Cassia alata Leaf Extract Induces Cytotoxicity in A549 Lung Cancer Cells via a Mechanism that is Caspase 8 Dependent

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ABSTRACT

Objective: To evaluate the cytotoxic effect of a hexane extract of Cassia alata leaves in A549 lung cancer cells.
Method: Parental A549 lung cancer cells were exposed to various concentrations (100–180 µg/ml) of Cassia alata leaf extract for 24 hours. Following treatment, the cells were evaluated using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to determine the cytotoxic effect of the extract. Caspase 8, 3 and 9 negative A549 cells were also prepared using lentiviral based shRNA knockdown of the caspase 8, 3 and 9 genes, respectively. The cytotoxic effect of Cassia alata leaf extract was then evaluated in these knockdown cells using the MTT assay. Chemical analysis was performed on the extract using high performance liquid chromatography (HPLC).
Results: Cassia alata extract was cytotoxic in parental and caspase-9 negative, but not caspase 3 and 8 negative A549 cells. The IC₅₀ values were 143 µg/ml and 145 µg/ml in parental and caspase 9 negative A549 cells respectively. The flavanoid kaempferol was identified as a constituent of Cassia alata leaf extract.
Conclusions: Cassia alata produces cytotoxicity in A549 cancer cells that is mediated by caspase 8 activation. This effect may be attributable to kaempferol.

Keywords: Caspase 8, Cassia alata, cytotoxicity, lung cancer

El Extracto de la Hoja de Cassia alata Induce Citotoxicidad en las Células A549 del Cáncer Pulmonar Humano a Través de un Mecanismo Dependiente de la Caspasa 8

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RESUMEN

Objetivo: Evaluar el efecto citotóxico de un extracto de hexano de hojas de Cassia alata en las células A549 del cáncer pulmonar.
Método: Células A549 parentales del cáncer pulmonar fueron expuestas a varias concentraciones (100–180 µg/ml) de un extracto de la hoja de Cassia alata durante 24 horas. Tras el tratamiento, las células fueron evaluadas usando el ensayo de bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) a fin de determinar el efecto citotóxico del extracto. También se prepararon células A549 negativas caspasa 8, 3 y 9 mediante silenciamiento génico vía ARN (shRNA knockdown) de los genes de las caspasas 8, 3 y 9 respectivamente, sobre la base de la inserción de vectores lentivirales. Entonces, usando un ensayo MTT se procedió a evaluar el efecto citotóxico del extracto de hojas de Cassia alata en éstas células genéticamente modificadas. Se realizó un análisis químico del extracto utilizando cromatografía líquida de alta eficacia. (HPLC).
Resultados: El extracto de Cassia alata resultó ser citotóxico en las células A549 negativas parentales y caspasa 9, pero no en las negativas caspasa 3 y 8. Los valores de IC₅₀ fueron 143 µg/ml y 145 µg/ml en las células A549 negativas parentales y caspasa 9 respectivamente. El flavonol kaempferol fue identificado como un constituyente del extracto de las hojas de Cassia alata.
**INTRODUCTION**

*Cassia alata* (*C. alata*) is a native plant of tropical America (1). Common names for *C. alata* include Wild Senna, Ringworm Cassia and King of the Forest. The plant is a large shrub belonging to the family Caesalpiniaceae and can be found in Indonesia, Bangladesh, Philippines, Africa and Jamaica (1−4). In these countries, various parts of the plants have been used traditionally for the treatment of boils, dysentery, itching, eczema, ringworm and scabies (4, 5). The plant has also been reported to possess several pharmacological properties including anti-inflammatory (2, 3) and analgesic activity (6). There is, however, limited information regarding the traditional use of *C. alata* for cancers and there is also limited scientific data available regarding cytotoxic properties of *C. alata* extracts. To date, there has only been one report of ethanol extracts of *C. alata* leaves exhibiting significant cytotoxic activity using the brine shrimp lethality bioassay (3). Compounds from *C. alata* have also exhibited anti-angiogenic activity and cytotoxic activity in breast cancer cell lines (7) as well as protective effects against pancreatic cancer (8). Based on the lack of scientific data regarding the activity of *C. alata* in lung cancer, the cytotoxic effect of an ethanol extract of *C. alata* leaves was evaluated in A549 lung cancer cells, a type of non-small cell lung cancer (NSCLC).

Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer deaths in males in 2008 globally (9). Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer deaths (9). Globally, lung cancer accounted for 13% (1.6 million) of the total cancer cases and 18% (1.4 million) of deaths in 2008 (9, 10). Additionally, 63% of cancer deaths occur in developing countries (10) and in Jamaica, lung cancer is the second highest type of cancer diagnosed (11).

The majority of lung cancer patients present with NSCLC for which therapeutic management continues to be immensely challenging (12−14). Efforts to improve the outcome of first-line therapy for advanced and metastatic NSCLC have primarily focussed on the addition of targeted agents to platinum-based two-drug regimens (15). Several new therapeutic options have emerged for advanced NSCLC, incorporating novel cytotoxic agents (taxanes, gemcitabine, pemetrexed) and molecular-targeted agents [erlotinib, bevacizumab] (15, 16). Despite the improvements in care and number of therapeutic agents available, the survival for patients with advanced-stage NSCLC remains modest and new modalities are still needed (12).

The major principle that drives the evaluation of potential anti-cancer agents is that tumour cells are susceptible to the cytotoxic activity of these agents and as such, theoretically, should arrest the growth and spread of neoplasms. Investigation of the involvement of apoptosis as a mechanism of executing cytotoxicity is now at the forefront of drug development, as this physiological process is absent in cancer cells (17, 18). In mammals, apoptosis is regulated by caspases and involves two major pathways: the intrinsic and extrinsic pathways. The extrinsic pathway can be triggered by ligation of death receptors and subsequent caspase-8 activation (19). Caspase-8 is a member of the cysteine proteases and like all caspases, caspase-8 is synthesized as an inactive single polypeptide chain zymogen procaspase and is activated by proteolytic cleavage, through either autoactivation after recruitment into a multimeric complex or trans-cleavage by other caspases (19). The intrinsic pathway is initiated by cellular stress followed by activation of caspase-9 (19). Each of these pathways converges to a common execution phase of apoptosis that requires proteolytic activation of caspases-3 and/or -7 from their inactive zymogens (19, 20). In the present study, we examined the involvement of caspases in the cytotoxic mechanism of the *C. alata* extract. The experiments were focussed specifically on evaluating the involvement of the key caspase players: caspase-8, the apical caspase of the extrinsic pathway, caspase-9, the initiator caspase of the intrinsic pathway and caspase-3, the executioner caspase of both pathways (20).

This study reports a novel cytotoxic activity exhibited by an extract of *C. alata* that is mediated by caspase 8.

**MATERIALS AND METHODS**

**Plant material and extraction**

The *Cassia alata* plant was obtained from Clarendon, Jamaica, and authenticated by Mr Patrick Lewis at the herbarium, The University of the West Indies (UWI), Mona Campus. A voucher specimen (#35349) was deposited at the herbarium. The leaves of *C. alata* were removed from the stems, washed with running water and allowed to drip dry. Leaves were homogenized in methanol (100 g leaves – 1000 ml methanol). The extract was filtered and concentrated *in vacuo* using a rotary evaporator. The methanol (Sigma Aldrich, United States of America (USA)) fraction was then partitioned between hexane (Sigma Aldrich) and water. The hexane fraction was then concentrated *in vacuo* and stored at 4°C for further studies.
Culture of the A549 lung cancer cell line
A549 lung cancer cells were kindly donated by Stupack Lab from the Moores Cancer Center, University of California, San Diego, California, USA. Cell lines were maintained and propagated in 90% Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured as an adherent monolayer and maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were harvested after subjecting them to brief trypsinization.

Cytotoxicity assay
Cytotoxic effects of *C. alata* extract were assessed by MTT assay (Bioassay systems). Cells (A549 parental, C3-knockdown, C8-knockdown and C9-knockdown) were plated in 96-well plates (5000 per well) in triplicate and incubated overnight at 37°C. After 24 hours, *C. alata* extract was added from a stock diluted to concentrations ranging from 100 to 180 µg/ml using DMSO (Sigma Aldrich). A volume of 20 µl of each concentration of *C. alata* extract was added in triplicate to selected wells respectively. Control wells received media only (20 µl) in triplicate. The cells were then incubated for 24 hours. Following incubation, 15 µl of the MTT labelling reagent was added to each well and incubated in a humidified atmosphere at 37°C for four hours. Following incubation, 100 µl of the solubilizing reagent was added to each well and mixed gently for one hour at room temperature. The absorbency of each well was measured at 540 nm using an ELISA reader (Lab Systems) and the percentage viability was calculated. The extract concentration that was cytotoxic in 50% of the cells (IC₅₀) was calculated from multiple runs.

