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Immunorestorative in immunosuppressed Balb/c mice and cytotoxic activity of water extract from *Trichilia hirta* root

[Actividad inmunorestauradora en ratones Balb/c inmunodeprimidos y citotóxica del extracto acuoso de raíz de *Trichilia hirta*]

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Abstract

Patients receiving chemotherapy treatment in Santiago de Cuba traditionally use water extracts from *Trichilia hirta* roots. The study aim was to evaluate the immunorestorative and cytotoxic activity of water extracts from *Trichilia hirta* root. Administration of root water extract increased the total and differential leukocyte counts in inmunosupressed Balb/c mice. Thymus weight recovered significantly as well as bone marrow cellularity. Moreover, water extract (125 ug/mL) showed selective cytotoxicity against cancer cells T-47D and SK-mel-3 in comparison with non-cancer cells (Vero). The results indicate that *Trichilia hirta* has significant immunorestorative effects *in vivo* and selective cytotoxicity *in vitro*. Therefore, it might be a promising alternative for cancer therapy.

Keywords: *Trichilia hirta*; immunorestorative; cytotoxicity; clonogenic assay; polysaccharides

Resumen

Pacientes bajo tratamiento quimioterpéutico tradicionalmente usan extractos acuosos de raíz de *Trichilia hirta* en Santiago de Cuba. El objetivo de este estudio fue evaluar la actividad inmunorestauradora y citotóxica de extractos acuosos de raíz de *Trichilia hirta.* La administración del extracto acuoso de raíz incrementó los conteos globales y diferenciales de leucocitos en ratones inmunodeprimidos. El peso del timo, así como, la celularidad de la médula ósea se recuperaron significativamente. Además, el extracto acuoso (125 ug/mL) mostró citotoxicidad selectiva contra las células tumorales T-47D y SK-mel-3 en comparación con la línea no tumoral (Vero). Los resultados indican que *Trichilia hirta* posee significativos efectos inmunorestauradores *in vivo* y citotoxicidad selectiva, por lo cual podría ser una promisoria alternativa para la terapia del cáncer.

Palabras Clave: *Trichilia hirta*; Inmunorestaurador; Citotoxicidad; ensayo clonogénico; polisacáridos.

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INTRODUCTION

Trichilia hirta L. (Meliaceae) is widely distributed in South America and Caribbean. This plant has been used in traditional medicine to treat cancer (Hartwell, 1967) external ulcers (Roig, 1974) and respiratory disorders (Beyra et al., 2004), it has also shown an antimalarial effect (MacKinnon et al., 1997). Organic extracts have shown anti-inflammatory activity related with COX-2 selective inhibition (Obukowicz et al., 2004). In Santiago de Cuba (Cuba), the local healers recommend this plant mainly to cancer patients receiving chemotherapy (Hernández et al., 2004b).

Phytochemical investigations on the components of the leaves of *Trichilia hirta* resulted in the identification of limonoids and protolimonoids (Cortez et al., 1992); polyphenols, saponins and terpenoids; flavonoids, saponins and tannins (Hernández et al., 2004a).

Popular reports indicate that oral administration of *Trichilia hirta* root extract improved the overall general well being in cancer patients. These observations are interesting because the patients were terminally ill and had exhausted all the modern conventional therapies that included surgery, radiation and/or chemotherapy. It also suggests that *Trichilia hirta* could have immunoprotective effect and selective cytotoxicity.

The development of agents capable of improving 'patients' immune systems from a stage of immune deficiency to one of more normal function would likely have a significant impact on disease and the affected patient (Diwanay et al., 2004). Herbal agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy.

Cytoprotective agents should reduce or prevent these toxicities. These agents should ideally be selective for normal cells versus cancer cells, be effective in reducing or preventing toxicity, should have no negative impact on anticancer therapy, and have minimal adverse effects (Hoekman et al., 1999). Therefore, this study was undertaken to explore immunopharmacological and cytotoxic activities of water extract from *Trichilia hirta* roots and evaluate their potential as supportive treatment with cancer chemotherapy.

