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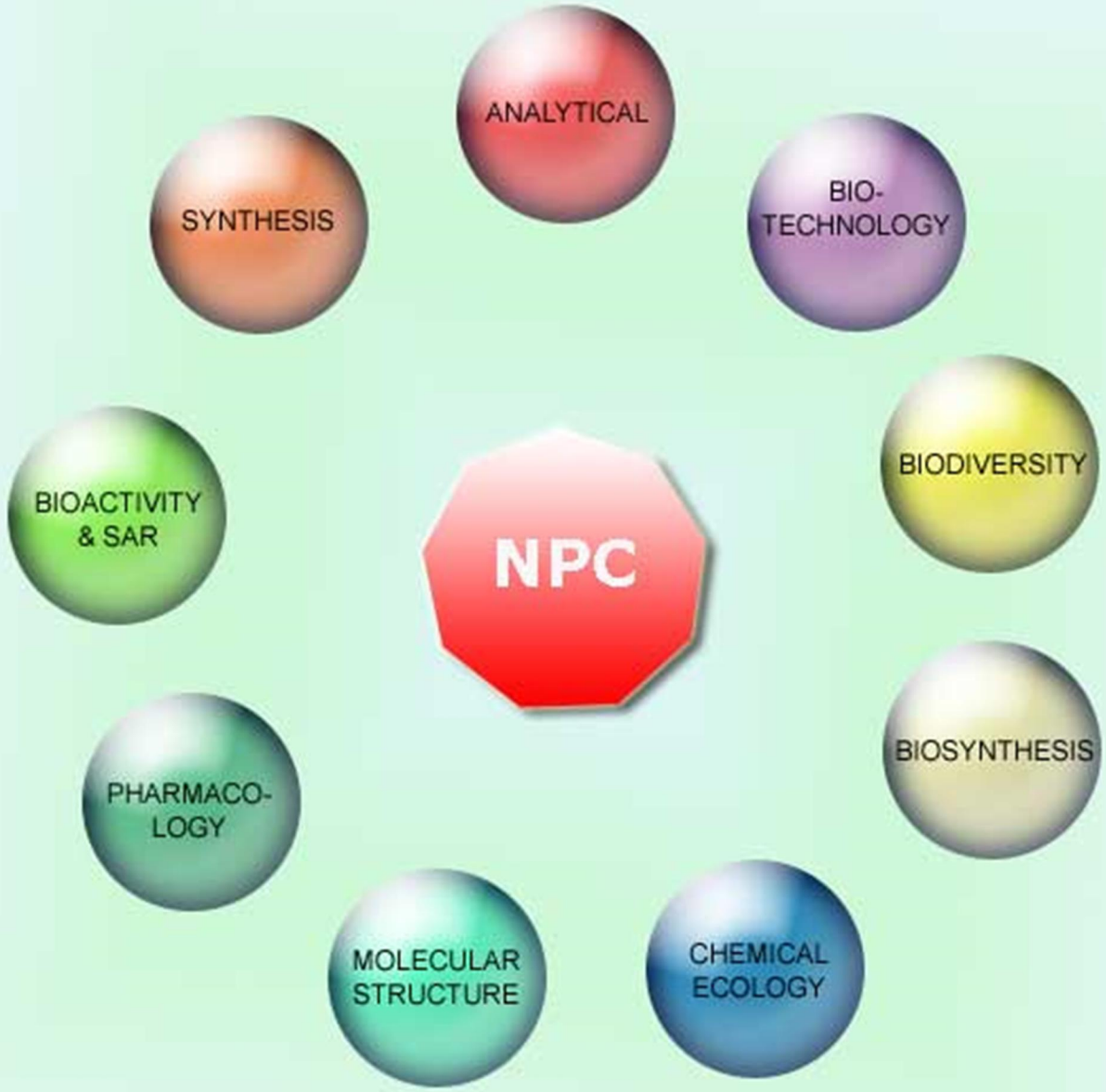

Volume 11. Issue 3. Pages 273-430. 2016
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2016
Vol. 11
No. 3
371 - 374

Cytotoxic Compounds from Aerial Organs of *Xanthium strumarium*

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Received: April 24th, 2015; Accepted: December 7th, 2015

