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ORIGINAL

In vitro antitumor effect of essential oil of leaves from *ñandypa (Genipa americana L.)* on human pancreatic carcinoma cell lines (MIA PaCa2)

Efecto antitumoral in vitro del aceite esencial de *ñandypa (Genipa americana L.)* en líneas celulares de carcinoma pancreático humano (MIA PaCa2)

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ABSTRACT

Introduction: South American biodiversity offers a source of new therapeutic compounds. Cancer remains a major global health issue with high morbidity and mortality. *ñandypa* (*Genipa americana L.*), a medicinal plant with various reported properties, was tested for citotoxic activity of the essential oil from their leaves. **Objective:** to determine the in vitro antitumor effect of an essential oil derived from *ñandypa* on human pancreatic carcinoma cell lines (MIA PaCa2). **Methodology:** Essential oils from *G. americana* leaves were obtained via hydrodistillation, and their chemical composition was analyzed using Gas Chromatography-Flame Ionization Detection-Mass Spectrometry (GC-FID-MS). Pancreatic tumor cells and normal fibroblast cells were then tested for antitumor activity. The cells were treated with serial dilutions of the essential oil (0.1%, 0.5%, 1%, 1.5%, 5%, and 10%). Cell viability was measured using an MTT assay, with results read on a spectrophotometer at 570nm and 630nm. **Results:** GC-FID-MS analysis revealed the oil's primary components were alcohols (17.83%), aldehydes (9.58%), and esters (5.18%). Exposure to the oil for four hours resulted in a high rate of tumor cell mortality, with the effect being more pronounced at higher concentrations. Importantly, the oil was not found to be toxic to normal cells (MRC-5). **Conclusion:** The essential oil of *Genipa americana L.* exhibits significant, dose-dependent antitumor activity against human pancreatic carcinoma cells (MIA PaCa-2). Notably, it showed no toxicity toward normal human fibroblast cells (MRC-5). These findings, supported by the presence of bioactive compounds like BHT, α-pinene, and β-caryophyllene, suggest its potential as a selective therapeutic agent for pancreatic cancer.

Keywords: phytoteraphy; *Genipa americana L.*; MRC-5 cells; antitumoral; essential oil.

RESUMEN

Introducción: La biodiversidad sudamericana ofrece una fuente de nuevos compuestos terapéuticos. El cáncer sigue siendo un importante problema de salud global, con alta morbilidad y mortalidad. El *ñandypa* (*Genipa americana L.*), una planta medicinal con diversas propiedades reportadas, fue evaluado por su actividad citotóxica mediante el aceite esencial obtenido de sus hojas. **Objetivo:** Determinar el efecto antitumoral in vitro de un aceite esencial derivado de *ñandypa* sobre líneas celulares de carcinoma pancreático humano (MIA PaCa-2). **Metodología:** Los aceites esenciales de hojas de *G. americana* se obtuvieron mediante hidrodestilación, y su composición química se analizó mediante Cromatografía de Gases con Detector de Ionización de Flama acoplada a Espectrometría de Masas (GC-FID-MS). Posteriormente, se evaluó la actividad antitumoral en células tumorales pancreáticas y en fibroblastos normales. Las células se trataron con diluciones seriadas del aceite esencial (0,1%, 0,5%, 1%, 1,5%, 5% y 10%). La viabilidad celular se midió mediante el ensayo MTT, y la lectura se realizó en un espectrofotómetro a 570 nm y 630 nm. **Resultados:** El análisis por GC-FID-MS reveló que los componentes principales del aceite fueron alcoholes (17,83%), aldehídos (9,58%) y ésteres (5,18%). La exposición al aceite durante cuatro horas produjo una alta tasa de mortalidad de células tumorales, con un efecto más marcado a concentraciones más altas. De manera importante, no se encontró toxicidad del aceite sobre células normales (MRC-5). **Conclusión:** El aceite esencial de *Genipa americana L.* presenta una actividad antitumoral significativa y dependiente de la dosis contra células de carcinoma pancreático humano (MIA PaCa-2). Además, no mostró toxicidad sobre fibroblastos humanos normales (MRC-5). Estos hallazgos, respaldados por la presencia de compuestos bioactivos como BHT, α-pineno y β-cariofileno, sugieren su potencial como agente terapéutico selectivo para el cáncer de páncreas.

