Advance Access Publication 12 December 2006

eCAM 2007;4(3)355–359
doi:10.1093/ecam/nel102

Original Article

Indigofera suffruticosa: An Alternative Anticancer Therapy

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Indigofera suffruticosa Mill (Fabeceae) occurs in the Northeast countryside and has intensive popular use in the treatment of infectious, inflammatory and other processes. The main aim of the present work was to investigate the cytotoxic and antitumor effects of aqueous extracts of leaves of *I. suffruticosa* obtained by infusion and maceration as well as to evaluate the toxicological properties. Aqueous extracts did not exhibit cytotoxicity against HEp-2 (human epidermoid cancer cell) cell lines by MTT method. From the aqueous extract by infusion, the toxicological assay showed low order of toxicity. The antitumor effect of aqueous extracts by infusion (64.53%) and maceration (62.62%) against sarcoma 180 in mice at a dose of 50 mg kg^{-1} (intraperitoneally), based on low order of toxicity was comparable to the control group, which showed 100% development. Considering the low order of toxicity and that it is highly effective in inhibiting growth of solid tumors, the aqueous extracts of leaves of *I. suffruticosa* may be used as an alternative anticancer agent.

Keywords: antitumor – aqueous extract – cytotoxicity – *Indigofera suffruticosa* – toxicity

Introduction

Cancer is the leading cause of mortality worldwide and the failure of conventional chemotherapy to effect major reduction in the mortality indicates that new approaches are critically needed (1). An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents to block the development of cancer in humans (2). A variety of bioactive compounds and their derivatives have been shown to inhibit carcinogenesis in a number of experimental systems involving initiation, promotion and progression (3,4). Plants, vegetables and herbs used as folk and traditional medicine have been accepted currently as one of the main sources of cancer chemoprevention drug discovery and development (5). In Brazil traditional system of medicine supports a growing interest in the pharmacological evaluation of various plants.

Species belonging to the Fabaceae, the *Indigofera suffruticosa* Mill (Fig. 1) have been used as an infusion or decoct (flavor extract by boiling 1 liter of hot water per 5 g of leaves) (6). This plant is found in tropical and subtropical areas and well adapted to growth in semi-arid regions and soil of low fertility (7–9). It occurs in Northeast Brazil and has intensive popular use in the treatment of inflammation (10,11) and other diseases such as epilepsy in human (12) and in animal models (13–15). Recently, embryotoxic effects and antimicrobial activity have been reported (16,17). A chemical investigation of extract of leaves of *I. suffruticosa* in Natural Products Alert (18) and Chemical Abstracts databases have revealed the presence of alkaloids, flavanoids, steroids, proteins, carbohydrates and indigo. Antitumor activities have been reported in several plant species (2,19,20–24), however, up to now, few researches have been done to investigate this traditionally used plant in the recognition of their mechanism, guaranteeing in the future its scientific and therapeutic use. For allopathic drug development, even when traditional

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Figure 1. *Indigofera suffruticosa* Mill. Aspect of blossomed branch.

formulations are taken into consideration, traditional medical systems are very rarely regarded in the same way (25). The aim of the present study was to carry out a brief basic toxicological analysis and establish the safety of aqueous extract of leaves of *I. suffruticosa* focusing on its cytotoxic and antitumor activities.

Methods

Plant Material

The leaves of *I. suffruticosa* were collected in June 2000 in São Caetano, State of Pernambuco/Brazil and authenticated by the Biologist Marlene Barbosa from the Botany Department, Federal University of Pernambuco (UFPE), Brazil. A voucher specimen number 32 859 has been deposited at the Herbarium of the Botany department.

Mouse Experiments

Male Swiss albino mice weighing 20–25 g were purchased from the animal house of Centro de Pesquisas Aggeu Magalhães–Pernambuco, Brazil. They were housed in standard environmental conditions of temperature, humidity and under clear and dark cycles of 12 h. The mice were fed on diet of the biotery (LABINA Purina Brazil) and water *ad libitum*. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Cell Lines

Sarcoma 180, solid tumor and HEp-2 (human epidermoid cancer cell) cell lines were obtained from Department of Antibiotics/UFPE, Brazil. The solid tumor was maintained in Swiss albino mice and the HEp-2 cells in minimum essential medium, DMEM (Dulbeccos' Modified Eagle medium).

