3-fold) to each of the extracts. *Pluchea sagittalis* has previously been shown to have anti-inflammatory and anti-oxidant activities (Perez-Garcia et al., 1996). This is the first report demonstrating *Pluchea sagittalis* to have anti-proliferative activity against human tumour cell lines. Both the organic and aqueous extracts of a mixture of leaves and stems from both *Baccharis cordifolia and Baccharis ochracea* also showed variability in the activity between the three cell lines as did the organic stems extract from *Eupatorium laevigatum.* The later two species also have no previous records of anti-proliferative activity.

In summary, the screening of Asteraceae species collected from the State of Rio Grande do Sul, Brazil, has identified a number plant species whose extracts exhibit potent anti-proliferative activity, for the first time, against human tumour cell lines. Further studies are currently being undertaken to determine the compounds responsible for the activity seen *in vitro* and we are continuing our program of collection of Asteraceae species in the search for new pharmacophores.

Acknowledgements

The authors would like to thank the SOAD foundation for providing the funding necessary for the screening program. The authors are indebted to many colleagues for their contribution, specifically Dr. Dennis Mans, Denise Faria, Rafael Lopes and Silvana de Silva.

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