Palabras clave: Fitoterapia; *Genipa americana L.*; células MRC-5; antitumoral; aceite esencial.

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INTRODUCTION

Although rare, pancreatic cancer has a poor prognosis and is the fourth leading cause of cancer mortality in developed countries (1). In the United States, it accounts for 3% of all cancers and 7% of cancer deaths (2). In Latin America and the Caribbean, it has an incidence of 7.5% (3), and is the 7th most common cancer in Brazil (4). In Paraguay, it ranks 4th in men and 5th in women (5).

Cancer treatment relies on surgery, radiotherapy, and chemotherapy, often in combination (6). While surgical removal is the only curative option, other alternatives exist for unresectable tumors (7). Gemcitabine is a common chemotherapy drug for pancreatic cancer (9,10), but many patients develop resistance, leading to treatment failure (11). Therefore, the search for new, less invasive chemotherapeutic agents is essential.

One of the sources of new drugs is plants, in Paraguay, native plants typically have more ethnopharmacological than scientific background; for example, one of the plants with multiples therapeutic uses is the *ñandypa* (*Genipa americana L.*) that is a tree widely distributed in the American tropics, popularly referred in Paraguay as hypocholesterolemic (12). Native tribes have long used its leaves for various therapeutic effects, including antiseptic, antifungal, antidiarrheal, depurative, weight-reducing, and cholesterol-lowering activities, treatment of cancer, diabetes, gastrointestinal, renal, uterine, and ovarian disorders, as well as serving as anthelmintic and antibiotic agents (13–22). Considering that a substantial proportion of phytochemical compounds is common to both foliar and carpological tissues, Genipin (GNP), a natural derivative in fruits and leaves of *Genipa americana L.* (*G. americana L.*), is a highly biocompatible cross-linker with 10-fold lower cytotoxicity than glutaraldehyde. It effectively reacts with proteins and polysaccharides to enhance their physical and emulsifying properties. Beyond its structural utility, GNP exhibits proven medicinal benefits, including anti-inflammatory, antibacterial, and antioxidative effects.As the primary bioactive compound, it mediates the anti-inflammatory and hepatoprotective properties observed in these plant extracts (23-26). Scientific studies have confirmed the presence of active compounds like terpenes, steroids, and flavonoids, especially, HPLC of the *G. americana L*. leaf aqueous extract confirmed the presence of flavonoids and iridoids, supplementing the initial phytochemical finding of high-intensity phenolic compounds, flavonoids, and triterpenes (27–31). In the chronic epilepsy model (kindling), the polysaccharide-rich extract of *G. americana L.* leaves demonstrated a potent neuroprotective effect by significantly inhibiting inflammation (reducing TNF-α) and malondialdehyde (MDA), reversing oxidative stress, and reducing glial fibrillar acid proteins (GFAP and Iba-1) in the hippocampus (32).

Given its widespread availability, low toxicity, and historical medicinal use, *G. americana L.* is a promising source for new drugs and due to the lack of studies regarding this specific oil-based pharmaceutical dosage form derived from its leaves. The limited success and side effects of current chemotherapy treatments (33) highlight the need for new, effective alternatives. This study aims to evaluate the in vitro antitumor activity of *G. americana L.* essential oil on human pancreatic carcinoma cells (MIA PaCa-2).

METHODOLOGY

G. americana L. leaves samples were collected from the Itaipu Binacional Environmental Center located on the Superhighway in the city of Hernandarias - Paraguay (with coordinates - 25.446729484527378, -54.63425964110454). Samples of leaves, flowers, and fruits were sent to the botany laboratory of the National University of Asunción, where they were identified by the botanist and stored in the university's herbarium.

The leaves were dried at room temperature for 4 days. Approximately 186.63 g of ground dried leaves were extracted by hydrodistillation for 3 h in a Clevenger-type apparatus. The essential oil sample were extracted using ethyl ether, the organic phases were dried with anhydrous Na2SO4, filtered, and evaporated under vacuum and at low temperature, yielding 0.198 g considering the dry mass of starting material (34,35).

