

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

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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 *C. 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-picrylhydrazyl). Light microscopy (eosin and hematoxylin 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 (IC₅₀) of the ethanol extract was found to be 73.9 µg/mL against leukemic U-937 cells. Good antioxidant activity (IC₅₀ = 18.89 µg/mL) of the ethanol extract indicated the potential of *C. 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 (IC₅₀ 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 *C. agatiflora* possesses potential chemopreventative and therapeutic properties. The exact mechanism of action should still be determined in future studies. It is hypothesised that the ethanolic extract as well as madurensine induces autophagy, which in prolonged circumstances may lead to autophagic cell death.

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 otitis media, a bacterial infection of ears, as well as for treating sexually transmitted diseases (Njoroge and Bussmann, 2006 and Njoroge *et al.*, 2004). 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* subsp. *agatiflora* for its cytotoxic activity. Between 19 and 35% of cancer-related mortalities are associated with nutritional factors (Russo, 2007 and WHO, 2008) and thus the 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* subsp. *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 BDBiosciences.

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* subsp. *agatiflora* were homogenized with distilled water and extracted for 24h 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 minutes). 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) (Rahman 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% CO₂.

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 1x10⁵ cells/mL) into a microtitre plate and incubated for 24h to allow the cells to attach. Samples were diluted (1.563-400 µg/ml), added to the plates and incubated for 72h. The positive drug control, actinomycin D was included. After 72h incubation XTT was added at a final concentration of 0.3 mg/ml and incubated for 2-3 hours. 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 (IC₅₀ values) were 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 10mg/mL. The concentrations tested for the plant extracts ranged between 3.906 - 500 µg/mL and the concentration of vitamin C between 0.781 - 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 minutes. The radical scavenger capability of the samples were determined by using a multi-well plate reader to measure the decolouration of DPPH at 515nm, using KC Junior software. The IC₅₀ 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 (NH₄OH) 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 x 20cm) 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 x 15 cm) using EtOH as an eluent. Collected fractions were spotted on TLC plates using CHCl₃: MeOH: NH₄ (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 CHCl₃: MeOH: NH₄OH (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 - 100 $\mu\text{g/mL}$.

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

Leukemic U-937 cells were exposed to 73.9 $\mu\text{g/mL}$ (IC_{50}) and 147.8 $\mu\text{g/mL}$ (2IC_{50}). Vero cells were exposed to ethanol extract at 73.9 $\mu\text{g/mL}$ (IC_{50}) and 147.8 $\mu\text{g/mL}$ (2IC_{50}) and additionally to 352.4 $\mu\text{g/mL}$ (IC_{50}) and 704.8 $\mu\text{g/mL}$ (2IC_{50}). Madurensine, one of the isolated compounds, was retained for more crucial analysis. U-937 cells are suspension cells and therefore it was necessary to manipulate the cells to adhere to the coverslips. U-937 cells were washed three times with buffer to and resuspended in complete medium lacking FBS. This treatment allowed U-937 cells to adhere to the coverslip. Exponentially growing U-937 and Vero cells were seeded at one million and 250,000 cells per well respectively on sterilized coverslips. After 24h incubation (37°C , 5% CO_2), U-937 cells were exposed to 73.9 $\mu\text{g/mL}$ (IC_{50}) and 147.8 $\mu\text{g/mL}$ (2IC_{50}) of ethanolic extract including vehicle-treated control (0.74%), actinomycin D (2.51 mM) and cells propagated in growth medium. Vero cells were exposed to 73.9 $\mu\text{g/mL}$ (IC_{50} of U-937 cells) and 147.8 $\mu\text{g/mL}$ (2IC_{50} of U-937 cells), 352.4 $\mu\text{g/mL}$ (IC_{50}) and 704.8 $\mu\text{g/mL}$ (2IC_{50}) of ethanolic extract including vehicle-treated control (3.5%), actinomycin D (100.43 mM) and cells propagated in growth medium. The cells were incubated for 72h at 37°C . Cells were fixed in Bouin's fixative (60 minutes) and stained using standard haematoxylin and eosin staining procedures (Stander *et al.*, 2009). The cells were investigated using Nikon Stereo Light microscope equipped 1.4 Apo oil lense (Microscopy Unit, University of Pretoria). The magnification was x 1000.

