Food Chemistry 141 (2013) 196–200

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Antitumour properties of the leaf essential oil of *Xylopia frutescens* Aubl. (Annonaceae)

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ARTICLE INFO

Article history:
Received 20 December 2012
Received in revised form 22 February 2013
Accepted 26 February 2013
Available online 7 March 2013

Keywords:
Xylopia frutescens
Annonaceae
Essential oil
Chemical composition
Antitumour

ABSTRACT

The aim of this study was to investigate the chemical composition and anticancer effect of the leaf essential oil of *Xylopia frutescens* in experimental models. The chemical composition of the essential oil was analysed by GC/FID and GC/MS. *In vitro* cytotoxic activity of the essential oil was determined on cultured tumour cells. *In vivo* antitumour activity was assessed in Sarcoma 180-bearing mice. The major compounds identified were (*E*)-caryophyllene (31.48%), bicyclogermacrene (15.13%), germacrene D (9.66%), δ-cadinene (5.44%), viridiflorene (5.09%) and α-copaene (4.35%). *In vitro* study of the essential oil displayed cytotoxicity on tumour cell lines and showed IC_{50} values ranging from 24.6 to 40.0 μg/ml for the NCI-H358M and PC-3M cell lines, respectively. In the *in vivo* antitumour study, tumour growth inhibition rates were 31.0–37.5%. In summary, the essential oil was dominated by sesquiterpene constituents and has some interesting anticancer activity.

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1. Introduction

Xylopia frutescens Aubl. (Annonaceae) is a medicinal plant found in Central and South America, Africa and Asia (Braga, 1976). In Brazil, it is popularly known as "embira", "embira-vermelha" and "pau carne", and its seeds are used in folk medicine as a bladder stimulant, to trigger menstruation, and to combat rheumatism, halitosis, tooth decay and intestinal diseases (Correa, 1984; Takahashi, Boaventura, Bayma, & Oliveira, 1995). The seeds have an acrid, aromatic taste and are used instead of pepper in Guyana. In Panama, its leaves are used to treat fever (Joly et al., 1987). Studies examining the biological properties of *X. frutescens* have demonstrated antibacterial, antifungal, anti-viral, antiplasmodial and anti-inflammatory activities (Braga, Wagner, Lombardi, & Oliveira, 2000; Fournier et al., 1994; Jenett-Siems, Mockenhaupt, Bienzle, Gupta, & Eich, 1999; Matsuse, Lim, Hattori, Correa, & Gupta, 1999).

Numerous studies have demonstrated anticancer activity for the essential oils obtained from medicinal plants (Asekun & Adeniyi, 2004; Britto et al., 2012; Quintans et al., 2013; Ribeiro et al., 2012; Sœur et al., 2011). In this work, we evaluated the chemical composition and *in vitro* and *in vivo* anticancer effects of the leaf essential oil of *X. frutescens.*

2. Materials and methods

2.1. Cells

The cytotoxicity assay was performed using OVCAR-8 (ovarian adenocarcinoma), NCI-H358M (bronchoalveolar lung carcinoma) and PC-3M (metastatic prostate carcinoma) human tumour cell lines, all obtained from the National Cancer Institute, Bethesda, MD. Cells were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were maintained at 37 °C in a 5% CO_2 atmosphere.

Sarcoma 180 tumour cells, which had been maintained in the peritoneal cavity of Swiss mice, were obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará, Brazil.

2.2. Animals

A total of 40 Swiss mice (males, 25–30 g), obtained from the central animal house at the Federal University of Sergipe, Brazil,

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http://dx.doi.org/10.1016/j.foodchem.2013.02.114

were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12:12 h light–dark cycle (lights on at 6:00 a.m.). Animals were treated according to the ethical principles for animal experimentation of SBCAL (Brazilian Association of Laboratory Animal Science), Brazil. The Animal Studies Committee at the Federal University of Sergipe approved the experimental protocol (number 08/2012).

