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Ethnopharmacological evaluation of *Trichilia hirta* L. as anticancer source in traditional medicine of Santiago de Cuba

[Evaluación etnofarmacológica de *Trichilia hirta* como una fuente anticancer en la medicina tradicional de Santiago de Cuba]

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Abstract
Trichilia hirta L. (Meliaceae) is traditionally used as antitumor source in Santiago de Cuba. Therefore, the aim of this study was to document and analyze the traditional medicinal use of this plant by cancer patients in Santiago de Cuba and to evaluate its antiproliferative activity on human normal and cancer cells. Cancer patients consuming *Trichilia hirta* extracts (Jubabán) were randomly selected and interviewed. The antiproliferative activity of a polysaccharide-rich fraction from leaves was evaluated against normal (MRC-5) and cancer cells (A-549, HeLa and Hep-2) by MTT assay. The study revealed that *Trichilia hirta* extracts are mainly used as anticancer source (46%). Moreover, the majority of cancer patients consuming *Trichilia hirta* extracts had carcinoma (86%). In particular, the most frequent were lung (26%) and prostate (18%) carcinoma. The majority (90%) of patients were consuming the extracts simultaneously, or after the chemotherapy and radiotherapy treatment. The polysaccharide-rich fraction showed antiproliferative activity against human lung cancer cells (A-549) and human cervix carcinoma (HeLa) cancer cells. However, no toxicity was observed in human normal fibroblasts (MRC-5). These results suggest that polysaccharide-rich fraction from *Trichilia hirta* contribute to the antitumor properties of this specie.

Keywords: *Trichilia hirta*; ethnopharmacology; antitumor; cytotoxicity; MTT.

Resumen
Trichilia hirta L. (Meliaceae) es tradicionalmente usada como recurso antitumoral en Santiago de Cuba. Por lo que, el objetivo de este estudio fue documentar y analizar el uso tradicional de esta planta por pacientes con cáncer en Santiago de Cuba y evaluar su actividad antiproliferativa sobre células humanas normales y tumorales. Pacientes con cáncer consumiendo los extractos de *Trichilia hirta* (jubabán) fueron aleatoriamente seleccionados y entrevistados. La actividad antiproliferativa de la fracción rica en polisacáridos de hojas fue evaluada en células normales (MRC-5) y en células tumorales (A-549, HeLa y Hep-2) a través del ensayo con MTT. El estudio reveló que los extractos de *Trichilia hirta* eran usados mayoritariamente como recurso antitumoral (46%). Además, la mayoría de los pacientes consumiendo extractos de *Trichilia hirta* presentaron carcinoma (86%). En particular, los más frecuentes fueron carcinomas de pulmón (26%) y próstata (18%). También la mayoría de los pacientes (90%) consumieron los extractos simultáneamente o después de tratamientos con quimioterapia y radioterapia. La fracción rica en polisacáridos mostró actividad antiproliferativa contra las células de cáncer de pulmón humano (A-549) y carcinoma de cerviz humano (HeLa). Sin embargo, no se observó toxicidad en fibroblastos humanos normales (MRC-5). Estos resultados sugieren que la fracción rica en polisacáridos de hojas de *Trichilia hirta* contribuye a la actividad antitumoral de esta especie.

Palabras Clave: *Trichilia hirta*; ethnofarmacología; antitumoral; citotoxicidad; MTT.

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INTRODUCTION

The documentation of knowledge is an essential step in ethnobiology as it provides data for further studies that may be driven by either philosophical or mostly utilitarian questions. The broad perspective of ethnopharmacology contextualizes ecology and addresses the perception of plants, plant use, pharmacology and physiology in human communities. Ethnopharmacology can be specifically relevant with respect to further developing and evaluating indigenous pharmacopoeias (Leonti, 2011). Medicines from biologically active plants have a long history of use in both traditional and modern societies as herbal remedies or crude drugs, purified Food and Drug Administration (FDA)-approved compounds, and as starting materials for further medicinal chemistry modifications. Moreover, the majority of new medicines derived from plant secondary metabolites have been applied toward the treatment and/or prevention of cancer (Balunas *et al*., 2006).