MATERIAL AND METHODS

Plant material, extract preparation and phytochemical screening

Trichilia hirta roots were collected in February, before flowering, from El Caney district, Santiago de Cuba (Cuba) and identified by a specialist at the Eastern Center for Ecosystem and Biodiversity (BIOECO). A voucher specimen (BIOECO No. 1078) is deposited at the herbarium of this institution. Roots (20 g) were extracted with diethyl ether, ethanol and water respectively. The resulting extracts were subjected to preliminary phytochemical screening for detection of several plant phytoconstituents (Trease and Evans, 1989).

Water extracts were prepared to approximate the traditional medicinal preparation of this plant. Air-dried roots (75.3 and 225.9 g, respectively) were stirred in 500 mL of distilled water for 10 min at 100 ^{o}C followed by rapid filtration through gauze and then through Whatman filter paper #1.

For immunological evaluation, extracts were standardized on the base of total carbohydrate content to 4.63 mg/kg and 13.89 mg/kg respectively using distillated water. The total carbohydrate content of root water extracts was determined by phenol-sulphuric acid method and absorbance was read at 492 nm using glucose as a standard (Dubois et al., 1951).

5-Fluorouracil-induced immunosuppression

The assay was carried out according to the work of Aportela with some modifications (Aportela et al., 2001). Briefly, Balb/c mice (female, 5–6 weeks old) were randomly distributed into four groups consisting of five animals. The animal quarter was maintained at 22 ± 2 $^{\circ}$C and 63% relative humidity with a 10h/14h light/dark cycle. Immune system suppression was achieved in all animals by a single intraperitoneal injection (150 mg/kg) at day five of study. First and second groups were used as the negative and positive control. The negative control was group treated with NaCl solution (0.9 %) and the positive group was treated with 5-fluorouracil (150 mg/kg, i.p.). Two experimental groups received a pre-treatment during four days with standardized root water extracts at 4.63 mg/kg and 13.89 mg/kg equivalent of carbohydrates respectively (p.o.). After application of 5FU, the experimental groups received a post-treatment with extracts for another four days. On day nine, blood samples were collected from retro-orbital plexus of all animals with the aid of hematocrit tubes into EDTA

heparinized tubes and analyzed for hematological parameters. The percentage immunorestoration was calculated using the formula as:

Percentage immunorestoration (%)

= [Cell counts (treatment) − Cell counts (positive control)] / Cell counts (positive control) x 100

All experiments were approved by the institutional Ethical Committee (TOXIMED) and have been performed in accordance with Cuban legislation and the National Research Council Guidelines for the Care and Use of Laboratory Animals.

Determination of hematological and immunological parameters

The total and differential leukocyte count (TLC and DLC, respectively) was carried out per standard protocol (Talwar, 1983). Samples of bone marrows were obtained from the left femur of each animal with 500 µl of phosphate buffer saline (PBS). Bone marrow cell counts (BMCC) were completed using a hemocytometer. Thymus and spleen were extracted, the excess water was absorbed with filter paper and the organs were weighted.

Cytotoxic (antiproliferative) activity by MTT assay

SK-mel-3 (human melanoma, ATCC HTB-69), T-47D (human breast adenocarcinoma, ATCC HTB-133) and Vero (kidney epithelial cell of African green monkey *Cercopithecus aethiops*, ATCC CCL-81^{TM}) cell lines were used in this study. The 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).

The cell lines were grown in RPMI 1640 (Sigma) containing 2 mM L-glutamine and 10% heat inactivated fetal bovine serum (FBS) at 37 ^{o}C in humidified 5% CO_2. Cells were seeded in 96 well-plates (100 µl/well at a density of 1 $\times 10^5$ cells/ml) for 24 h. After the cell adherence, the supernatant was discarded and the root water extracts were added at concentrations of 31.3, 125 and 500 µg/mL into the wells for 72 h.