2.2.8 Apoptosis detection – flow cytometry (Annexin-V and Propidium iodide staining)

Exponentially growing U-937 cells were seeded at 0.5×10^6 cells per 25 cm^2 flask. Cells were exposed to 73.9 $\mu\text{g/mL}$ (IC_{50}) and 147.8 $\mu\text{g/mL}$ (2IC_{50}) of the ethanolic extract and exposed to 47.97 M madurensine respectively and incubated for 72h. The analysis included vehicle-treated control (0.74%) and actinomycin D (0.25 M) treated cells. One million cells were double-stained with annexin-V and propidium iodide, according to the manufacturer's instructions. Annexin-V and propidium iodide fluorescence were measured with a BD FACS Aria flow cytometer (BD

Biosciences) equipped with an air-cooled argon laser excited at 488nm (Stander et al, 2009) at the Department of Biochemistry, University of Pretoria, with guidance from Wayne Barnes. The annexin-V signal was detected using the 530/30 BP filter and the PI signal using 585/42 BP filter. Data from at least 10,000 cells were analyzed with BD FACS Diva Software Version 6.1 (BD Biosciences) (Kang *et al.*, 2009).

3. Results

2.1 Cytotoxicity of crude extracts

The National Cancer Institute of the United States of America (2010) suggested that IC₅₀ values below 20 µg/mL was active, while many authors have suggested extracts have anti-cancer potential when the IC₅₀ value is below 100 µg/mL. Infusion and decoction extracts were not active, with IC₅₀ values higher than 400 µg/mL. The ethanol extract was the most active with varying IC₅₀ values between 74.94 and 243.3 µg/mL. U-937 cells were the most sensitive cancerous cells treated with the ethanolic extract and positive control. It was also found that the ethanol extract was the most selective comparing IC₅₀ values of U-937 and Vero cells. The selectivity index (SI) of extracts was defined as the ratio of cytotoxicity on normal healthy cells to cancerous cells. In general it is considered that the biological efficacy is not due to cytotoxicity when the SI value is ≥ 10 (Mena-Rejon *et al*, 2008). The Infusion and Decoction extracts didn't show any preference to any of the cell lines. The ethanolic extract had the best selectivity values as compared to all of the samples tested with U-937 (4.77), SNO (3.11), HeLa (2.30) and MCF-7 (1.45), excluding actinomycin D that had an SI value of 40 using U-937 cells. Actinomycin D had SI values lower than 1.2 on all the rest of the cancerous cells. The pure compound's toxicity is not due to cytotoxicity, but rather due to another type of mechanism, such as the induction of apoptosis by forming stable complexes with DNA and interfering with DNA-dependent RNA synthesis.

Table 1: Cytotoxicity of extracts

Sample / Cell line	IC ₅₀ ^a (µg/mL)				
	U-937	HeLa	SNO	MCF-7	Vero
Infusion	>400	>400	>400	>400	>400
Decoction	>400	>400	>400	>400	>400
Ethanol	73.94 ± 1.06	153.3 ± 0.75	113.2 ± 2.43	243.3 ± 6.2	352.4 ± 5.9
Actinomycin D ^b	2.51 ± 0.06 mM	94.16 ± 1.25 mM	90.39 ± 5.0 mM	150.65 ± 1.25 mM	100.43 ± 36.41 mM

^a Fifty percent inhibitory concentration

^b Positive drug control

3.2 Determination of antioxidant activity

All three extracts showed dose-dependent responses. Both water extracts showed nearly identical capacity of DPPH reduction, while the ethanolic extract was the most effective in free radical scavenging. All three samples demonstrated dose-dependent responses (Fig. 1). The IC₅₀ values were as follows: Ethanolic 18.89 ± 0.305 µg/mL, Decoction 27.31 ± 1.59 µg/mL and Infusion 29.63 ± 1.59 µg/mL. Vitamin C, the positive control had an IC₅₀ of 240.94 ± 0.18 mM.