Xanthium strumarium L., the main species of the genus *Xanthium*, is ubiquitously distributed. The aim of this study was to determine the cytotoxic effect of aerial organs of *X. strumarium*, grown in Cuba, against cancer cell lines and the isolation of compounds potentially responsible for this activity. Initially, an ethanol partitioning procedure yielded the XSE extract that was subsequently fractionated with chloroform resulting in a XSCF fraction. Both, XSE and XSCF fractions exhibited cytotoxic effects on MDA MB-231, MCF7, A549 and CT26 cell lines by using the MTT assay. Above all, the XSCF fraction was more active than XSE. For this reason, XSCF was subsequently fractionated by silica gel chromatography and the active fractions submitted to semi-preparative HPLC for isolation of bioactive compounds. Six sub-fractions (SF1 to SF6) were recovered. Sub-fractions 3 and 6 were the most active on each assayed cell line, while sub-fractions 4 and 5 were only active against A549 and CT26 cell lines. In each case, sub-fraction 6 showed the strongest inhibitory effect. The HPLC-DAD fingerprint of sub-fraction 6 showed a single peak that was identified by GC-MS as (-) spathulenol, a sesquiterpene with reported antitumor activity.

Keywords: *Xanthium strumarium*, Cytotoxic, Bioassay-guided fractionation, (-) Spathulenol.

The genus *Xanthium* (Asteraceae, Compositae), represented by 25 species, exhibits a global distribution and is ubiquitously found throughout Eurasia and America. This genus has been the subject of several studies that led to the isolation of sesquiterpene lactones (xanthanolides) [1a], terpenoids [1b-1d], thiazinediones [1e, 1f], sterols and caffeoylquinic acids [1g], as the main secondary metabolites. Xanthium species have long been used as traditional herbal medicines in eastern countries. The whole plant has been used to treat bacterial infections, diabetes, skin pruritus and inflammatory diseases such as rhinitis and rheumatoid arthritis [1h].

X. strumarium L. has also been used in folk medicine in Latin-America against different types of cancers [2a]. In alternative Brazilian medicine, *X. strumarium* has been employed to treat dermatitis [1d], and its leaves have been used by various native American tribes to relieve constipation, diarrhea and vomiting [2b]. Several activities have been confirmed by experimental pharmacology in samples of *X. strumarium* collected in different sites: anti-inflammatory, analgesic [2c, 2d], anticancer [2e-2g], antifungal [2h], antiulcerogenic [2i], antimitotic [2j], antitrypanosomal, antimalarial [2k] and diuretic [2l]. The chemical composition of *X. strumarium* has been found to include phenolic compounds, such as chlorogenic and ferulic acids, triterpenoid saponin, and xanthanolide sesquiterpene lactones (8-epi-xanthatin and 8-epi-xanthatin epoxide) [1d].

However, the chemical composition and concentration of these compounds from the same plant species may vary in response to ecological and plant growth regulators [3]. So far, the chemical composition of *X. strumarium* extracts obtained from Cuban plants has not been reported. In a previous report we demonstrated the inhibitory effect of Cuban *X. strumarium* on tubulin polymerization [4]. Due to the importance of antimitotic agents in anticancer therapy, the aim of this study was to determine the cytotoxic effect of extracts obtained from aerial organs from Cuban *X. strumarium* against cancer cell lines and describe the isolation of some compounds potentially responsible for this activity.

In this paper we elucidate the anticancer potential of *X. strumarium* from Cuba through the evaluation of the cytotoxic effect by using four cancer cell lines corresponding to the most prevalent human cancers (colon, breast, and lung) presenting difficult prognosis.

X. strumarium aerial parts (leaves and stems) were extracted with 70% EtOH; the extract (XSE) was further subjected to chloroform solvent-solvent partitioning, resulting in XSCF fraction. Both, XSE and XSCF samples were assayed for their cytotoxic effects against four cancer cell lines. XSCF showed higher cytotoxic activity than XSE in the assayed cell types. Above all, the XSE and XSCF fractions were more active on the CT26 cell line (IC_{50} values, as µg/mL, of 58.9±3.2 and 25.3±1.4, respectively) than on others cell lines; MDA MB 231 (IC_{50} = 61.2±1.0 and 53.3±2.3), MCF7

(IC_{50}=86.1±4.5 and 70.6±0.1) and A549 (IC_{50}=64.2±2.0 and 52.2±3.2). Results are consistent with previous reports using the chloroform fraction from the same source obtained in other locations [2e, 2g, 2n].