The essential oil sample were performed on a Shimadzu GCMS-QP2010 Plus Gas Chromatograph with Rtx- 5MS non-polar column (30 m x 0.25 mm x 0.25 µm and the following analytical conditions were used:1/20 split ratio and the temperature requirements: 280 °C (interface), 250 °C (ion source), and 250 °C (injector). During the first 5 minutes, the oven temperature was kept at 60°C, gradually increasing the temperature (3°C/min) until the desired temperature of 240 °C was obtained. For component identification, a series of n-alkanes (C8-C19) was used by comparing the calculated relative retention indices with mass spectra from the NIST library spectrometer database, contrasted with data available in the literature (36).

Quantitative analyses were performed using a

Shimadzu 2010 Gas Chromatograph with flame ionization detector (GC-FID) and an OV-5 column (30 m × 0.25 mm × 0.25 µm). The carrier gas helium at a flow rate of 1mL/min, split ratio of 1/20, sample diluted in ethyl ether with an injection volume of 1 µL, with the injector at 250 °C and detector at 280 °C. During the first 5 minutes, the oven temperature was kept at 60°C, gradually increasing the temperature (3°C/min) until the desired temperature of 240 °C was obtained.

The biological material consisted of human pancreatic epithelial carcinoma cell lines (Homo sapiens), derived from a 65-year-old caucasian male. Prior to the procedures, the cells were stored in the vapor phase of liquid nitrogen, the cell line exhibited epithelial morphology, characterized by adherent cells along with floating rounded cells, existing both as individuals and loosely attached clusters (37).

Pancreatic tumor cells (ATCC CRL- 1420) were cultured in DMEM medium (SigmaAldrich, USA) containing 10% SFB (Gibco, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 26.4 mM sodium bicarbonate. These cells were grown in an incubator (SS SCIENTIFIC, Brazil) with a 5% CO2 atmosphere at 37°C. Unless otherwise specified, these cells were always maintained in this environment.

Additionally, to compare the cytotoxicity of *G. americana L.* essential oil in non-carcinogenic cells, that is, normal cells, the human fibroblast cell line MRC-5 was used. The MRC-5 line, established by J.P. Jacobs in 1966, consists of diploid fibroblasts derived from the lung tissue of a 14-week-old Caucasian male fetus (38,39).

To perform the cell viability test, the colorimetric assay was performed, the 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium (MTT) (40). The MTT test determines the salt uptake by viable cells and its reduction in the mitocondrial interior to originate formazan crystals. The methodology was based on adding 10µL of diluted MTT (Sigma-Aldrich, USA) to the treated cells. Approximately, after 4 hours of incubation, the medium was removed and the formazan crystals were diluted in 100µL of DMSO (NEON, Brazil) (36) and was used as the positive control, while culture medium served as the negative control, all assays were performed in triplicate.Thus, the results were read in a spectrophotometer (Loccus, Brazil) at 570nm and 630 nm.

For the evaluation of the antitumor activity of *G. americana L.* oil, the essential oil was diluted in DMSO and culture medium to obtain the range of concentrations: 0.1% - 0.5% - 1% - 1.5% -5% and 10% (v/v), then the effects on cell viability were evaluated by MTT assay. The rate of cell viability was calculated as a percentage compared to the controls (100% cell viability), which corresponded to cells treated only with 0.05% DMSO, by measuring the absorbance resulting from the MTT assay, after three hours of incubation with the substrate, the absorbance was determined by measuring them at to calculate the percentage of cytotoxicity (%C) of each concentration of the extract of *G. americana L.* at 570 and 630 nm in a microplate reader. The percent cytotoxicity was calculated by dividing the percent reduction of the cells under treatment and the percent reduction of the control cells (41).

The same protocol was followed for the human pancreatic carcinoma cell line (MIA PACA2) and the human fibroblast cell line (MRC-5).

Calculations of the mean, standard deviation, and standard error and statistical analysis for comparison of each set of experimental means were performed using Graph Pad Prism 5.