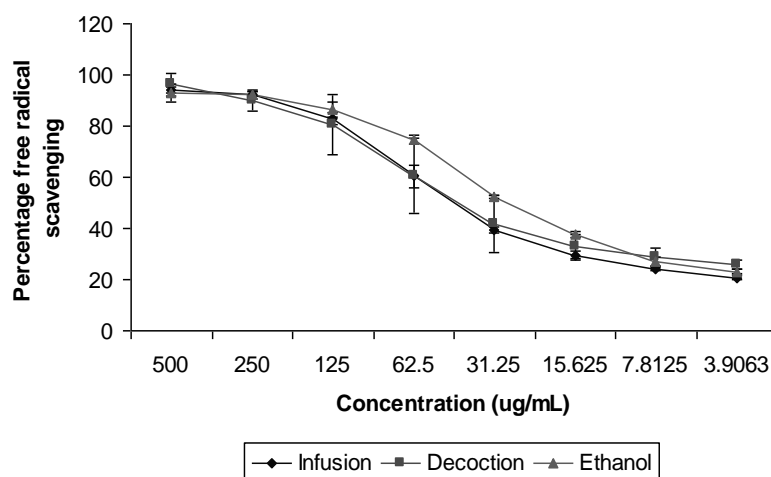


Fig. 1 Anti-oxidant activity of extracts

3.3 Isolated compounds via bioassay-guided fractionation

Two compounds were isolated, both belonging to pyrrolizidine alkaloids. Compound 1 was isolated from the total alkaloidal fraction using silica column chromatography. The compound was identified as doronenine (1,2 – Dihydro bulgarsenine), based on NMR data (¹H and ¹³C). The NMR data for the compound was similar with those reported for the same compound in literature (Roder *et al.*, 1980). This is the first report of doronenine being isolated from *Crotalaria agatiflora* subsp. *agatiflora*. Compound II was isolated from Fraction 4 and identified as madurensine based on spectroscopic analysis reported by previous researchers (Verdoorn and Van Wyk, 1992). Madurensine had been previously identified in *Crotalaria agatiflora*, *Crotalaria rosenii*, *Crotalaria madurensis*, *Crotalaria laburnifolia* and *Crotalaria agatiflora* subsp *imperialis* (Atal and Kapur, 1966, Abegaz *et al.*, 1987 Asres *et al.*, 2004 and Flores *et al.*, 2009) and was found together with trans-anacrotine to be the only alkaloids in the seeds of *Crotalaria capensis* (Verdoorn and Van Wyk, 1992) (Fig. 2).

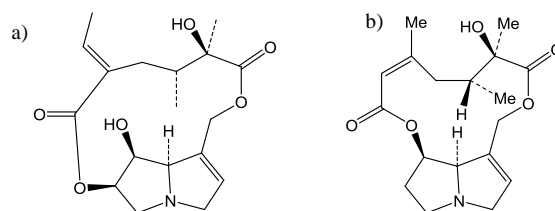


Fig. 2 Chemical structures of madurensine (a) and doronenine (b)

Madurensine had an IC_{50} value of 47.97 ± 6.3 M, while doronenine had an IC_{50} value of 29.57 ± 0.916 M against U-937 cells (Fig. 3a). Actinomycin D had an IC_{50} value of 2.51 ± 0.063 mM. Madurensine had been screened for anti-cancer activity by the National Cancer Institute (NCI). Different yeast stains such as *mlh1 rad18*, *bub3*, *cln2 rad14*, *sgs1 mgt1*, *mec2-1* and *rad50* were used to test the compound's anti-cancer activity. The bioassay is based on growth inhibition of yeast strains with defined genetic alterations. Compound treatments which inhibited the growth of the yeast by 70% were considered active. All strains tested negative for anti-cancer activity (PubChem, 2009). To our knowledge no data is available for any biological activity of doronenine. Vero cells were less susceptible to the influence of doronenine and madurensine as compared to that of the compounds on U-937 cells (Fig. 3b). Madurensine and doronenine exhibited an estimated IC_{50} value of 7443.69 ± 1.17 and 946.79 ± 0.58 M respectively (calculated with GraphPad Prism 4). Actinomycin D had an IC_{50} value of 100.43 ± 36.41 mM. Madurensine had a selectivity index (SI) value of 155.2 while doronenine had an SI value of 32. Although doronenine was more active than madurensine against U-937 cells, it was less selectively cytotoxic. Both compounds showed weak DPPH scavenging potential at the highest concentration tested. Both these compounds' IC_{50} values were higher than $100 \mu\text{g/mL}$.