For isolation of metabolites with cytotoxic activity, the XSCF was submitted to a silica gel column and the resulting fractions pooled into 5 groups, designated as F1 to F5. The cytotoxicity of the five fractions was measured against A549 and CT26 cell lines. Among them, three fractions viz., F3, F4 and F5 showed high cytotoxic activity against both assayed cells lines (Figure 1A) and F3 was the most active fraction. For this reason, this fraction was further fractionated by HPLC in an attempt to purify the bioactive compound(s). The cytotoxic effect of each obtained sub-fraction (SF1 to SF6) was assayed against each cell line (MDA MB-231, MCF7, A549 and CT26). Sub-fractions 3 and 6 were more active on each cell line assayed, whereas sub-fractions 4 and 5 were only active against A549 and CT26 cells. Sub-fraction 6 had the strongest inhibitory effect, 90%, 70%, 50% and 40% for CT26, A549, MDA MB231 and MCF7, respectively (Figure 1B).

Thirteen chemical species were identified following chemical characterization of fraction F3 by GC–MS (Table 1), among them, benzoic acid, fatty acid derivatives, flavonoids and sesquiterpenic compounds.

The fingerprint by HPLC-DAD of sub-fraction 6 showed a single peak that was identified by GC-MS as (-) spathulenol. The presence of this compound in *X. strumarium* was previously reported [1c, 1d]. In our assaying conditions this sesquiterpene metabolite was responsible for the cytotoxic activity found in the chloroform fraction of *X. strumarium*.

(-)-Spathulenol isolated from leaves of *Dasymaschalon dasymaschalum* exhibited potent cytotoxic activities against human lung cancer cell lines (NCI-H187) with IC_{50} values of 6.6 µg/mL [5a]. Several authors have shown that the essential oils (EO) from many plants enriched in (-) spathulenol exhibit moderate to potent cytotoxic activity against several cancer cell lines, such as: CACO-2 (colorectal cancer), Hep-G2 (human hepatocellular carcinoma), HeLa (human ovarian carcinoma), Bel-7402 (human hepatocellular carcinoma), A-549, MCF-7 and A2780 (ovarian carcinoma) [5b-5e]. Other biological activities have been associated with (-)-spathulenol such as, antitumor, immunomodulatory and antibiotic and as an inhibitor of the human ABCB1 efflux pump [5f, 5g].

The present paper reports for the first time the isolation and identification of (-)-spathulenol, a sesquiterpene metabolite, as a cytotoxic compound of aerial organs of *X. strumarium* grown in Cuba.

Table 1: Compounds identified in fraction F3 obtained from *X. strumarium* chloroform fraction (XSCF) by GC-MS

	Retention time	Relative percentage	Identified compounds (nist2008)
C1	9.84	2.4	Limonene
C2	11.80	7.1	Benzoic acid, methyl ester
C3	13.97	0.9	Benzoic acid, ethyl ester
C4	16.98	0.1	Hexanoic acid, 1,1-dimethyl ester
C5	28.28	3.0	Coniferol (*trans*)
C6	32.78	2.3	Palmitic acid
C7	33.03	2.7	(-) Spathulenol
C8	35.22	9.5	Arachidonic acid, ethyl ester
C9	35.25	1.3	Linoleic acid, methyl ester
C10	41.58	trace	Pinocembrin
C11	41.78	trace	5-Methoxy- 3,7-dihydroxyflavanone
C12	42.10	2.8	Hexadecanoic acid, 2,3-dihydroxypropyl ester
C13	43.16	trace	Naringenin