There are two main strategies for the selection of plant species in drug discovery: random screening and ethnomedical knowledge. The second approach includes plants used in organized traditional medical systems like herbalism, folklore and shamanism (Pieters and Vlietinck, 2005).

Trichilia hirta L. (Meliaceae) is an important medicinal plant that is gaining popularity for treating various ailments, particularly against cancer. This plant is commonly known as *Jubabán* in Santiago de Cuba. It is also known in different countries as Broomstick, broomwood, cabo de hacha, cedrillo, cedrillo colorado, cedro macho, napahuite, red cedar and trompillo (Little and Wadsworth, 1964; Liogier, 1988).

This specie has traditionally been used as a remedy to treat cancer (Pettit *et al*., 1983), external ulcers (Roig, 1974) and respiratory disorders (Beyra *et al*., 2004). Its organic extracts have shown anti-inflammatory activity related with COX-2 selective inhibition (Obukowicz and Hummert, 2004). The administration of *Trichilia hirta* extracts to Balb/c mice indicated that ethanol extract from this plant could exhibit leukocyte-stimulating properties that makes it a promising alternative for the development of an immunoprotective agent (Sosa *et al*., 2011).

Popular information suggests that oral administration of *Trichilia hirta* root and leaf extracts improved the overall general well being in cancer patients (Hernández *et al*., 2004). These observations are motivating because the patients were terminally ill and had exhausted all the modern conventional therapies that included surgery, radiation and/or chemotherapy.

In the current study, we investigated in detail and documented the treated illness with *Trichilia hirta* extracts, as well as the plant parts used, ways of remedy preparations, route of administration and dosage, side effects, and simultaneous use with oncological treatment. We also evaluated the cytotoxic activity of a polysaccharide-rich fraction from *Trichilia hirta* leaves on human normal and cancer cells *in vitro.*

MATERIALS AND METHODS

Study area

Santiago de Cuba is the capital city of Santiago de Cuba Province in the south-eastern area of the island nation of Cuba, some 540 miles (870 km) south-east of the Cuban capital of Havana. It is situated between latitude 20° N, 01 N, longitude 75° W, and 50° W. The municipality extends over 1,023.8 square kilometers (395.3 sq mi). Historically Santiago de Cuba has long been the second most important city on the island after Havana, and remains the second largest. It is on a bay connected to the Caribbean Sea and is an important seaport. In 2009 the city of Santiago de Cuba had a population of about 727.449 people (National office of statistics. Republic of Cuba, 2009).

Data collection

The study was conducted in eight local communities of Santiago de Cuba municipality. The communities were El Caney, Chicharrones, Versalles, Martí, Abel Santamaría, Vista Hermosa, Antonio Maceo and 30 de Noviembre. A number of 53 cancer patients and herbalist consuming *Trichilia hirta* extracts (jubabán) were randomly selected and interviewed between February-May 2009 mainly through individual interviews using a semi-structured interview format. During the interview with each patient, information regarding the illness treated with *Trichilia hirta* extracts, the plant parts used, ways of remedy preparations, route of administration and dosage, maximum time of consumption, side effects observed during extract use, simultaneous application of oncological treatment (chemotherapy, radiotherapy, surgery) was gathered. Details about types of cancer and conventional treatments of patients were obtained

in collaboration with the Oncological Hospital of Santiago de Cuba.

Plant material, extract preparation and phytochemical screening

Trichilia hirta leaves were collected in Santiago de Cuba (Cuba) and identified by a specialist at the Eastern Center for Ecosystem and Biodiversity (BIOECO). A voucher specimen (BIOECO No. 1078) was deposited at the herbarium of this institution.