2.3. Plant material

X. frutescens leaves were collected in July 2011 at "Mata do Junco" in the Municipality of Capela, Sergipe State, Brazil, coordinates: S 10° 57′ 52″ W 37° 04′ 65″. The species was identified by Dr. Ana Paula do Nascimento Prata. Voucher specimen number 22178 was deposited at the Herbarium of the Federal University of Sergipe, Brazil. The leaves were obtained from plants that were flowering and in fructification.

2.4. Hydrodistillation of the volatile constituents

X. frutescens leaves (200 g) were dried in an oven with circulating air at 40 °C for 24 h and submitted to hydrodistillation for 4 h using a Clevenger-type apparatus (Amitel, São Paulo, Brazil). The essential oil was dried over anhydrous sodium sulfate and the percentage content (v/w) was calculated on the basis of the dry weight of plant material. The essential oils were stored in a freezer until analysis. Hydrodistillation was performed in triplicate.

2.5. GC analysis

GC analyses were carried out using a Shimadzu GC-17A fitted with a flame ionisation detector (FID) and an electronic integrator (Shimadzu, Kyoto, Japan). Separation of the compounds was achieved using a ZB-5MS fused capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Phenomenex, Torrance, CA). Helium was the carrier gas at 1.0 ml/min flow rate. The column temperature program was: 40 °C for 4 min, a rate of 4 °C/min to 240 °C, then a rate of 10 °C/min to 280 °C, and then 280 °C for 2 min. The injector and detector temperatures were 250 °C and 280 °C, respectively. Samples (10 mg/ml in CH_2Cl_2) were injected with a 1:50 split ratio. The injection volume was 0.5 μl. Retention indices were generated with a standard solution of *n*-alkanes (C_8–C_{20}). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without FID response factor correction.

2.6. GC/MS analysis

GC/MS analyses were performed on a Shimadzu QP5050A GC/MS system equipped with an AOC-20i auto-injector. A J&W Scientific DB-5MS fused capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Agilent, Santa Clara, CA) was used as the stationary phase. MS data were taken at 70 eV with a scan interval of 0.5 s from *m*/*z* 40 to 500. All other conditions were similar to the GC analysis.

2.7. Identification of constituents

Essential oil components were identified by comparing the retention times of the GC peaks with standard compounds run under identical conditions and by comparison of retention indices (Van Den Dool & Kratz, 1963) and mass spectra (Adams, 2007) with those found in the literature, and by comparison of mass spectra with those stored in the NIST 107 and NIST 21, and Wiley 229 libraries.

2.8. In vitro cytotoxic activity assay

Tumour cell growth was determined by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a purple formazan product, as described by Mossman (1983). For all experiments, cells were seeded in 96-well plates (0.7×10^5 cells/ml for adherent cells or 0.3×10^6 cells/ml for suspended cells in 100 μl of medium). After 24 h, the drugs (0.78–50 μg/ml) were dissolved in pure DMSO and added to each well using HTS – High-Throughput Screening (Biomek 3000, Beckman Coulter Inc., Fullerton, CA). Then, the cells were incubated for 72 h. Doxorubicin (purity > 98%; Sigma Chemical Co., St. Louis, MO) was used as positive control. At the end of incubation, the plates were centrifuged, and the medium was replaced by fresh medium (150 μl) containing 0.5 mg/ml MTT. Three hours later, the formazan product was dissolved in 150 μl DMSO, and absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter Inc.). The drug effects were expressed as the percentage of control absorbance of reduced dye at 595 nm.