Preparation of Extracts

Two extracts were prepared by infusion and maceration from 150 g of leaves of *I. suffruticosa*. The leaves were weighed; chopped and extracted with solvents and water. The infusion was prepared with 75 g of fresh leaves in 2 × 200 ml of increasing polarity solvents (hexane, ethyl acetate and methanol) at 40°C for 10 min and removing solid matter by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water using the same conditions, and the maceration obtained following the above-mentioned process at room temperature (28°C) overnight. The solvents were removed by rotary evaporation. The yields (w/w) of the infusion and the maceration were hexane (0.67 and 0.74%), ethyl acetate (0.39 and 0.34%) methanol (3.9 and 1.88%) in terms of newly collected plant material. After lyophilization, the aqueous extracts yielded 4.20 and 1.75% and the dried material was stored at −20°C (26,27). The aqueous extracts by infusion and maceration were used for cytotoxic and antitumor activities.

In vitro Cytotoxicity

The HEp-2 cells (human epidermoid cancer cells) were investigated by the MTT method [3-(4,5-dimetyl (thiazol-2-yl)-2,5 diphenyltetrazolium bromide)]. Extracts with concentrations more than 30 mg ml^{-1} are considered citotoxic (28). They were trypsinized, counted and prepared in a suspension with 10^5 cells per ml of DMEM and distributed in a plate with 96-wells, which was incubated at 37°C in a humidified atmosphere for 24 h. The aqueous extracts of leaves of *I. suffruticosa* obtained by infusion and maceration were dissolved in DMSO (dimethylsulfoxide) in concentrations of 6.25, 12.5, 25 and 50 μg ml^{-1} and put in the wells with the HEp-2 cells. Each concentration was tested in quadruplicate. As the control, DMEM with DMSO was used. After 72 h, 25 μl of MTT and 5 mg ml^{-1} of PBS was added to the wells and the plate was incubated for 2 h. The optical density was measured at 550 nm with ELX 800 reader.

Acute Toxicity Study in Mice

Thirty-six healthy Swiss albino mice male, weighing 20–25 g, were divided in groups of six. The animals were on fasting for 18 h before being submitted to the experiment. The aqueous extract of *I. suffruticosa* by infusion was dissolved/suspended in distilled water and administered by the intraperitoneal (i.p.) route in doses of 50, 150, 300, 600, 1200 and 2400 mg kg^{-1}. The general behavior of mice was observed continuously for 1 h after the treatment and then intermittently for 4 h, and thereafter over a period of 24 h (29). The mice were further observed for up to 14 days following treatment for any signs of toxicity and deaths. The LD_{50} value was determined according to the method of Litchfield and Wilcoxon (30).

Antitumor Assays

Male albino Swiss mice were divided into six groups of five animals. In all groups 0.3 ml of Sarcoma 180 cells from a solid tumor (around 3×10^6 cells) i.p were injected. After 48 h of the implant, 0.2 ml kg^{-1} i.p. of saline solution was administered in control group (groups 1 and 2). The chemotherapy was initiated making use of the aqueous extract of leaves of *I. suffruticosa* by infusion (groups 3 and 4) and by maceration (groups 5 and 6) in daily concentration of 50 mg kg^{-1} i.p. for seven consecutive days. The dose of the extract was based on the $LD_{50.}$ On the 8th day, the mice were sacrificed for analysis of tumor development.

Statistical Analyses

The experimental results of antitumor assays were expressed as Median (min–max). Data were assessed by ANOVA followed by Kruskal–Wallis. $P < 0.001$ was considered as statistically significant.

Results

Cytotoxic Effect of Aqueous Extracts of *I. suffruticosa*

Aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration in concentrations of 6.25–50 μg ml^{-1} were tested on HEp-2 cell lines by MTT method. The aqueous extracts did not produce any cytotoxic effect to HEp-2 cell lines (>30 μg ml^{-1}) when compared with control DMEM and DMSO (Table 1).