The polysaccharide fraction of *Trichilia hirta* leaves was isolated according to a published (Li *et al*., 2007) with some modifications. Briefly, 100 g of *Trichilia hirta* leaves were air-dried and extracted with boiling water (at a ratio of 1:10, w/v) for 30 min according to the traditional method used in Santiago de Cuba. The suspension was filtered under suction to remove the insoluble matter. The supernatant was then concentrated to about 50 mL by rotary evaporation (IKA RV Basic, Germany). Concentrated extracts were successively fractionated to remove lipids by maceration with chloroform and ethyl acetate, respectively. Both fractions of chloroform and ethyl acetate were eliminated and the water-soluble residue was re-suspended in five volumes of 95% ethanol and allowed to stand overnight to precipitate crude polysaccharides. The precipitate was finally centrifugated (300 × *g* for 20 min) to remove the supernatant. A stock solution (250 μg/mL) was prepared in dimethyl sulphoxide (DMSO) diluted in minimum essential medium (MEM) supplemented with 1x pen.strepto-fungizone and 10% heat inactivated fetal bovine serum (FBS). The final concentration of DMSO in each sample did not exceed 0.05% v/v. The total content of polysaccharides was measured by phenol-sulfuric method (Dubois *et al*., 1951). The crude polysaccharides contained 90% of total carbohydrate.

IN VITRO DETERMINATION OF ANTIPROLIFERATIVE ACTIVITY

Cell culture and treatment of HeLa, A-549 and MRC-5 cells

Human cervix carcinoma (HeLa), lung carcinoma (A-549), larynx carcinoma (Hep-2) and one type of normal human cell line, fibroblast cells (MRC-5), from the American Type Culture Collection (ATCC) were kindly provided by Alexis Díaz García from National Laboratory of Microbiology (Institute of Tropical Medicine ¨Pedro Kourí¨. Cuba). The cells were cultured as monolayer in MEM and grown at 37 ºC in 5% CO_2 with humidified air atmosphere.

Cells were seeded in 96 well-plates (50 μL/well at a density of 2.5×10^5 cells/mL) for 24 h. After the cell adherence, the supernatant was discarded and the extracts were added at concentrations of 8, 16, 30, 60 and 120 μg/mL into the wells for 72 h. Cells growing in MEM with corresponding concentrations of DMSO (0.05%) were used as control.

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the conversion of the yellow tetrazolium salt-MTT, to purple-formazan crystals by metabolically active cells, provides a quantitative determination of viable cells (Shriram *et al*., 2010). After 72 h of treatment, 10 μL of MTT solution (5 mg/mL) were added to each well and incubated at 37º C for another three hours. At the end of incubation the medium was discarded. The formed formazan crystals were dissolved with 150 μL of DMSO and the absorbance was read on a spectrophotometer plate reader at 560 nm (BIO-RAD) with 630 nm reference wavelengths. The percent of cell viability was obtained using the following formula:

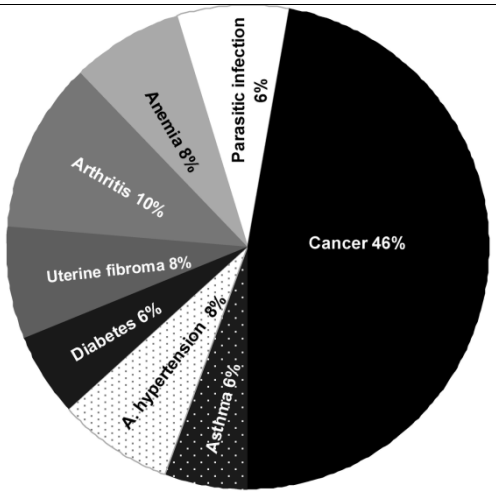
Inhibition of cell viability (%) = (1- Treated cells/ Untreated cells) x 100

RESULTS AND DISCUSSION

Extracts from *Trichilia hirta* are traditionally used to treat different diseases (Fig. 1). A previous study indicated that aqueous extracts from this plant were used to treat uterine fibroma, asthma, diabetes, arterial hypertension and cancer in Santiago de Cuba (Hernández *et al*., 2004). However, the spectrum of medicinal uses of these extracts has currently been expanded to treat arthritis, anemia, and parasitic infections (Figure 1).