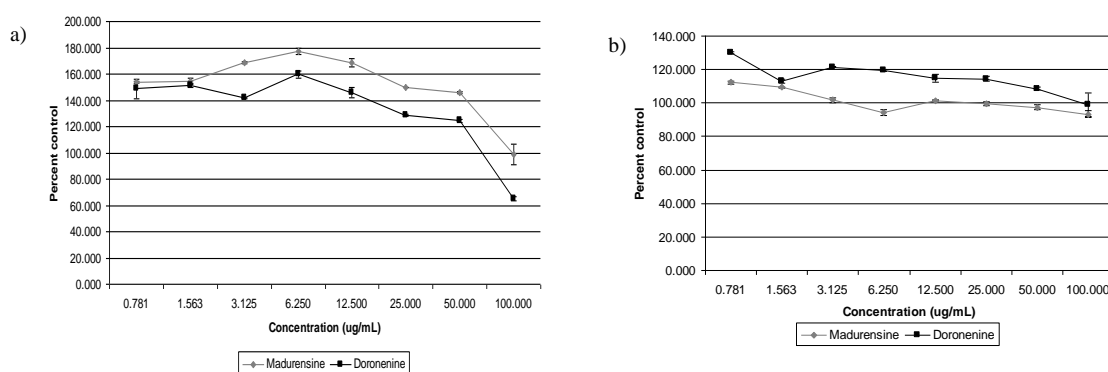


Fig. 3 Dose-response curves of madurensine and doronenine on U-937 cells (a) and Vero cells (b)

3.4 Light microscopy

3.4.1 U-937 cells

Large multiple nuclei were observed in the present study (Fig. 4a). The cells had intact cell membranes and large amounts of cytoplasm. Vehicle control cells were viable and still able to grow (Fig. 4b). Actinomycin D (2.51 mM) showed severe signs of cell death (Fig. 4c) and the density of cells decreased as compared to the untreated cells which was an indication that cells detached during incubation. Nuclear material of treated cells, chromatin condensation and fragments were visible. *Crotalaria agatiflora* treated U-937 cells revealed an increase in morphological features of cell death in a dose-dependent manner, which included decreased cell density, hypercondensed chromatin, apoptotic bodies and shrunken cells (Fig. 4d and e). Those features are characteristic of apoptosis and autophagy.