2.9. In vivo antitumour activity assay

The *in vivo* antitumour effect was evaluated using Sarcoma 180 ascites tumour cells, following protocols previously described (Bezerra et al., 2006, 2008; Britto et al., 2012). Ten-day-old Sarcoma 180 ascites tumour cells (2×10^6 cells per 500 μl) were implanted subcutaneously into the left hind groin of mice. The essential oil was dissolved in 5% DMSO and given to mice intraperitoneally once a day for 7 consecutive days. At the beginning of the experiment, the mice were divided into four groups of 8–15 animals as follows: Group 1: animals treated by injection of vehicle 5% DMSO ($n = 15$); Group 2: animals treated by injection of 5-fluorouracil (5-FU, purity > 99%; Sigma Chemical Co.) (25 mg/kg/day) ($n = 9$); Group 3: animals treated by injection of the essential oil (50 mg/kg/day) ($n = 8$); Group 4: animals treated by injection of the essential oil (100 mg/kg/day) ($n = 8$). The treatments were started one day after tumour injection. The dosages were determined based on previous articles. On Day 8, the animals were sacrificed by cervical dislocation, and the tumours were excised and weighed. The drug effects were expressed as the percent inhibition of control.

Body mass loss, organ weight alterations and haematological analysis were determined at the end of the above experiment, as described by Britto et al. (2012). Peripheral blood samples of the mice were collected from the retro-orbital plexus under light ether anaesthesia, and the animals were sacrificed by cervical dislocation. After sacrifice, the livers, kidneys and spleens were removed and weighed. In haematological analysis, total leukocyte counts were determined by standard manual procedures using light microscopy.

2.10. Statistical analysis

Data are presented as mean ± SEM/SD or half maximal inhibitory concentration (IC_{50}) values and their 95% confidence intervals (CI 95%) obtained by nonlinear regression. The differences between experimental groups were compared by ANOVA (analysis of variance) followed by the Student–Newman–Keuls test ($p < 0.05$). All statistical analyses were performed using the GraphPad program (Intuitive Software for Science, San Diego, CA).

3. Results and discussion

Hydrodistillation of *X. frutescens* leaves gave a colourless crude essential oil with a yield of 1.00 ± 0.09%, in relation to the dry

weight of the plant material. As shown in Table 1, it was possible to identify 34 compounds according to GC/MS and GC/FID analysis. The major compounds identified were (*E*)-caryophyllene (31.48%), bicyclogermacrene (15.13%), germacrene D (9.66%), δ-cadinene (5.44%), viridiflorene (5.09%) and α-copaene (4.35%). Some phytochemical studies on the stem bark and fruit from *X. frutescens* have been previously reported (Fournier et al., 1994; Leboeuf, Cave, Provost, Forgacs, & Janquemin, 1982; Melo, Cota, Oliveira, & Braga, 2001; Rocha, Silva, & Panizza, 1980; Sena-Filho, Duringer, Craig, & Schuler, 2008; Takahashi et al., 1995). Particularly, germacrene D (24.2%), linalool (12.1%), β-pinene (8.0%), *cis*-sabinene hydrate (7.9%), *trans*-pinocarveol (7.8%), α-copaene (7.0%) and limonene (5.6%) were the major compounds identified in *X. frutescens* fruits (Sena-Filho et al., 2008). α-Cubebene (25.2%) and δ-cadinol (27.4%) were the compounds identified in its stem bark (Fournier et al., 1994).

In genus *Xylopia*, bicyclogermacrene (36.5%), spathulenol (20.5%) and limonene (4.6%) were found in leaf essential oil of *Xylopia aromatica*. *Xylopia cayennensis* was composed of α-pinene (29.2%), β-pinene (16.5%), caryophyllene oxide (14.5%), bicyclogermacrene (14.5%), germacrene D (4.7%) and 1,8-cineole (4.5%). *Xylopia emarginata* was dominated by spathulenol (73.0%). For *Xylopia nitida*, γ-terpinene (44.1%), *p*-cymene (13.7%), α-terpinene (12.6%) and limonene (11.3%) were identified (Maia et al., 2005). In another study with leaf essential oil of *X. aromatica* the major compounds were α-pinene (26.1%), limonene (22.3%), bicyclogermacrene (20.4%) and β-pinene (19.0%) (Lago et al., 2003). The essential oil of *Xylopia sericea* contained cubenol (57.4%) and α-epi-muurolol (26.1%) as the main compounds found in the leaves, while β-pinene (45.6%) and α-pinene (17.2%) were the main compounds found in the fruits (Pontes et al. (2007). Tavares et al. (2007) investigated the chemical constituents from leaves of *Xylopia langsdorffiana* and observed that the major compounds were germacrene D (22.9%), *trans*-β-guaiene (22.6%), (*E*)-caryophyllene (15.7%) and α-pinene (7.3%).