Ethnopharmaceutical investigations of *Trichilia hirta* extracts have indicated its use as abortifacient, astringent, emmenagogue, insecticide, poison, stimulant and Sudorific (Duke, 2012). Also, water extracts from *Trichilia hirta* leaves have been used as cicatrizing in Cuba (Roig, 1974), and anticatarrhal (Beyra *et al*., 2004).

Figure 1
Illness treated with *Trichilia hirta* water extract in Santiago de Cuba.



The information was obtained mainly through individual interviews with selected subjects using a semi-structured interview format.

The survey indicated that the traditional medicine users of Santiago de Cuba (Figure 1) mainly use *Trichilia hirta* extracts as anticancer source. This plant has similarly been used for the traditional treatment of cancer in Venezuela (Pettit *et al*., 1983). This result is coherent with the global increase of cancer incidence (Ferlay *et al*., 2010; Siegel *et al*., 2011) and concurrent arise of herbal medicines use by cancer patients (Gratus *et al*., 2009; Cheng *et al*., 2010; Ali-Shtayeh *et al*., 2011).

Despite of the traditional use of *Trichilia hirta*, little is known about the patterns of use of this plant as anticancer source. Following this approach, the results of interviewing cancer patients in collaboration with the Oncological Hospital of Santiago de Cuba allowed us to know that *Trichilia hirta* extracts have mainly been used to treat carcinomas (86%) and leukemia (14%) (Figure 2A). Moreover, the results showed that extracts from this plant are generally consumed by patients with lung (26%) and prostate (18%) carcinomas, although patients with different anatomical location of carcinomas also consumed these extracts.

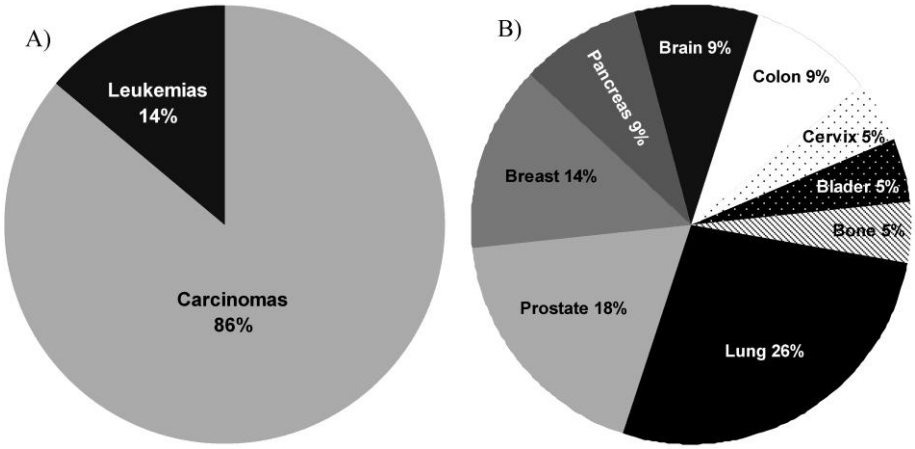
The pattern of extracts preparation started with dry or fresh materials, although 70% of the plant material was reported to be dry and stored for a future use. The extracts were mostly prepared as decoction (100%) using water as solvent of extraction. Nevertheless, the quantities of plant material used to prepare the remedies and the time of decoctions were different for leaves and roots. People applied a decoction time of 5-10 minutes to prepare the extracts generally using a small quantity of leaves. However, in case of roots, people prepared the extracts applying a decoction time of 30-40 minutes and used a defined quantity of ¼ kg of roots.