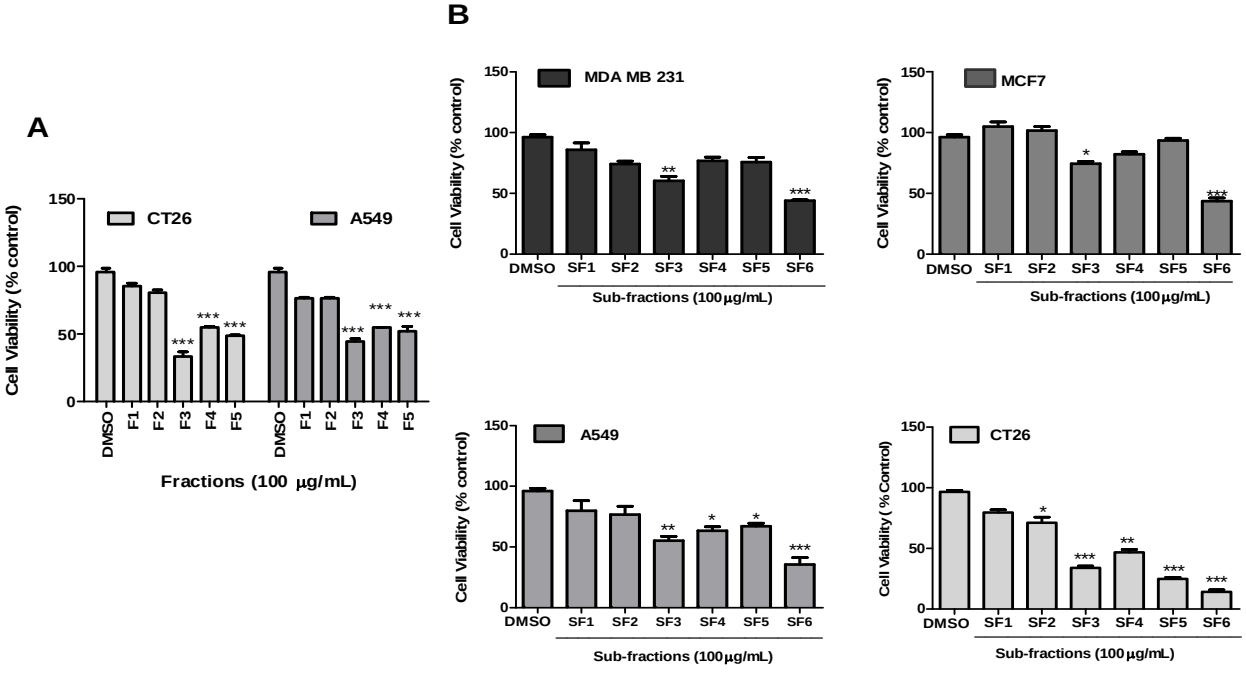

Figure 1: Cytotoxic effects measured with MTT assay of A) fractions (F1-F5) from XSCF on CT26 and A549 cancer cells lines, and B) sub-fractions (SF1 to SF6) obtained from active fraction (F3) of XSCF on MDA MB 231, MCF7, A549 and CT26 cancer cells lines. The cells were treated for 72 h with either fractions or sub-fractions (100 µg/mL). Data are presented as means ± SD in three independent experiments. Significance: *$p < 0.05$, **$p < 0.01$, ***p vs. the negative control.

Experimental

Plant material and extraction: *X. strumarium* was collected at the Medicinal Plants Experimental Station "Dr. Juan Tomás Roig" in Artemisa, Cuba. A voucher specimen, number ROIG 4594, was deposited at the herbarium of this institution. The leaves and stems were dried in a dark place at room temperature. The ground material (500 g) was extracted with 70% ethanol by percolation. The solvent was evaporated under vacuum providing 39 g of total extract (yield, 7.8%). The ethanolic extract (XSE) was re-suspended in distilled water (200 mL) and extracted 3 times with benzene to remove non-polar substances (1:3). The remaining aqueous phase was extracted 3 times with chloroform. The resulting fraction was dried under vacuum and termed the chloroform fraction (XSCF) (1.32 g, 3.38%). Both, the ethanolic and chloroform fractions were kept refrigerated at 4°C until use.

Compound isolation: XSCF was dissolved in MeOH and fractionated through a silica gel column (30 x 1.8 cm) with a stepwise elution gradient composed of chloroform: ethyl acetate: methanol (90:10:0; 80:20:0; 70:30:0; 50:50:0; 40:60:0; 20:80:0; 0:100:0; 0:70:30; 0:50:50; 0:0:50 v/v/v). Twenty fractions were collected and pooled into 5 fractions (F1-F5) based on their TLC mobility profiles (Kieselgel G60 F254 0.2 mm, Merck; mobile phase: chloroform: methanol 9:1, v/v) revealed with Natural Products reagent (NP – 1% methanolic solution of diphenylboric acid aminoethyl ester) and a vanillin-sulfuric acid reagent. Fraction F3 (28 mg), was further chromatographed by semi-preparative HPLC using a Phenomenex Ib-Sil C18 column (10 mm × 250 mm, 5 µm) at 40ºC. Chromatography was carried out in a HPLC-DAD system consisting of Waters 1525 Binary HPLC Pumps with a 1500 Series Column Heater, a manual injection valve with a 50 µL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA). Gradient elution was carried out with water-0.1% acetic acid (solvent A) and methanol (solvent B) at a constant flow of 1.5 mL/min. The solvent composition was as follows. Initial: 50% B, 50% A, changing to 100% B at 50 min by 5 min for a total running time of 55 min. Six sub-fractions/peaks (SF1 - SF6) were obtained and their cytotoxic activities were measured against 4 cancer cell lines: MDAMB-231 (breast), MCF7 (breast), A549 (lung) and CT26 (colon).

