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In vitro chemo-preventative activity of *Crotalaria agatiflora* subspecies *agatiflora* Schweinf

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ABSTRACT

Ethnopharmacological relevance: *Crotalaria species* have been widely used in Chinese traditional medicine to treat several types of internal cancers. *Crotalaria agatiflora* is used as a medicinal plant in several African countries for the treatment of bacterial and viral infections as well as for cancer.

Materials and methods: Water and ethanol extracts of the leaves of *Crotalaria agatiflora* were evaluated for cytotoxicity on four cancerous and one noncancerous cell lines, using XTT (Sodium 3′-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) colorimetric assay. Antioxidant activity was determined using DPPH (1,1-diphenyl-2-picryl hydrazyl). Light microscopy (eosin and haematoxylin staining) and flow cytometry (Annexin-V and propidium iodide) were used to evaluate the mechanism of action of the ethanol extract and one of the isolated compounds.

Results: The 50% inhibitory concentration (IC_{50}) of the ethanol extract was found to be 73.9 μg/mL against leukemic U-937 cells. Good antioxidant activity (IC_{50} = 18.89 μg/mL) of the ethanol extract indicated the potential of *Crotalaria agatiflora* as chemo-preventative supplement. A bioassay guided fractionation of the ethanol extract led to the isolation of two pure compounds, namely madurensine and doronenine. Madurensine and doronenine showed moderate cytotoxicity on cancerous U-937 cells (IC_{50} values: 47.97 and 29.57 M respectively). The crude extract treated U-937 cells showed definite signs of cell death during light microscopic investigation, while little apoptosis (10–20%) and necrosis (<2%) were detected in cells treated with the extract or madurensine.

Conclusions: The results indicated that *Crotalaria agatiflora* possesses potential chemopreventative and therapeutic properties. The exact mechanism of action should still be determined in future studies. It is hypothesized that the ethanolic extract as well as madurensine induces autophagy, which in prolonged circumstances may lead to autophagic cell death.

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1. Introduction

Crotalaria L. is one of the largest genera in tropical Africa. The genus includes 690 species that are mainly situated in Africa and Madagascar (Le Roux et al., 2009). Species have also been found in India, United States of America (USA) and China. African countries use *Crotalaria* species (aerial parts), such as *Crotalaria caudate* Welw. Ex. Baker, *Crotalaria retusa* L., *Crotalaria emarginella* Vatke. and *Crotalaria mesopontica* Taub. for treating several types of bacterial and viral infections as well as for wound healing and for the treatment of skin conditions (Vlietinck et al., 1995; Bahar et al., 2006; Maregesi et al., 2007).

Similar uses of the genus are found in India, where the flowers are used to treat eczema and the leaves are placed on cuts to aid the healing process (Ram et al., 2004). Unspecified species of the genus are being used traditionally as decoctions in Ecuador to treat cancer (Tene et al., 2007). In the USA, *Crotalaria pumila* Ortega (aerial parts) is used to treat yellow fever and skin rashes (Adonizio et al., 2006). All plant parts of *Crotalaria sessiliflora* Vatke., *Crotalaria assamica* Benth. and *Crotalaria ferruginea* are being used traditionally in China to treat cancer (Graham et al., 2000). Aerial parts of *Crotalaria agatiflora* Schweinf. are used in Kenya for the treatment of ortitis media, a bacterial infection of ears, as well as for treating sexually transmitted diseases (Njoroge and Bussmann, 2006; Njoroge et al., unpublished data). Researchers had found that this species relieved spasms in dogs, found to be a good relaxant and lowered blood pressure during treatment (Sharma et al., 1967). Due to the variety of biological activity of the genus most importantly being anti-cancer activity, it was decided to focus investigations on *Crotalaria agatiflora* subspp. *agatiflora* for its cytotoxic activity. Between 19 and 35% of cancer-related mortalities are associated with nutritional factors (Russo, 2007; WHO, 2008) and thus the

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cancer preventative activity was also investigated. The aims of the study were to determine the chemo-preventative (anti-cancer and cancer preventative) activity of *Crotalaria agatiflora* subspp. *agatiflora*. In the present study the bioactive principles of the extract were also identified and the mechanism of action of selected samples was investigated.

2. Materials and methods

2.1. Chemicals and reagents

All cell lines, media, trypsin-EDTA, fetal bovine serum (FBS) and antibiotics (penicillin, streptomycin and fungizone) were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA). All plastic consumables used for culturing and analysis were supplied through Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). Vanillin, sephadex, Bouin's fixative, haematoxylin, eosin and xylene were of analytical grade and supplied by Sigma–Aldrich (St. Louis, MO, USA). Solvents, silica and TLC plates were purchased from Merck (Germany). BD Biosciences' Annexin-V-FITC apoptosis kit was purchased from BD Biosciences.

2.2. Methods

2.2.1. Plant material

Crotalaria agatiflora subsp. *agatiflora* leaves were collected in Pretoria, South Africa during February 2009. The plant material was identified by Ms. Magda Nel at the University of Pretoria, and voucher specimen (PRU 096454) was deposited in the Schweickerdt Herbarium (PRU), Pretoria, South Africa.