The water extracts of *Trichilia hirta* leaves and roots were taken orally (100%). This further highlights the importance of documentation of the intake mode of these substances as part of routine clerking and assessment of patients in order to avoid potential drug-herb-vitamin interactions, particularly in patients undergoing chemo or hormonal therapy (Chang *et al*., 2011). The majority of interviewed cancer patients (70%) consumed 6-8 ounce of *Trichilia hirta* extract three times per day, while the other 30% showed differences in frequencies and volume of extract consumption.

The majority of cancer patients (90%) were taking the extracts before the meals; that empirically established pattern of consumption might contribute to a rapid absorption of active compounds of the extracts. In addition, the subjects suggested that taking the remedy daily for long periods helps to increase the effectiveness of the extracts. Consequently, the majority of patients (93%) were taking the *Trichilia hirta* extract daily up to four months. Although some people (7%) consumed the

extracts for a variable period from 15 days and up to six months.

Figure 2
Traditional use of *Trichilia hirta* extracts against cancer.



A) Types of cancer traditionally treated with *Trichilia hirta* (Jubabán) extracts. B) Location of carcinomas traditionally treated with *Trichilia hirta* extracts. The information was obtained mainly through individual interviews with selected patients using a semi-structured interview format. Details about types of cancer and conventional treatments of patients were obtained in collaboration with the Oncological Hospital of Santiago de Cuba.

The observed effects during the consumption of *Trichilia hirta* extracts provide important information about the pharmacological and toxicological effects of these extracts. Patients described several positive effects: hyperactivity, euphoria, increased appetite. However, some patients (15%) described side effects like nausea, increased transpiration, pain and small bleeding. Previous studies support that herbal medicine are seen as being less likely to give rise to adverse side effects (Lynch and Berry, 2007). Regarding the previous idea, the majority of interviewed patients (70%) informed the decrease or loss of the side effects in a period of seven days, while some patients kept these negative symptoms during 15 days. However, herbal medicines are usually a mixture of many active ingredients, which increases the likelihood of harm (Izzo and Ernst, 2009).

Cancer patients are commonly under a state of immunosuppression induced by tumor itself (Williams *et al*., 2004; Whelan and Scadden, 2006) and chemotherapy increases this altered state. Thus, the common purposes of concurrent administration of an herb with chemotherapy are to enhance the efficacy and/or to reduce the side effects of chemotherapy (Cheng *et al*., 2010). In this context, decoctions of *Trichilia hirta* roots have shown immunoprotective activity on Balb/c mice treated with 5-Fluorouracil (Hernández *et al*., 2010). Moreover, reports indicate that cancer patients perceive that boosting of the immune system will increase the chances of healing or survival treatment (Humpel and Jones, 2006). Thus, the immunological effect of *Trichilia hirta* extracts suggests a positive herb-drug interaction and supports the use of those extracts by cancer patients under chemotherapy treatment.

Most of interviewed patients (90%) were taking the extracts of *Trichilia hirta* simultaneously, or after the chemotherapy and radiotherapy treatment. Similar surveys have shown an increased popularity of concurrent application of herbal remedies with conventional treatment (chemotherapy, radiation, or surgery) among cancer patients (Richardson *et al*., 2000; Engdal *et al*., 2008). However, herbal remedies are usually complex mixtures of chemical entities that may display positive or negative herb-drugs interactions (Ernst, 2004; Unger, 2010). In consequence, the concern for these potential interactions is growing (Beijnen and Schellens, 2004; Izzo and Ernst, 2009) and *Trichilia hirta* is not and exception. Therefore, the mentioned side effects