The determination of fingerprints of XSCE and each fraction obtained from the silica column was carried out using a HPLC-DAD system consisting of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, a manual injection valve with a 20 µL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA). An XBridgeTM C18 column (4.6 mm × 150 mm, 5 µm; Waters Corporation, Milford, MA) was used as stationary support. Compounds were monitored at 280 nm and 330 nm. Gradient elution was carried out with 0.1% aqueous acetic acid (solvent A) and methanol- 0.1% acetic acid (solvent B) at a constant flow of 0.5 mL/min. The gradient program was as follows: Initially, 10% B, 90% A, changing to 57% B at 45 min and to 100% B at 60 min, and 5 min before returning to the initial condition. The concentration of injected sample was 3 mg/mL. Data collection was carried out with EmpowerTM 2 software.

Compounds identification by gas chromatography/mass spectrometry (GC-MS) analyses: GC-MS analyses were performed on a Trace GC and Polaris Q ion trap mass spectrometer (Thermoelectron) with Xcalibur 1.4 SR1 software.

HPLC sub-fractions/peaks obtained from fraction F3 were dissolved in deuterated chloroform for this analysis. The MS detection was performed in the positive ion mode using mass acquisition between 40-500 amu. The temperature program was: 60°C for 3 min, then increased at a rate of 5°C/min to 280°C and the final temperature was held for 10 min. The split-splitless injection port was set at 280°C in the split mode at a split ratio of 1:10. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. Compounds were identified by comparison with profiles from literature in the NIST 2008 library and by injection using authentic standards, when available.

Screening for cytotoxicity of the total extract, chloroform fraction and sub-fraction from X. strumarium: A stock solution of 2 mg/mL of XSE and XSCF and 1 mg/mL of sub-fractions/isolated compounds were re-suspended in dimethyl sulfoxide (DMSO) for cytotoxic evaluation. The highest concentration of extracts, fractions and compounds tested was 100 µg/mL, as recommended by the NCI's Developmental Therapeutics Program protocol for prescreening of natural products [6].

Cell lines: Four tumor cell lines were used to screen plant extracts for anticancer effects: MDA MB-231 (breast), MCF7 (breast), A549 (lung) and CT26 (colon). MCF7, MDA MB-231 and A549 cells were grown in DMEM medium and CT26 in RPMI 1640 with L-glutamine (Sigma), containing 10% FBS (Sigma) and 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma), in either T-75 cm^2 or T-25 cm^2 tissue culture flasks and cultured at 37°C in a humidified incubator containing 5% CO_2. The medium was changed twice a week. Cell stocks were stored in liquid nitrogen for prolonged preservation.

MTT cytotoxicity assay: The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cytotoxicity assay was carried out in accordance with the protocol described by Mosmann [7] with some modifications. In each well of a culture microplate $1x10^4$ cells were added. After being cultured overnight, cells were exposed to the extract, the chloroform fraction or the isolated compounds for 72 h. At the end of this period, the cells were incubated with MTT (5 mg/mL) for 4 h. The plates were read in a microplate spectrophotometer (Omega) at 550 nm. The samples IC_{50} values were determined from a dose–response curve obtained by using 5 different sample concentrations (6.25, 12.5, 25, 50, and 100 µg/mL). Analyses were made in triplicate for each condition.

Statistical analysis: All data were expressed as means ± SD. The data were analyzed by a one-way ANOVA. Mapping used GraphPad Prism 5 (GraphPad Software, San Diego, CA). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered statistically significant.

Acknowledgments - The authors acknowledge the financial support from Ministerio de Ciencia y Tecnología (MINCyT), Argentina, Ministerio de Ciencia Tecnología y Medio Ambiente (CITMA) and Centro de Investigación y Desarrollo de Medicamentos (CIDEM) Cuba. Consejo de Investigación de la Universidad Nacional de Tucumán (CIUNT), Argentina, and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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2016
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