Acute Toxicity in Mice

There were no deaths but some low signs of toxicity were observed after i.p. injection of aqueous extracts of leaves of *I. suffruticosa* by infusion at any dose level up to the highest dose tested (2400 mg kg^{-1}) whose effects were more pronounced (Table 2). Some adverse effects, such as agitation, piloerection, exhaustion and sleepiness, were seen immediately after the i.p. injection while others (irritability, exhaustion, agitation and spasm) were observed later, and they were more pronounced at the higher dose. The acute toxicity (LD_{50}) of aqueous extract of leaves of *I. suffruticosa* by infusion at different doses in mice did not show rates of mortality during 72 h of observation in the preliminary assay.

Antitumor Effects of Aqueous Extracts on Sarcoma 180

The effects of the aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration on Sarcoma 180 is shown in Fig. 2.

Table 1. Cytotoxic activity of aqueous extracts of leaves of *I. suffruticosa* obtained by infusion and maceration on HEp-2 cells

	Infusion				Maceration			
Concentration (μg ml^{-1})	6.25	12.5	25	50	6.25	12.5	25	50
Inhibition (%)	15.87	12.92	17.35	19.93	15.87	19.56	22.15	17.35

The extract by infusion reduced significantly the mean volume of Sarcoma 180 via i.p. administration in dose of 50 mg kg^{-1} 64.53% [0.64 mg (0.01–22.10)] and the maceration extract reduced 62.62% [(0.64 mg (0.01–2.10)] at the same dose when compared to the mean volume of the tumor of control group treated with saline solution which showed a tumor development of 100% [1.97 (1.27–2.87)]. As shown, the inhibition was dose-dependent and similar to that promoted by the maceration and infusion extracts at the same dose.

Discussion and Conclusions

The treatment of cancer may benefit from the introduction of novel therapies derived from natural products. Natural products have served to provide a basis for many of the pharmaceutical agents in current use in cancer therapy (31). The use of chemotherapeutic drugs in cancer involves the risk of life threatening host toxicity. The search, therefore, goes on to develop the drugs, which selectively act on tumor cells. The plants belonging to family Fabacea have medicinal properties, especially the plant *I. suffruticosa* (11,16,17). The present investigation shows that aqueous extracts revealed absence of

Table 2. Acute toxicity of lyophilized aqueous extract of leaves of *I. suffruticosa* by infusion

Dose (mg kg^{-1})*	D/T	Toxic symptoms
50	0/6	Agitation, piloerection, exhaustion, sleepiness
150	0/6	Piloerection, exhaustive agitation
300	0/6	Irritability, exhaustive agitation, spasms
600	0/6	Irritability, exhaustion agitation, spasms
1200	0/6	Irritability, exhaustion agitation, spasms
2400	0/6	Irritability, exhaustive agitation, spasms

D/T = Dead/treated mice: 0/6 = toxic symptoms during the observation period after the injection. Mice in each dose group ($n = 6$) were carefully examined for any signs of toxicity (behavioral changes) for 14 days.
*The lyophilized aqueous extract of leaves of *I. suffruticosa* was dissolved in distilled water and administered as single i.p. dose to group of mice.

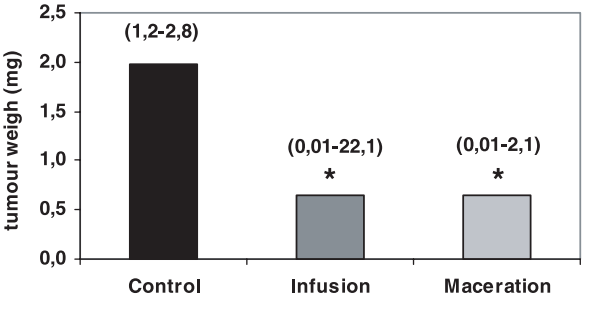

Figure 2. Antitumoral activity of the aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration in Swiss albino mice after implant of the Sarcoma 180. In the control animals, 0.2 ml of saline solution was injected and in the treated groups (infusion and maceration), 50 mg kg^{-1} i.p. during 7 days. The results of the antitumoral activity are expressed as the median (min–max). $n = 30$ mice. *$P < 0.001$.