Quintans et al. (2013) analysed the chemical composition of three specimens of *X. laevigata* and observed that γ-muurolene (0.60–7.99%), δ-cadinene (1.15–13.45%), germacrene B (3.22–7.31%), α-copaene (3.33–5.98%), germacrene D (9.09–60.44%), bicyclogermacrene (7.00–14.63%) and (*E*)-caryophyllene (5.43–7.98%) were the major constituents in all samples of the essential oils. Although some chemical constituents present in the leaf oil of *X. frutescens* have been found in the essential oils from other Brazilian *Xylopia* species, recent studies as described above have demonstrated significant variations in the essential oils from the various species belonging to this genus. However, (*E*)-caryophyllene, bicyclogermacrene, germacrene D and α- and β-pinene, present in high concentration in most of the species investigated, appear to be the main compounds in the essential oil from the Brazilian *Xylopia* species.

Cytotoxicity was assessed against OVCAR-8 (ovarian adenocarcinoma), NCI-H358M (bronchoalveolar lung carcinoma) and PC-3M (metastatic prostate carcinoma) human tumour cell lines using the thiazolyl blue test (MTT) assay. Table 2 shows the obtained IC_{50} values. The essential oil showed IC_{50} values ranging from 24.6 to 40.0 μg/ml for the NCI-H358M and PC-3M cell lines, respectively. Doxorubicin, used as positive control, showed IC_{50} values from 0.9 to 1.6 μg/ml for the NCI-H358M and PC-3M cell lines, respectively. According to Suffness and Pezzuto (1990), those extracts presenting IC_{50} values below 30 μg/ml in tumour cell line assays are considered promising for anticancer drug development. Thus, the essential oil obtained from *X. frutescens* presented promising results. Interestingly, cytotoxic activities have also been reported for the essential oils from some plants belonging to the *Xylopia* species, such as *X. aethiopica* (Asekun & Adeniyi, 2004). These effects have been associated with a mixture of the major and minor constituents of these essential oils.

The leaf essential oil of *X. frutescens* was also able to inhibit tumour growth in mice in a dose-dependent manner. In the *in vivo* antitumour study, mice were subcutaneously transplanted with Sarcoma 180 cells and treated by the intraperitoneal route once a day for 7 consecutive days with the essential oil. The effects of the essential oil on mice implanted with Sarcoma 180 tumour cells are presented in Fig. 1. On Day 8, the average tumour weight of the control mice was 1.93 ± 0.13 g. In the presence of the essential oil (50 and 100 mg/kg/day), the average tumour weights were 1.33 ± 0.19 and 1.20 ± 0.10 g, respectively. Tumour growth inhibition rates were 31.0–37.5%. 5-FU (25 mg/kg/day), used as positive control, reduced tumour weight by 63.2%.

Table 1
Chemical composition of the leaf essential oil of *Xylopia frutescens*.