After 72 h of treatment the medium was discarded and 200 uL of MTT solution (1 mg/mL) in RPMI 1640 (Sigma) were added to each well and incubated at 37 °C for another four hours. Formazan crystals were dissolved in 150 µl of dimethyl sulphoxide (DMSO) by incubating in shaking condition at room temperature for 15 min. The absorbance was read on a spectrophotometer plate reader at 540 nm (Lab systems Multiskan Plus). Three controls were used; negative control that contained the medium and cells, positive control with Sodium Dodecyl Sulphate (SDS, 100 µg/mL), the other one contained each extract and medium without cells to check the effect of the extracts color on the optical density. The inhibition rate was obtained using the following formula:

Grow inhibition (%)

= 100 − (Sample absorbance − blank absorbance) / (Control absorbance − blank absorbance) x 100

Clonogenic assay

The assays were performed as described below. In a 24 well plate duplicates of approximately 100 cells in 1 mL of culture medium were seeded and let grow for 7 days with 125 µg/mL of root extract at 37 $^{\circ}$C and 5% CO_2 humidified atmosphere. Two controls were used; negative control that contained the medium and cells, positive control with Sodium Dodecyl Sulphate (SDS, 100 µg/mL). At the end of the incubation period when colonies were visible, the cells were fixed with methanol and stained with Giemsa. The effect of root water extracts on cancer cell was determined by means of Plating efficiency (PE) (Mather and Roberts, 1998) using the following formula:

PE (%) = Colonies Counted / Cells Inoculated x 100.

Statistical analysis

One-way ANOVA followed by Turkey's test were used to determine the statistical significance between values of the various experimental and control groups. Data are expressed as means ± standard errors (SEM). A difference was considered significant at $P<0.05$.

RESULTS

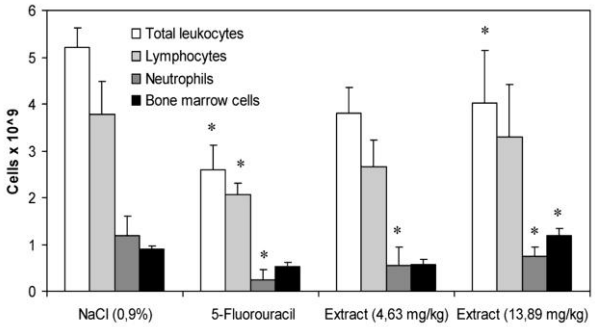
The result of phytochemical screening showed that ethanol and water extracts from *Trichilia hirta* roots exhibited positive reactions to coumarins, lipids, saponins, phenols and tannins, reducing carbohydrates, amines and free aminoacids, flavonoids and glycosides. However, diethyl ether root extract only displayed coumarins and lipids in this study. All extracts contained mucilage.

As shown in fig. 1, treatment with 5-fluorouracil (5FU) significantly reduced the total leukocyte (P = 0.0003), lymphocyte (P = 0.0285) and neutrophil counts (P = 0.0004) compared to control group treated with NaCl 0.9%. The group treated with water extracts

from *Trichilia hirta* roots (4.63 mg/kg) showed a slight recovery on TLC (46.15%) compared with the 5FU-treated group. However, the recovery on TLC was significantly higher at a dose of 13.89 mg/kg (P = 0.0003) with a 54.61% of cell population recovery (Fig 1).

No statistical differences were found in lymphocyte counts between extract-treated groups and NaCl-treated group (P = 0.0285), which suggest both doses (4.69 and 13.89 mg/kg) stimulated the recovery of lymphocyte population (fig. 1). Groups treated with extracts showed high percent of neutrophils recovery (116 and 200%, respectively) compared with 5FU-treated group (Fig 1). However, they were significantly lower than the NaCl-treated group (P = 0.0004).

Figure 1. Total leucocytes, lymphocyte, neutrophil and bone marrow cell counts of Balb/c mice.



Two experimental groups were orally fed during eight consecutive days with 4.63 and 13.89 mg/kg of water extracts standardized by carbohydrates content and compared with two control groups, NaCl-treated group and 5FU-treated group. Blood and bone marrow samples were collected on day nine. The cell counts were carried out according to the standard protocol with Neubauer chamber. Values are expressed as the mean ± SEM. Statistical analysis were performed by one way Anova followed by Turkey´s test at P<0.05.

In this study, water extract from *Trichilia hirta* root standardized by its carbohydrate content (13.89 mg/kg) resulted in significant (P = 0.0011) dose-dependent recovery of bone marrow cellularity compared with the 5FU-treated group. Moreover, the treated group with 13.89 mg/kg of extract displayed a total recovery in bone marrow cellularity (121.4%) compared with NaCl-treated group (Fig 1).