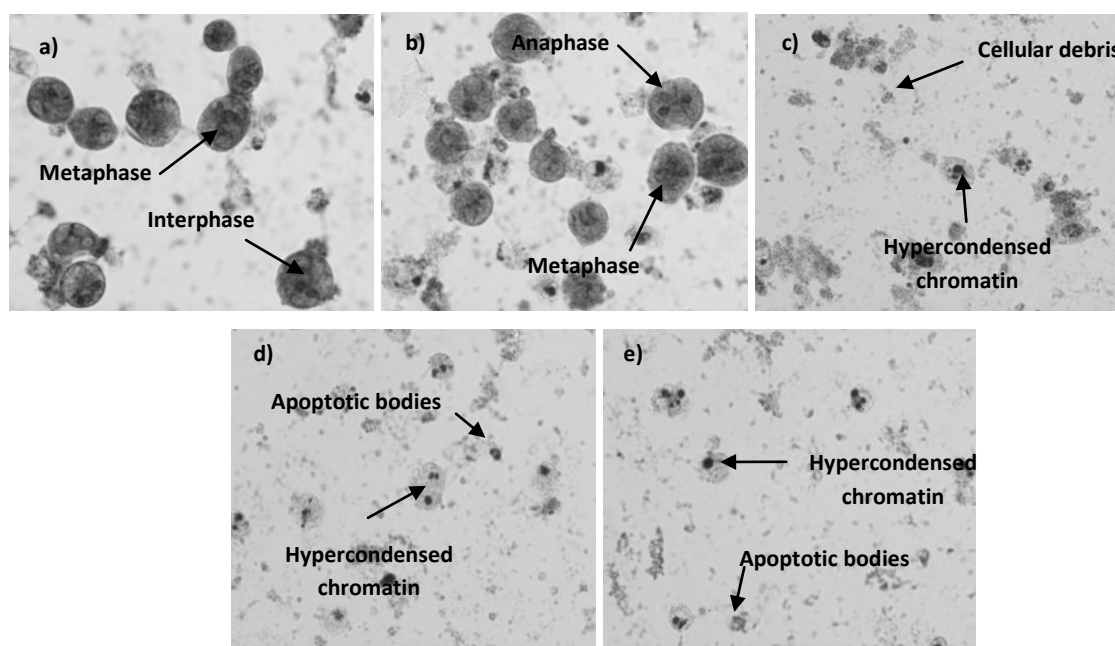


Fig. 4 Haematoxylin and eosin staining of U-937 cells, medium control (a), DMSO (b), actinomycin D (c), 73.9 µg/mL extract treated (d) and 147.8 µg/mL extract (e) treated cells.

3.4.2 Vero cells

Vehicle control cells (3.5%) were viable (Fig. 5b) as compared to untreated Vero cells (Fig. 5a). Actinomycin D (100.43 mM) showed severe signs of cell death (Fig. 5c). Non-cancerous Vero cells revealed minimal signs of cell death when the cells were treated with 73.9 µg/mL and 147.8 µg/mL (IC₅₀ and twice the of IC₅₀ of U-937 cells) of the ethanolic extract (Fig. 5d and 5e). Cells treated with 352.4 µg/mL and 704.8 µg/mL of the ethanolic extract showed dose-dependent signs of

cell death. Those signs included reduction in cell size and hypercondensed chromatin (Fig. 5f and 5g).