Absorbance results were converted to percentage survival. The linear regression technique was applied to fit the response curve to then determine the 50% inhibitory concentration, the data were analyzed for significant statistical differences within the groups using one-way ANOVA.

RESULTS

By exposing the cells to four hours of treatment, viability decreased at *G. americana L.* oil concentrations of 10; 5; 2.5; 1.25; 0.625; 0.3125; 0.15625 and 0.078125 µM ($p < 0.05$), with IC 50 values of 275 µM. All concentrations have demonstrated a high rate of cell mortality, which was more pronounced at high concentrations of the oil. (Figure 1,2).

FIGURE 1. EFFECT OF THE ESSENTIAL OIL FROM *G. AMERICANA* LEAVES ON THE VIABILITY OF MIA PACA 2 CELLS.

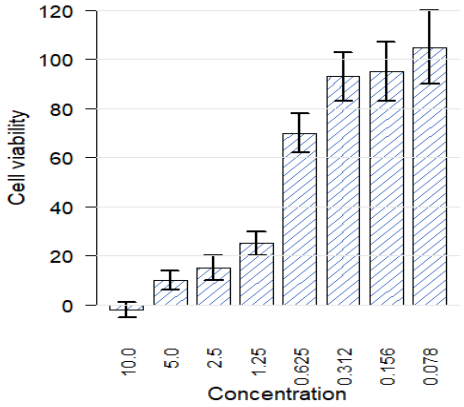
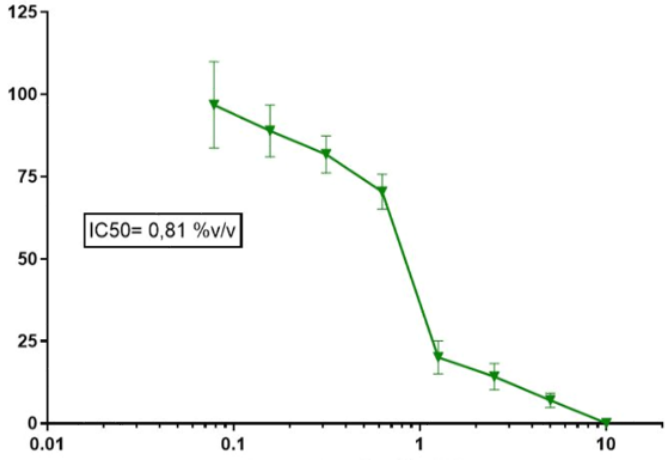
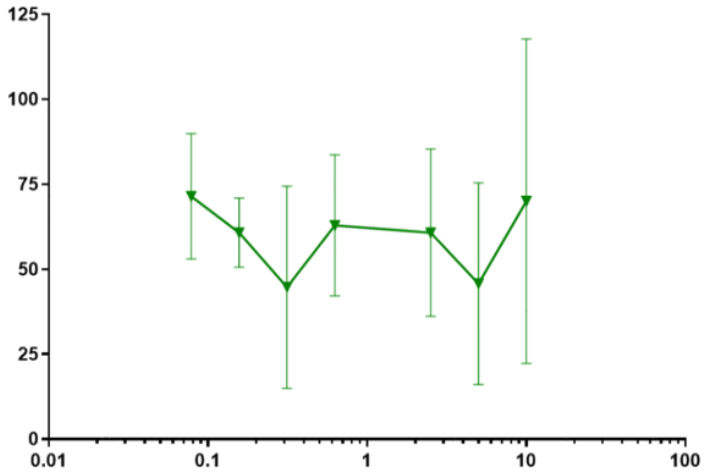


FIGURE 2. PROLIFERATION GRAPH OF MIA PACA TREATED WITH THE ESSENTIAL OIL OF *G. AMERICANA L.*



Regarding the results of the cytotoxicity of the essential oil of *G. americana L*. on the human fibroblast cell line MRC-5, as seen in Figure 3, it is inferred that the essential oil of *G. americana L*. is not toxic to normal cells.