Compound		RI[a]	RI[b]	Leaf oil%
1	(*E*)-β-Ocimene	1047	1044	0.41 ± 0.70
2	α-Cubebene	1348	1345	0.92 ± 0.03
3	α-Ylangene	1370	1373	0.64 ± 0.03
4	α-Copaene	1377	1374	4.35 ± 0.27
5	β-Elemene	1390	1389	0.93 ± 0.20
6	α-Gurjunene	1408	1409	0.47 ± 0.04
7	(*E*)-Caryophyllene	1422	1417	31.48 ± 1.47
8	β-Copaene	1431	1430	0.53 ± 0.05
9	Aromadendrene	1440	1439	3.21 ± 0.28
10	*trans*-Muurola-3,5-diene	1450	1451	0.40 ± 0.03
11	α-Humulene	1457	1452	2.60 ± 0.21
12	*allo*-Aromadendrene	1461	1458	0.74 ± 0.03
13	*cis*-Cadina-1(6),4-diene	1464	1461	0.15 ± 0.03
14	γ-Muurolene	1476	1478	3.26 ± 0.25
15	Germacrene D	1483	1484	9.66 ± 2.18
16	Viridiflorene	1492	1496	5.09 ± 0.46
17	Bicyclogermacrene	1497	1500	15.13 ± 2.44
18	δ-Amorphene	1504	1511	0.19 ± 0.17
19	γ-Cadinene	1514	1513	1.69 ± 0.25
20	δ-Cadinene	1519	1522	5.44 ± 0.91
21	*cis*-Calamenene	1523	1528	0.79 ± 0.06
22	*trans*-Cadina-1,4-diene	1534	1533	0.53 ± 0.10
23	α-Cadinene	1538	1537	0.32 ± 0.08
24	Germacrene B	1561	1559	0.40 ± 0.12
25	(*Z*)-Dihydro-apofarnesal	1573	1571	0.45 ± 0.07
26	Spathulenol	1579	1577	1.35 ± 0.27
27	Caryophyllene oxide	1585	1582	0.61 ± 0.02
28	Globulol	1588	1590	1.36 ± 0.50
29	Viridiflorol	1597	1592	0.19 ± 0.33
30	Rosifoliol	1609	1600	0.54 ± 0.15
31	1-*epi*-Cubebol	1630	1627	0.45 ± 0.10
32	*epi*-α-Cadinol	1644	1638	0.97 ± 0.50
33	α-Muurolol	1648	1644	0.24 ± 0.25
34	α-Cadinol	1658	1652	1.00 ± 0.68
	Monoterpene identified			0.41
	Sesquiterpene identified			96.10
	Total identified			96.51

Data are expressed as mean ± SD of three analyses. RI (retention indices).
[a] Calculated on DB-5MS column according to Van Den Dool and Kratz (1963), based on a homologous series of normal alkanes.
[b] According to Adams (2007).

Table 2
In vitro cytotoxic activity of the leaf essential oil of *Xylopia frutescens*.

Cell lines	Histotype	Doxorubicin	Essential oil
OVCAR-8	Ovarian adenocarcinoma	1.2	33.9
		0.9–1.6	24.9–46.3
NCI-H358M	Bronchoalveolar lung carcinoma	0.9	24.6
		0.6–1.3	14.9–40.7
PC-3M	Metastatic prostate carcinoma	1.6	40.0
		1.1–2.4	31.3–51.2

Data are presented as IC_{50} values in μg/ml and their 95% confidence interval obtained by nonlinear regression from two independent experiments performed in duplicate, measured by MTT assay after 72 h of incubation. Doxorubicin was used as positive control.