cytotoxic effects, low toxicity dose-dependent and antitumor effect. Our results concerning the aqueous extracts of *I. suffruticosa* leaves obtained by infusion and maceration on HEp-2 cell lines did not reveal cytotoxic potential. Reports showed that cytotoxicity with extracts of leaves of *I. suffruticosa* were not encountered in the literature and it confuses the comparison of these results with others using the same conditions. Extracts of Umbelliferous plants retarded the development of solid and ascites tumors and increased the life span of these tumor-bearing mice. Tritiated thymidine uridine and leucine incorporation assay suggested that fraction acts directly on DNA synthesis (20). Extract of *Emilia sonchifolia* was cytotoxic on tumor cells with reduction in tritiated thymidine but not to normal human lymphocytes (32). Many pharmacological effects observed in animals can extend result in high value of application for the human species. Based on cytotoxicity bioassays, over 400 compounds have been isolated from plants, marine organisms and microorganisms between 1996 and 2000 (33). As a result, this is the main reason for large use of toxicological test for determination of toxicity and safety when using drugs (34). The aqueous extract of *I. suffruticosa* leaves obtained by infusion showed low order of toxicity in mice at least up to the maximum dose of 2400 g kg^{-1} and no death but some low signs of toxicity.

A number of species of *Indigofera* contain an amino acid indospicine (35,36), and nitropropanoyl esters of glucose (37,38) as some natural toxic product. Phytochemical analysis suggests that the presence of biologically activity compounds (alkaloids, steroids, flavanoids, proteins—lectin, carbohydrates, indigo, etc.) in the aqueous extracts of leaves of *I. suffruticosa* could be correlated to anti-inflammatory and antimicrobial activities (11,21). Biological activities of the compounds detected in the aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration could be linked to antitumor activity.

Aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration were used on Sarcoma 180 in mice at dose of 50 mg kg^{-1} i.p. The aqueous extracts showed a tumor reducing activity. Our results concerning the aqueous extracts of leaves of *I. suffruticosa* corroborate with those found in aqueous extract of *Emblica officinalis* (Eupherbiacase), which showed a reduction of solid tumor in mice. The extract inhibits cell cycle regulating enzymes cdc 25 phosphatase (16,21).

Actual mechanism by which aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration showed antitumor activity is not known. The results of the present study indicate that antitumor activity of aqueous extracts of *I. suffruticosa* may be due to its interference with cell development. This will be the object of future researches as modulating lipid peroxidation and augmenting antioxidant defense system (superoxide dismutase and catalase) (2,19,24), DNA synthesis (20), interaction with cell cycle regulation (21) and non-steroidal anti-inflammatory activity (1). More recently, molecular biomarkers have been used for oncological management as prohibitin, mortalin and HSP 60/HSP 10 (39); hyaluronic acid (HA) (40); epidermal growth factor receptor (EGFR) and cytokeratins (41); research of tumor angiogenesis with CD105 antigen (42); alpha-fetoprotein (AFP) (43); cyclooxygenase-2 (COX-2) (44) and immunohistochemical markers, such as CD34 and CD117 (45). Besides assessment of prognostic factors as clinical (tumor growth rate and inflammatory signs), to also analyze parameters like hemoglobin content and red blood cell count, and histological (tumor stage, grading, tumor necrosis, lymph nodes status and margins status).

Same mechanisms of aqueous extract of leaves of *I. suffruticosa* by infusion may be involved in the embryo development in mice (16). The first cleavages in mammalian embryogenesis are symmetrical mitotic divisions that increase the number of blastomeres by partitioning the oocyte without a net change in embryo size. Through the 8-cell stage the blastomeres of murine embryos are totipotent (46–48). The cell cycle of the two-cell embryos in the mouse has duration of 20–26 h with G_1 and S phases that last ~1–2 and 6–7 h; these phases are followed by an extended G_2 phase (12–15 h) and M (mitosis) phase of 1–2 h (49). Many plant extracts have been used as a source of medicinal agents to cure urinary tract infections, cervicitis vaginitis, gastrointestinal disorders, respiratory diseases, cutaneous problems, helmintic infections, parasitic protozoan diseases, and antitumor and antimicrobial (17,50–53). The results of this investigation may improve our understanding in usage of this plant as an alternative anticancer therapy. Identification of the active principles and their mechanisms of action remain to be studied. This is a promising plant for further studies toward drug development.

Acknowledgment

We wish to express our appreciation to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for its financial support.

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Received July 5, 2006; accepted October 30, 2006