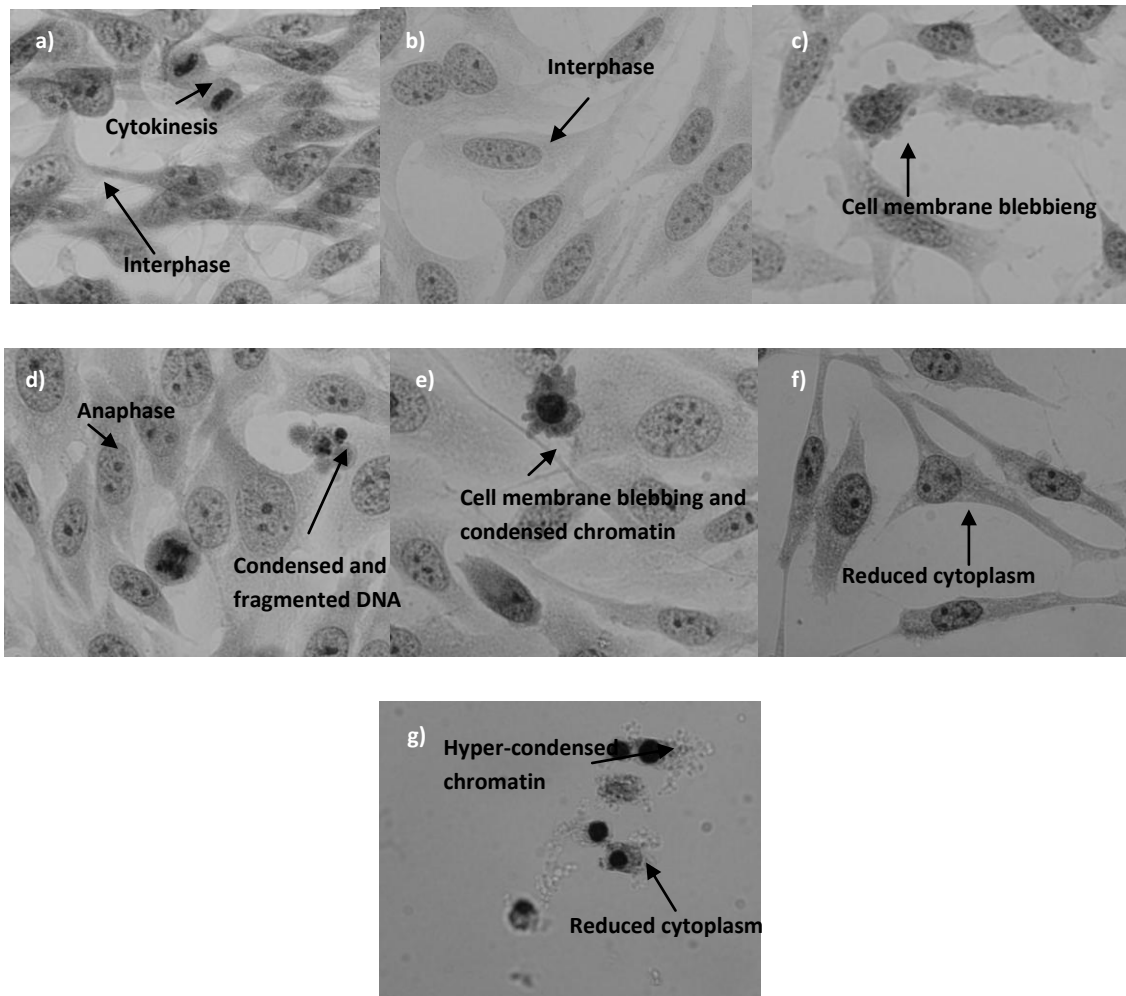


Fig. 5 Haematoxylin and eosin staining of Vero cells in medium (a), DMSO (b), actinomycin D (c), 73.9 µg/mL extract (d) 147.8 µg/mL extract (e), 352.4 µg/mL extract (f) and 708.4 µg/mL extract treated cells (g)

3.4.3 Apoptosis detection analysis after 72h incubation

Annexin-V can be detected in both early and late stages of apoptosis, while PI intercalates DNA during late stages of apoptosis and necrosis. Viable cells were negative for both Annexin-V and PI (lower left quadrant), early apoptotic cells were positive for Annexin-V and negative for PI (lower right quadrant), late apoptotic cells displayed both positive Annexin-V and PI binding (upper right quadrant) and necrotic cells were positive for PI binding and negative for Annexin-V (upper left quadrant). After treatment for 72 hours the percentages of combined early and late induced apoptosis by 73.9 µg/mL and 147.8 µg/mL of the crude ethanolic extract and 47.97 M madurensine,

were 6.7%, 17.6% and 3.5% respectively, while vehicle treated (0.74%) apoptotic cells was 2.3%. Apoptosis was thus insignificantly induced in all samples tested. These results suggested that the anti-proliferation effect of the samples were mediated insignificantly by the induction of apoptosis (Fig. 6).