Application of 4.63 and 13.89 mg/kg of root extracts during eight days resulted in significant increase of thymus (P = 0.0001) weight compared with 5FU-treated group (Table 1). Although, all groups were significantly lower than NaCl-treated group. Positive control showed a significant decrease on spleen weight (P=0.04). However, both dose of root extracts displayed a total recovery of spleen weight compared with NaCl-treated group (P = 0.04).

Table 1. Effects of standardized water extract of *Trichilia hirta* on the recovery of lymphoid organs of Balb/c mice.

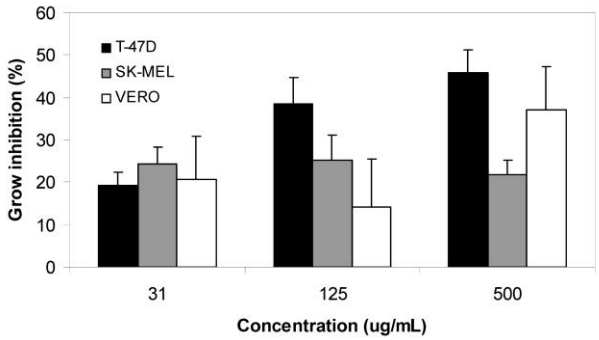
Groups	Dose (mg/kg)	Weight[a]	
		Thymus	Spleen
I. Control	-	7.90 ± 0.71	9.62 ± 0.91
II. 5FU	150	0.33 ± 0.05	5.20 ± 1.34*
III. Extract + 5FU	4.63	4.69 ± 0.68*	6.45 ± 1.05
IV. Extract + 5FU	13.89	4.91 ± 1.03*	6.57 ± 0.56

[a] Two experimental groups were orally fed during eight consecutive days with 4.63 and 13.89 mg/kg of water extracts standardized by carbohydrates content and compared with two control groups, NaCl-treated group and 5FU-treated group. At day nine of study thymus and

spleen were extracted, the excess of humidity was eliminated with filter paper and the organs were weighted. Values are expressed as the mean ± SEM. Statistical analysis were performed by one way Anova followed by Turkey´s test at P<0.05.

Trichilia hirta have been used to treat cancer (Hartwell, 1967). We investigated the differential sensitivity of various cancer cells to water extracts from root of this plant. Extracts showed dose-dependent cytotoxicity on T-47D with 19, 38 and 45% of grow inhibition at 31, 125, and 500 μg/mL respectively. However, cytotoxicity on SK-mel-3 showed any change (24-21%) compared with the dose increase. The root extracts displayed higher cytotoxicity on cancer cells at 125 μg/mL in comparison with Vero cells (kidney epithelial cell of monkey *Cercopithecus aethiops*). Similar effect was observed with the application of 500 μg/mL on human breast carcinoma. This suggests that root extracts of *Trichilia hirta* have a selective cytotoxicity (Fig. 2).

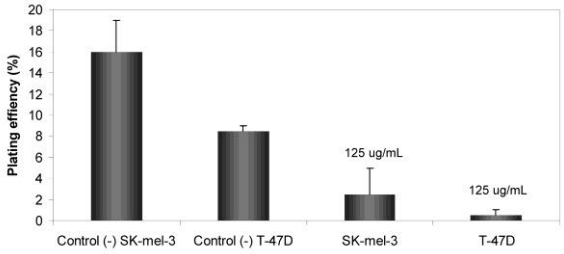
Figure 2. Antiproliferative action of water extracts from *Trichilia hirta* roots.



Human melanoma (SK-mel-3), human breast adenocarcinoma (T-47D), and kidney epithelial cell of African green monkey *Cercopithecus aethiop* (Vero) were treated with different concentrations (31, 125 and 500 μg/mL) for 72h. Each value represents the mean ± SEM.

Plating efficiency assay was performed as detailed in Section 2.5. SK-mel-3 and T-47D showed a plating efficiency of 16% and 8.5% respectively. However, application of 125 μg/mL of extract displayed an important inhibition of plating efficiency (2.5% and 0.5% in SK-mel-3 and T-47D respectively) (Fig. 3).

Figure 3. *Trichilia hirta* root extract (125 μg/mL) has cytotoxicity on cultured cancer cell lines *in vitro*.