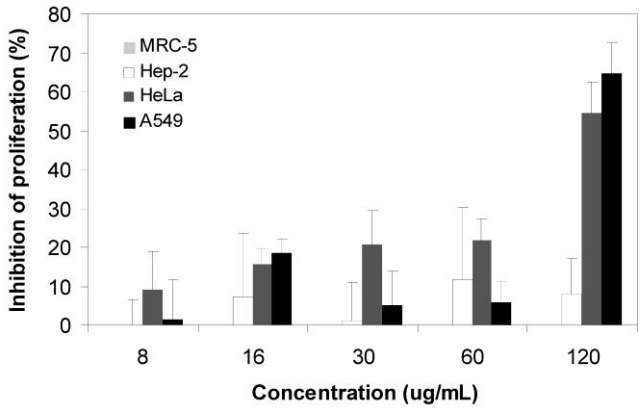
during the consumption of *Trichilia hirta* extracts and possible herb-drugs interactions indicate the need of future toxicological studies to determine the safety of *Trichilia hirta* extracts.

Most of the interviewed persons mentioned the assumed anticancer effect of these extracts. Moreover, a small part (10%) of interviewed cancer patients was taking *Trichilia hirta* roots and leaf extracts without any oncological treatment. This observation is motivating because these patients were terminally ill and had exhausted all the modern conventional therapies, that is, surgery, radiation and/or chemotherapy.

The results indicated that *Trichilia hirta* roots are generally used to prepare the aqueous extracts (80%). In addition, cancer patients are currently using leaves to prepare extracts (20%). The incorporation of *Trichilia hirta* leaves to medicinal practices against cancer could be due to its relative ease to collect in comparison with the huge effort required to collect roots. Interestingly, the use of leaves in the preparation of remedies is common elsewhere (Liogier, 1974; Beyra *et al*., 2004). Besides, leaves remain green and plenty for most months of the year in Cuba. Therefore, the patients informed that use of leaves as anticancer source is rapidly growing.

Our research group identified in previous studies that aqueous extracts from *Trichilia hirta* leaves extracts have antiproliferative activity on human melanoma cells (SK-mel-3) and human adenocarcinoma cells (T-47D) (data not shown). However, the contribution of some polar fractions to this activity needed further research. In order to assess this activity, a polysaccharide-rich fraction from leaves was assayed on human cervix carcinoma (HeLa), lung carcinoma (A-549), larynx carcinoma (Hep-2) and one type of normal human cell line, fibroblast cells (MRC-5).

Figure 3
Cell viability of human normal and cancer cells treated with polysaccharide rich fraction of *Trichilia hirta.*



Cells were treated with 8, 16, 30, 60 and 120 µg/mL of extract for 72 h and the cell viability was determined by MTT assay. Cells without treatment and normal human fibroblast cells (MRC-5) were used as controls. The results were expressed as percent of inhibition of proliferation.

The polysaccharide-rich fraction showed antiproliferative activity against human carcinomas cell lines of lung (A-549) and cervix (HeLa). The higher inhibition of the proliferation was observed at 120 µg/mL on A549 (54%) and HeLa (67%) cancer cell lines (Fig 3). Moreover, a slight antiproliferative effect was displayed on HeLa with 30 µg/mL and 60 µg/mL respectively. Although, the laryngeal carcinoma cells (Hep-2) were less sensitive to the extract, a previous study showed *in vitro* cytotoxic activity of aqueous extract from *Trichilia hirta* roots on human T-47D breast carcinoma and SK-mel-3 human melanoma (Hernández *et al*., 2010).

The observed inhibitory activity on human lung carcinoma cells is encouraging because the majority of interviewed cancer patient currently consuming extracts from *Trichilia hirta* had lung carcinoma (Figure 2). These results suggest the hypothesis that *Trichilia hirta* extracts could be useful for treating cancer, particularly lung carcinomas.

Despite of those antiproliferative activities, the effect of polysaccharide-rich fraction from

Trichilia hirta leaves was highly selective for cancer cells. This observation was supported by the lack of toxicity on human normal fibroblast. These cells showed a normal proliferation compared with control when they were treated with the polysaccharide-rich fraction. Similar results have been observed in a previous study, where aqueous extracts from *Trichilia hirta* showed a selective antiproliferative activity against cancer cells (Hernández *et al*., 2010).