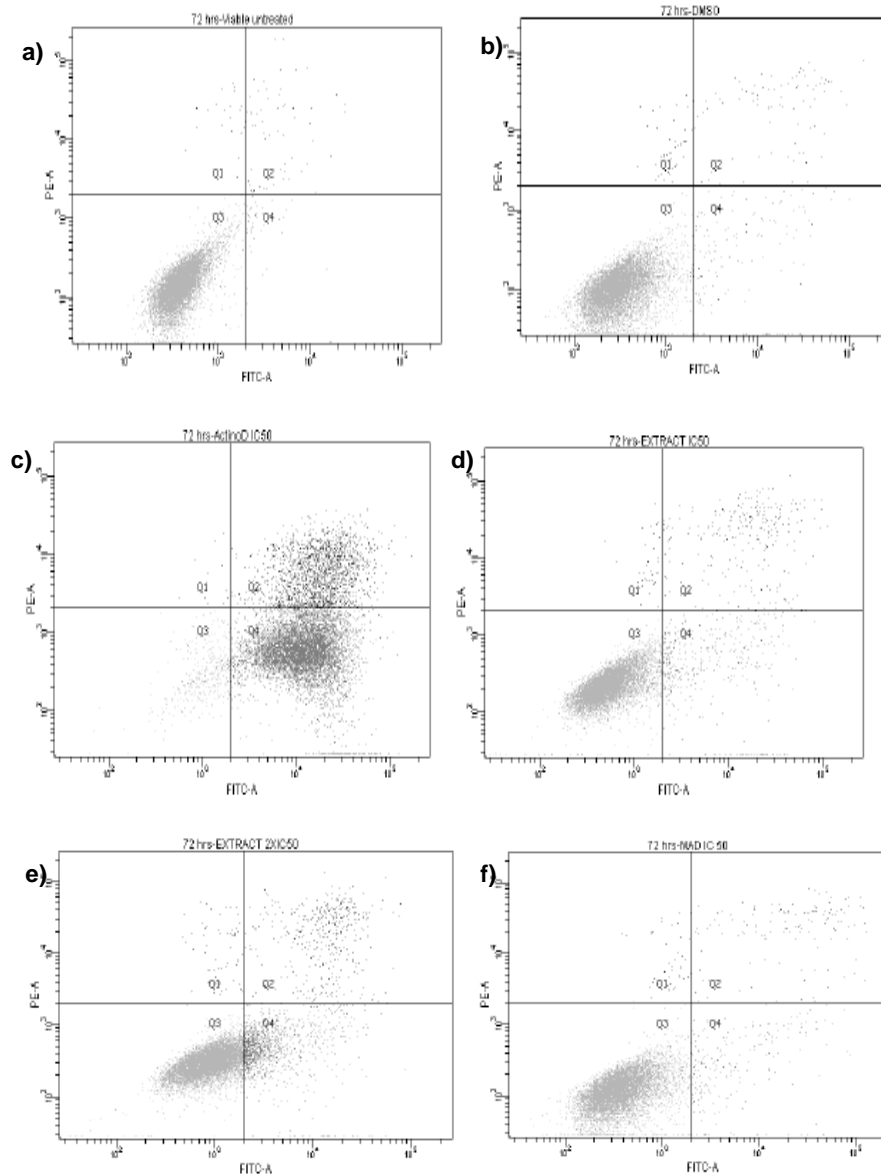


Fig.6 Annexin-V (FITC) versus Propidium iodide (PE) dot plots of: a) untreated U-937 cells, b) DMSO, c) actinomycin D, d) 73.9 µg/mL extract, e) 147.8 µg/mL extract and f) madurensine treated cells

4. Discussion

By using the IC₅₀ values obtained for the ethanolic extract it can be said that the survival rate of the cells was MCF-7 > HeLa > SNO > U-937. The Vero cells were perceived as normal healthy cells, although these cells have been transformed to immortalize them. Overall the water extracts of *Crotalaria agatiflora* performed poorly during the determination of cytotoxicity having similar IC₅₀ values, being higher than 400 µg/mL. This inability of water extracts to kill cancerous cells at low concentrations may be due to the type of compounds extracted during the extraction process.

Water is a polar molecule which in theory will then be able to be used when polar compounds are being extracted, such as sugars, amino acids and glycosides (Houghton, 2008). At the end it was determined that using water as extraction solvent for *Crotalaria* leaves will have poor anti-cancer activity. These findings are in contrast with the traditional uses of *Crotalaria spp.* in Ecuador for the use of fresh leaves that are infused and used to treat cancers (Tene *et al.*, 2007). In China a variety of *Crotalaria spp.* are used for treating cancers. Unfortunately little information for preparation of extracts for treatments had been documented. Ethanol is a very good extractant, thus it can be postulated that alkaloids and pyrrolizidine alkaloids may have caused the cytotoxicity of the tested cells. *Crotalaria* is known to have high concentrations of alkaloids (Graham *et al.*, 2000). The ethanol extract had the highest SI value on U-937 cells, as compared to the other extracts and against the other cell lines.