FIGURE 3. PROLIFERATION GRAPH OF MRC-5 TREATED WITH THE ESSENTIAL OIL OF *G. AMERICANA L.*



The essential oil from leaves of *G. americana* presented 0.10% yield based on dry weight. The chemical composition found in this work were analysed by the GC-FID-MS technique and is shown in Table 1. It was possible to identify 22 compounds, representing 39.98% of the total composition. The main compounds

found were 17.83% alcohols (Hexenol <2E>, n-hexenol,1-Octen-3-ol, Alcohol benzyl alcohol); 9.58% aldehydes (Hexenal, Hexadienal <(2E,4E)>benzene acetaldehyde and Hexenal <(2E)>, diethyl acetal); 5.18% esters (methyl salicylate, prenyl benzoate, hexenyl benzoate <(3Z)>, hexenyl benzoate <(n)>, Hexenyl Benzoate <(2E)> and Benzyl Benzoate); 4.09% phenols (Butylated Hydroxytoluene); 1.26% sesquiterpenoids (β-caryophyllene, β-Ionone <(E)> and epi- αBisabolol); 0.86% monoterpene (α-Pinene and β-Damascenone <(E)>); 0.62% furan (2-pentyl furan); and 0.56 isobornyl isobornyl isobutanoate <5-oxy->.

TABLE 1: RELATIVE COMPOSITION (%) OF THE COMPONENTS IDENTIFIED IN THE *GENIPA AMERICANA* ESSENTIAL OIL ANALYSIS.

Componentes	RI[a]	RI[b]	*Genipa americana*
Hexenal	844	855	8,35
Hexenol <2E>	858	862	6,38
n-hexenol	860	870	9,82
Hexadienal <(2E,4E)>	909	909	0,5
α-Pinene	931	939	0,41
1-Octen-3-ol	977	979	0,9
2-pentyl furan	992	988	0,62
Benzyl alcohol	1034	1031	0,73
Benzene acetaldehyde	1043	1042	0,58
Hexenal <(2E)>, diethyl acetal	1100	1098	0,15
Methyl salicylate	1193	1191	0,4
β-Damascenone <(E)>	1384	1384	0,45
β-carophyllene	1418	1419	0,38
Prenyl benzoate	1478	1486	0,4
β-Ionone <(E)>	1486	1488	0,51
Butylated hydroxytoluene	1513	1515	4,09
Hexenyl benzoate <(3Z)>	1570	1566	1,92
Hexyl benzoate <(n)>	1577	1580	1,01
Hexenyl benzoate <(2E)>	1585	1587	0,9
Isobornyl isobutanoate <5-oxy->	1596	1603	0,56
epi-α-Bisabolol	1684	1684	0,37
Benzyl benzoate	1766	1760	0,55
Total identified (%)			39,98

DISCUSSION

While specific data on the essential oil (EO) yield from *G. americana* leaves remains scarce in the literature, related studies provide a basis for comparison. For instance, methanolic leaf extracts have reported yields of 0.10% (28), whereas the EO yield from the fruit is significantly lower (9x106) (42). This discrepancy suggests that the secondary metabolism of *G. americana* favors the accumulation of volatile compounds in the foliar tissue, making leaves a more viable source for therapeutic EO extraction.

Our findings regarding the low cytotoxicity of *G. americana* leaf extracts align with previous reports. Ethanolic extracts have shown no significant cytotoxicity across various dilutions, leading to the establishment of 0.2 mg/mL as a safe concentration for antiviral assays (40). While some studies describe severe cellular alterations ranging from vacuole formation to complete monolayer destruction (43), our results align with studies demonstrating that Vero cell line remains unaffected by leaf extracts (25). This discrepancy in "cellular stress" may be explained by the extraction solvent; EOs contain concentrated terpenes that interact differently with cell membranes compared to crude ethanolic extracts.

In stark contrast, conventional chemotherapeutics like Paclitaxel (Taxol), derived from *Taxus brevifolia*, are associated with severe systemic toxicities, including neurological and cardiac complications (44,45). The favorable safety profile of *G. americana* EO highlights its potential as a more biocompatible therapeutic alternative. While Gemcitabine remains the clinical gold standard for pancreatic adenocarcinoma, its high systemic toxicity and the emergence of chemoresistance (46,47) underscore the urgent need for natural adjuvants like *G. americana* EO, which demonstrate selective activity with reduced impact on healthy cell models.