Human melanoma (SK-mel-3) and human breast adenocarcinoma (T-47D) were treated with 125 μg/ml of the extract. Plating efficiency was determined as is described in materials and methods (Section 2.5).

DISCUSSION

Immunosuppression leads to greater susceptibility to infection and has been implicated to play a role in tumour development (De Souza and Bonorino, 2009; Sethi et al., 2009). In this context, pharmacological manipulation of the immune system is desirable. According to Osadebe (Osadebe and Omeje, 2009) immunostimulation is a desired physiological response if the overall process culminates in cure or quicker convalescence in diseased conditions.

The results indicate that *Trichilia hirta* water extracts (root) exerts a dose-dependent stimulating effect on

total leukocytes (fig. 1), this could have resulted from a direct effect of extracts on the stem cells of the bone marrow or due to blast transformation of the white blood cells in mice.

Bone marrow and thymus equip lymphocytes to mediate the responses of the mature immune system (Paul, 2003). The extracts showed a stimulating effect of bone marrow cellularity, approving the hypothesis of direct effect of on bone marrow. This result could be of practical importance because most of the synthetic chemotherapeutic agents available today are immunosuppressants, cytotoxic, and exert a variety of side effects. In this context, cytoprotective agents should reduce or prevent these toxicities (Diwanay et al., 2004).

Neutrophils are components of innate immune system and they are capable of a wide range of responses, in particular chemotaxis, phagocytosis, exocytosis and inflammation (Abbas et al., 2008). Root water extracts of *Trichilia hirta* significantly increased of neutrophils. This may potentially help increasing immunity of body against microbial infections (Abbas et al., 2008). A recovery of spleen weight was observed in extract-treated groups and no statistical difference was found compared with NaCl-treated group. This stimulating effect on spleen could provide a positive factor against infections, owing to the protective role of spleen against infectious diseases (Abbas and Lichtman, 2004).

Plants produce a wide variety of high molecular weight glycosides, i.e. phenolic and saponins. They have different biological and pharmacological properties, including immunomodulatory and antitumor (Lakshmi et al., 2003; Patwardhan and Gautam, 2005). The presence of these glycosides in roots of *Trichilia hirta* may be responsible for the immunopharmacological activities of its extracts.

Several studies have been carried out *in vivo* with plant polysaccharides (Cho and Leung, 2007; Laskova and Uteshev, 1992; Li et al., 2010) and saponins (Song and Hu, 2009) reporting immunostimulation, but these plant constituents were usually injected systemically (intraperitoneally), not by the more ethnopharmacologically relevant oral route. However, in this study oral application of water extract from *Trichilia hirta* roots showed an important dose-dependent immunorestorative effect in 5FU-immunosuppressed mice.

The genera *Trichilia* have been found ¨active¨, that means least one and less than three active plants (Cragg et al., 2006) . However, as far as we know there have not been any previous investigations on the cytotoxicity of *Trichilia hirta*. Our investigation demonstrated that root extract (125 μg/mL) of this plant was cytotoxic against human breast carcinoma (T-47D), moderate against human melanoma (SK-mel-3) and slight against kidney epithelial cell of monkey *Cercopithecus aethiops* (Vero).

Plating efficiency is a very sensitive test and is often used for determining the nutritional requirements of cells, testing serum lots, measuring the effects of growth factors, and for toxicity testing (Mather and Roberts, 1998). Therefore, at the assayed time point, the extract-treated cells SK-mel-3 and T-47D failed to undergo division to form colonies under clongenic assay (Fig. 3). Although the mechanism of root extract cytotoxicity is not known, the results indicate that root extract have selective cytotoxicity on SK-mel-3 and T-47D.

CONCLUSIONS

The administration of water extract from *Trichilia hirta* root displayed a stimulating effect on total and differential leukocyte counts, relative weight of thymus and spleen, and also restored the myelosuppressive effects induced by 5-fluorouracil in mice. Moreover, root extract (125 μg/mL) showed selective cytotoxicity on cancer cells. Therefore, the present investigation indicates that water extracts from roots of *Trichilia hirta* have a significant dose-dependent immunorestorative effect *in vivo* and cytotoxicity on cancer cells *in vitro*. Thus, support the traditional use of *Trichilia hirta* as immunoprotective, and could be a promising alternative for cancer therapy.

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