In previous studies conducted on *Bidens pilosa* it was found that the ethanol extract had an IC₅₀ value of 80.93 µg/mL using the DPPH assay (Chiang *et al.*, 2004). Many other crude extracts had been tested previously for their antioxidant activity, as reported briefly by Drewes *et al.* (2008). It was found that *Hypoxis hemerocallidea* extract; another traditionally used plant of South Africa had an IC₅₀ value of 75 µg/mL when it was determined by TBA assay. It has been reported that olive leaf oil has an IC₅₀ value of more than 30 µg/mL, while green tea has an IC₅₀ value of 16 µg/mL. Comparing all of the above mentioned results with *Crotalaria agatiflora*, it is clear that *Crotalaria agatiflora* had better antioxidant activity than *Bidens pilosa* and *Hypoxis. hemerocallidea*. On the other hand the water extracts of *Crotalaria agatiflora* had similar antioxidant potential as olive leaf extracts, while the ethanol extract had similar antioxidant activity as compared to green tea. Most chemotherapy drugs are inducing the production of reactive oxygen species within the human body, thus forming an important part of the mechanism of action of many of these drugs such as doxorubicin. Thus the question should be asked whether plant extracts could have the ability to be cytotoxic and at the same time have protective properties such as good antioxidant potential.

When cancerous U-937 cells and non-cancerous Vero cells morphological changes were compared, we found that at 73.9 $\mu\text{g/mL}$ the U-937 cells were much more susceptible and sensitive to the treatments compared to the same concentration on Vero cells. As observed by Chinkwo (2005), who explored cervical carcinoma (Caski) and Chinese hamster ovary (CHO) cells treated with *Sutherlandia frutescens* (popular anti-cancer plant), the cells in the present study at the respective IC_{50} values had condensed nuclei and decreased amount of cytoplasm. Conclusions are in agreement with the conclusions made by Stander *et al.* (2009) who observed similar selectivity between cancerous breast adenocarcinoma (MCF-7) and non-cancerous epithelial mammary gland (MCF-12A) cells treated with aqueous extracts of *Sutherlandia frutescens*. In the present study, the affects of treatment were much more severe in U-937 cells and thus the mechanism of action was determined in U-937 cells. It should be mentioned that the results found with light microscopy was insufficient in determining the type of cell death, due to the fact that apoptosis and autophagy looks very similar in light microscopy investigations.

To demonstrate the mechanism of cell death, the effect of the ethanolic extract was tested at 73.9 $\mu\text{g/mL}$ (IC_{50}) and 147.8 $\mu\text{g/mL}$ (2IC_{50}) and madurensine at 47.97 M (IC_{50}) to determine the percentage binding of Annexin-V-FITC and PI. After 72 hours, untreated cells were 98.8% unstained by Annexin-V and PI and thus viable, with only minute percentages of cells in stages of cell death which was similar to the findings observed by Stander *et al.* (2009) who explored MCF-7 cells during flow cytometric analysis. Viability obtained during the analysis of untreated MCF-7 cells was 91.4%. The increased viability in the U-937 cells could be due to the fact that MCF-7 cells were trypsinized to detach the cells from the flask surfaces. During trypsinaztion cells can be damaged due to the nature of the enzyme trypsin. It was found that the viability (97.3%) decreased slightly after 72h incubation with 0.74% DMSO in the present study. This decrease was small but confirms that DMSO had negative effects on cell cultures. Actinomycin D induced apoptosis. This was in agreement with Stander *et al.* (2009), which found that 5.8% cells were viable after 0.25 M actinomycin D treatment. Cells treated with different concentrations of *Crotalaria agatiflora* leaves' extract showed dose-dependent responses. The same scenario was seen when U-937 cells were treated with madurensine. Out of these results it is evident that cells' viability was not affected by the treatments and that little cell death via apoptosis and necrosis took place.

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

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