A comparison with the characterization by De Jesús (48) shows similarities in the presence of α-Pinene

(0.41%), β-caryophyllene (0.38%), and butylated hydroxytoluene (BHT, 4.09%), which were also identified in this study. However, significant discrepancies exist regarding major constituents like (2E,4E)-decadienal and linoleic acid, which were not prominent in our findings. These variations are likely attributable to phytochemical plasticity, a phenomenon well-documented in the Rubiaceae family. Environmental stressors, soil composition, and circadian rhythms can shift metabolic flux toward the lipoxygenase (LOX) pathway, this metabolic shunting favors the production of C6 Green Leaf Volatiles (GLVs), such as the hexenals identified in our study, over heavier sesquiterpenes or fatty acids (48,49).

Certain hexenal derivatives, such as 2-trans-hexenal, have been scrutinized for potential carcinogenicity (50). Conversely, BHT (identified at 4%) has demonstrated protective effects; in murine models, BHT administration (5000 ppm) reduced the incidence of hepatomas and mammary tumors by enhancing the excretion of carcinogenic metabolites through the induction of enzymes like glucuronyltransferase (51,52). Furthermore, BHT has shown direct cytotoxicity in mouse erythroleukemia, human maxillary cancer, and T-24 bladder tumor cell lines (53–55). Although high doses (≥ 250 mg/kg/day) have been associated with spontaneous neoplasms, at lower concentrations, BHT appears to act as a potent anticarcinogenic agent without genotoxic risk (56).

The biological efficacy of the EO is likely not due to a single compound but a synergistic "entourage effect", for instance, β-caryophyllene is known to act as a functional CB 2 receptor agonist and membrane permeabilizer, which may facilitate the intracellular uptake of major aldehydes (57).

Regarding the major GLVs identified Hexenal, (2E)-Hexenol, and n-hexenol, this study provides a novel contribution by evaluating them against the MIA PaCa-2 model. While GLVs are increasingly recognized for inducing mitochondrial dysfunction and ROS-mediated apoptosis in other cancer models (58), our work is among the first to explore this in pancreatic carcinoma. Finally, the absence of prior data on the antitumor potential of (3Z)-hexenyl benzoate and n-hexyl benzoate highlights a significant gap in the literature that our findings begin to address.

The results of this study demonstrate that the essential oil derived from the leaves of *Genipa americana L.* possesses significant in vitro antitumor activity against the human pancreatic carcinoma cell line (MIA PaCa-2). This effect is dose-dependent, with the highest concentrations of the oil resulting in the most pronounced rates of tumor cell mortality. Crucially, the essential oil exhibited no toxicity toward normal human fibroblast cells (MRC-5) across all tested concentrations, suggesting a favorable safety profile and selective action against malignant cells.

Chemical characterization of the oil revealed a complex mixture of alcohols, aldehydes, and esters, with butylated hydroxytoluene (BHT) identified as the most abundant bioactive component at 4.09%. While other identified compounds such as α-pinene, β-caryophyllene, and β-ionone have established antitumor properties, the biological efficacy of the oil may be the result of a synergistic "entourage effect". These findings position *G. americana* as a promising source for the development of new, biocompatible therapeutic agents for pancreatic cancer, which currently lacks effective, low-toxicity treatment options.

AUTHOR CONTRIBUTIONS

The corresponding author made substantial contributions to the conception, acquisition, analysis, and interpretation of the data, and to the drafting of the manuscript. The supervisor contributed to the study design, data analysis and interpretation, critically revised the manuscript, and approved the final version.

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EDITORIAL NOTE

The opinions expressed in this article, as well as the methodological approach and the results presented, are the sole responsibility of the authors. This work was reviewed and approved by external reviewers as part of the editorial process; however, it does not necessarily reflect the official position of the journal, its editorial board, or its Editor-in-Chief.

DATA AVAILABILITY

The data are available from the corresponding author upon reasonable request. Eva R. Montiel Fernández. Email: evam.investigacion@gmail.com

REVIEWER COMMENTS

The names of the external reviewers, as well as their reports, are available at the following link: Dictamen 699.pdf

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