2.2.2. Extraction

Air dried leaves were mechanically separated. Three different leaf extracts were prepared, i.e. decoction, infusion and ethanolic. The air dried leaves of *Crotalaria agatiflora* subspp. *agatiflora* were homogenized with distilled water and extracted for 24 h twice. The menstruum was freeze dried to yield a brown powder. For the infusion powdered leaves and a vacuum rotary evaporator (water bath 80 °C) were used for extraction (15 min). The menstruum was freeze dried to yield an orange powder. *Crotalaria agatiflora* leaves were exhaustively extracted with distilled ethanol, the menstruum filtered and concentrated under reduced pressure with a vacuum rotary evaporator (Buchi) (Raman and Kang, 2009). The plant extracts were stored in the cold room (0 °C).

2.2.3. Cell cultures

Cells were maintained in culture flasks in complete medium, supplemented with 10% heat-inactivated FBS and antibiotic cocktail (100 U/mL penicillin, 100 μg/mL streptomycin and 250 μg/L fungizone). Cells were grown and maintained in a humidified atmosphere at 37 °C and 5% CO_2.

2.2.4. Cytotoxicity of extracts using XTT kit

Cytotoxicity was measured by the XTT method using the Cell Proliferation Kit II as described by the method of Zheng et al. (2001). Briefly, cells (100 μL) were seeded (concentration 1×10^5 cells/mL) into a microtitre plate and incubated for 24 h to allow the cells to attach. Samples were diluted (1.563–400 μg/mL), added to the plates and incubated for 72 h. The positive drug control, actinomycin D was included. After 72 h incubation XTT was added at a final concentration of 0.3 mg/mL and incubated for 2–3 h. Absorbance of the developed colour was spectrophotometrically quantified using a multi-well plate reader, which measured the optical density at 450 nm with a reference wavelength of 690 nm. The samples were tested in triplicate. The inhibitory concentration of 50% of the cell population (IC_{50} values) was defined as the concentration of the sample at which absorbance was reduced by 50%. The results were statistically analyzed with GraphPad Prism 4 software. The selectivity index (SI) of the extract was defined as the ratio of cytotoxicity on Vero cells to cancerous cells (Mena-Rejon et al., 2008).

2.2.5. Antioxidant activity – DPPH radical scavenging

The method of Du Toit et al. (2001) was followed with some modifications. Briefly the samples were prepared as stock solutions of 10 mg/mL. The concentrations tested for the plant extracts ranged between 3.906 and 500 μg/mL and the concentration of vitamin C between 0.781 and 100 μg/mL. All the samples were prepared in triplicate. Ninety microlitres DPPH (0.04 mg/mL) was added to all of the wells, except for the colour control in which the DPPH was substituted with distilled water. The plates were left in the dark to develop at room temperature for 30 min. The radical scavenger capability of the samples was determined by using a multi-well plate reader to measure the decolouration of DPPH at 515 nm, using KC Junior software. The IC_{50} values for each sample were determined by using GraphPad Prism 4 software.

2.2.6. Isolation of bioactive compounds using bioassay-guided fractionation

A total of 50 g ethanolic extract was subjected to liquid–liquid partition. The extract was dissolved in 80% methanol. The filtrate was acidified using 5% HCl, shaken twice with dichloromethane (DCM) and then ammonia solution (NH_4OH) was added to the aqueous solution till pH ∼ 12.0. The aqueous solution was shaken twice again with DCM after which the DCM fractions were concentrated using a rotavapor. Sixteen grams alkaloidal fraction was subjected to silica gel column chromatography (CC, size 10 cm × 20 cm) using DCM/MeOH of increasing polarity (0–10%). A total of 40 fractions were collected and pooled based on their thin layer chromatography (TLC) profile (8 fractions). Based on the cytotoxicity results, fraction 3 and 4 were selected for the identification of bioactive principles. Fraction 3 was subjected to sephadex column chromatography (CC, 4 cm × 15 cm) using EtOH as an eluent. Collected fractions were spotted on TLC plates using $CHCl_3$:MeOH:NH_4 (95:5:0.1) as eluent. After the TLC plates were analyzed, similar fractions were combined which resulted in three major subfractions. Subfraction 3.3 contained only three major bands on the TLC plate. Subfraction 3.3 was further purified using preparative TLC. Thirty milligram of Subfraction 3.3 was spotted on three TLC plates and developed using $CHCl_3$:MeOH:NH_4OH (95:5:0.1) as eluent. Three different bands were observed under UV which was scratched off the aluminium plates using a blade. The silica gel powder was eluted twice with distilled ethyl acetate and three times with distilled MeOH. The structural elucidation of isolated compound (only Band III, 24 mg) was identified by physical (mp. $[\alpha]_D$) and spectroscopic (^1H and ^{13}C NMR) data (Compound I). Fraction 4 yielded a white crystalline compound which was washed first with ethyl acetate: hexane (50:50), followed by methanol (100%). The precipitated crystals were developed on TLC and showed one clear spot; hence the sample was subjected to NMR analysis (Compound II). Cytotoxicity was carried out against U-937 and Vero cells after which antioxidant activity was also conducted as previously described, with the exception that the compounds were tested between 0.781 and 100 μg/mL.

2.2.7. Cell morphology–light microscopy (haematoxylin and eosin staining)

Leukemic U-937 cells were exposed to 73.9 μg/mL (IC_{50}) and 147.8 μg/mL ($2IC_{50}$). Vero cells were exposed to ethanol extract at 73.9 μg/mL (IC_{50}) and 147.8 μg/mL ($2IC_{50}$) and additionally to 352.4 μg/mL (IC_{50}) and 704.8 μg/mL ($2IC_{50}$). Madurensine, one of

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