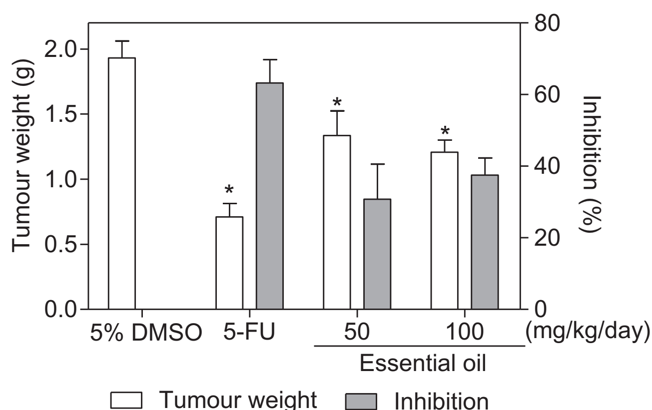

Fig. 1. *In vivo* antitumour effect of the leaf essential oil of *Xylopia frutescens*. Mice were injected with Sarcoma 180 tumour cells (2.0×10^6 cells/animal, s.c.). The animals were treated by intraperitoneal administration for seven consecutive days, starting one day after tumour implantation. 5-Fluorouracil (5-FU, 25 mg/kg/day) was used as positive control. Negative control was treated with the vehicle used for diluting the tested substance (5% DMSO). Data are presented as mean ± SEM of 8–15 animals. $^*p < 0.05$ compared with the 5% DMSO group.

Systemic toxicological parameters were also examined in essential oil-treated mice using the experimental protocol described above. Table 3 shows the obtained data. No significant changes in the weight of livers, kidneys or spleens were seen in the essential oil-treated groups ($p > 0.05$). No significant changes in body weight gain were observed either ($p > 0.05$). In addition, essential oil-treated animals showed a significant increase in total numbers of circulating peripheral leukocytes, compared to the control group ($p < 0.05$). These results indicate that the essential oil increased the cell types involved in the primary defence mechanism. In contrast, 5-FU, used as positive control, reduced the body weights and spleen organ weights and induced a decrease in total leukocytes ($p < 0.05$).

In conclusion, the leaf essential oil of *X. frutescens* is characterised by the presence of (*E*)-caryophyllene, bicyclogermacrene, germacrene D, δ-cadinene, viridiflorene and α-copaene. In addition, it exhibited *in vitro* and *in vivo* anticancer effects without an expressive toxicity. Further studies must be carried out to better understand the underlying mechanism involved in the anticancer activity of this essential oil.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This work was financially supported by Capes (Coordenadoria de Apoio a Pesquisa e Ensino Superior), CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico), FUNCAP (Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico) and FAPITEC/SE (Fundação de Amparo à Pesquisa e à Inovação Tecnológica do Estado de Sergipe).

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Table 3
Systemic toxicological assessment of the leaf essential oil of *Xylopia frutescens*.

Parameters	Treatments			
	5% DMSO	5-FU	Essential oil	
			50	100
Increase in body weight (g)	2.60 ± 0.85	−1.40 ± 1.03*	2.37 ± 1.70	2.25 ± 1.59
Liver (g/100 g body weight)	5.45 ± 0.40	5.10 ± 0.32	5.64 ± 0.27	5.75 ± 0.27
Spleen (g/100 g body weight)	0.65 ± 0.11	0.43 ± 0.08*	0.60 ± 0.05	0.73 ± 0.05
Kidney (g/100 g body weight)	1.44 ± 0.09	1.42 ± 0.08	1.36 ± 0.06	1.47 ± 0.06
Total leukocytes ($\times 10^3$ cells/μl)	10.9 ± 0.96	6.72 ± 0.84*	9.80 ± 0.78	19.5 ± 4.98*
Neutrophil (%)	38.6 ± 4.34	25.8 ± 6.10	34.0 ± 3.28	44.2 ± 4.29
Lymphocyte (%)	52.4 ± 5.19	68.2 ± 4.31	34.0 ± 3.28	56.8 ± 4.18
Eosinophil (%)	1.00 ± 0.44	1.80 ± 0.48	3.60 ± 1.20	0.80 ± 0.37
Monocyte (%)	8.00 ± 1.37	4.20 ± 2.49	9.00 ± 2.55	0.80 ± 0.37

Mice were implanted with Sarcoma 180 tumour cells (2.0×10^6cells/animal, s.c.). The animals were treated by intraperitoneal administration for seven consecutive days, starting one day after tumour implantation. 5-Fluorouracil (5-FU, 25 mg/kg/day) was used as positive control. Negative control was treated with the vehicle used for diluting the tested substance (5% DMSO). Data are presented as mean ± SEM. $^*p < 0.05$ compared with the 5% DMSO group by ANOVA followed by Student–Newman–Keuls.

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