The selectivity of *Trichilia hirta* polysaccharide-rich fraction could be associated to the diversity of biological activities that polysaccharides can exhibit. The polysaccharides are exogenous biological response modifiers (BRMs) and several exogenous BRMs have been reported to have anti-viral, anti-bacterial, anti-fungal, anti-parasitic, immunostimulant and anti-tumor activities *(Leung et al*., 2006). Thus, polysaccharides could affect the proliferation of cancer cells and protect normal cells.
Some studies have shown the potential anti-cancer activity of polysaccharides from medicinal plants, among them, crude *Orostachys japonicus* polysaccharide extract displayed remarkable anti-proliferative activity in HT-29 human colon cancer cells via apoptosis and various apoptosis-aiding activities (Deok-Seon *et al*., 2010). Crude polysaccharides from *Salicornia herbacea* showed anti-proliferative effect on HT-29 human colon cancer cells (Deok-Seon *et al*., 2009). Those studies suggested that polysaccharide extracts could have antiproliferative activity on cancer cells through the down-regulation of several genes involved in anti-apoptotic activity, cell proliferation and cell cycle regulation, and up-regulation of the expression levels of several genes involved in apoptosis, tumor suppression.

On the other hand, polysaccharides can stimulate or protect normal cells. For instance, polysaccharides isolated from *Salicornia herbacea* significantly induced NO production in the mouse macrophage line RAW 264.7. The effect of those polysaccharides suggested that those compounds may represent useful immunopotentiating agents (Lee *et al*., 2006). The *Lycium barbarum* polysaccharides can be used in compensating the decline in total antioxidant capacity, immune function and the activities of antioxidant enzymes and thereby reduces the risks of lipid peroxidation accelerated by age-induced free radical (Li *et al*., 2007). Polysaccharide-enriched fractions from the root of Radix *Angelica sisnensis* (APS) significantly enhanced not only the recovery of platelets, other blood cells and their progenitor cells, but also the formation of Colony Forming Unit (CFU) in the mouse model. In addition, APS showed antiapoptotic effect in a megakaryocytic cell line cells (M-07e) (Liu *et al*., 2010).

Currently, most medicines used in chemotherapy are combinations of chemical substances with no selectivity toward cancer cells and usually show toxicity to normal cells as well. Therefore, in recent years, many studies have attempted to identify new drugs candidates that are selective toward cancer cells to avoid damaging normal cells. The combination of the antiproliferative activity of *Trichilia hirta* polysaccharide-rich fraction on cancer cells and less cellular toxicity to normal cells (MRC-5) suggests that this fraction could be a possible candidate to be investigated for their synergistic effects in combination with chemical substances.

The anticancer activities of several genera belonging to Meliaceae have been studied and have yielded benzofurans and terpenoids as the major active constituents (Cragg *et al*., 2006). However, those compounds are mainly extracted in dichloromethane (Pupo *et al*., 2002) and have little solubility in water. Thus, our results suggest that polysaccharide-rich fraction from *Trichilia hirta* also contribute to the antitumor properties of this plant. Several polysaccharides derived from higher plants are relatively non-toxic and do not cause significant side effects. Therefore, plant polysaccharides could be good candidates for therapeutics with antitumor effects and low toxicity (Schepetkin and Quinn, 2006).

This study showed that *Trichilia hirta* (jubabán) is used by the traditional medicine of Santiago de Cuba for treating several illnesses. Besides, this plant is playing a significant role as complementary source for cancer patients with lung and prostate carcinoma. Roots and leaves are used for the preparation of extracts, which, are frequently taken together with chemotherapy and radiotherapy treatment as a complementary anticancer source. Moreover, the polysaccharide-rich fraction from *Trichilia hirta* leaves showed selective antiproliferative activity on human cancer cells.

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