Knockdown of caspases 3, 8 and 9 in A549 lung cancer cells
Caspase-8, caspase 9 and caspase 3 negative cells were prepared by Sh-RNA based knockdown of the caspase 8, caspase 3 and caspase 9 gene respectively. Silencing of caspase expression in the A549 cells was performed through the use of delivering shRNA in a lentiviral format. Briefly, 293 T cells were transfected with caspase 8, 3 and 9 (Open Biosystems) or scrambled (Addgene) shRNAs in pLKO.1 lentiviral vector, together with lentiviral packaging plasmids (PMLDL, VSV-G and RSV-REV). Lentiviral supernatants from 293 T cells were harvested after 48 hours and used to infect A549 cells. Viral constructs were incubated for 24 hours with A549 cell lines before replacing the media with selective media containing puromycin (1 µg/ml). The suppression of caspase 8, caspase 9 and caspase 3 was verified by Western blot analysis.

Western blot analysis
Cells were exposed to *C. alata* extract for 24 hours in 10 cm dishes. After the 24-hour incubation period, the cells were collected and washed twice with cold PBS. The cells were then lysed in lysis buffer and kept on ice for 30 minutes. The lysates were then centrifuged at 12,000 g at 4°C for 20 minutes; the supernatants were stored at -70°C until use. The protein concentration was determined by the Bradford method. Aliquots of the lysates (30 µg of protein) were separated by 12% SDS-PAGE and transferred on to a nitrocellulose membrane using transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8 and 20% methanol [v/v]). After blocking with 5% non-fat dried milk, the membrane was incubated for 2 hours with primary antibodies, followed by 30 minutes with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.5% Tween. Caspase 8 was probed with polyclonal antibody from (Millipore); caspase-3 (MAB4703, 1:500; Chemicon), caspase 9 (sc17784, 1:100; Santa Cruz Biotechnology) and actin (1:5000; Sigma). Secondary detection was achieved with horseradish peroxidase-conjugated secondary antibodies (BioRad) and ECL system (Pierce). Bands were detected using Enhanced Chemiluminescence.

HPLC analysis *C. alata* extract
One milligram of the *C. alata* extract was dissolved in methanol and injected into the HPLC system (Varian star). The HPLC detection system was a Prostar 325 UV-Vis Detector. The samples were injected into LUNAC18 column (5 µm particle size, 4.6 × 250 mm, Phenomenex, USA). The mobile phase consisted of acetonitrile/methanol/ammonium acetate (Ph 6.8) in a ratio 25:55:20 (v/v/v) and at a flow rate of 0.5 ml/min. All chromatographic procedures were performed at 25°C; the peaks were detected at 260 nm. Standard solutions of kaempferol (Sigma Aldrich, USA) were prepared at 5.0, 10.0, 25.0, 50.0 and 100.0 µg/ml by diluting with *C. alata*. A sample size of 10 µl was injected for the HPLC analysis.

Statistical analysis
The results of each series of experiments (performed in triplicates) are expressed as the mean values ± standard deviation of the mean (SD). Statistical significance of the data was determined using the independent-t-test; a value of *p* < 0.05 was accepted as statistically significant.

RESULTS
*C. alata* extract exhibits cytotoxic activity in parental A549 lung cancer cells
Figure 1 depicts the dose-dependent cytotoxic effect of the *C. alata* extract on the parental A549 cancer cells. The cells were exposed to concentrations of 100, 120, 140, 160, 180 µg/ml of *C. alata* extracts for 72 hours. Viability in *C. alata* treated cells was significantly lower than in untreated controls. IC₅₀ of the *C. alata* extract was determined to be 143 µg/ml.
Cytotoxic effect of *C. alata* extract in A549 cells is mediated by caspase 8

Caspase 3, 8 and 9 knockdown cells were prepared respectively by Sh-RNA based knockdown of the caspase 3, 8 and 9 gene (Figs. 2a, 2b and 2c). The cytotoxic effect of *C. alata* extract was abolished in caspase 3-knockdown cells (Fig. 3a) as well as caspase 8 knockdown cells (Fig. 3b). In caspase 9 knockdown cells (Fig. 3c), the cytotoxic activity of the extract was maintained with an IC$_{50}$ value of 145 µg/ml.

Chromatogram of *C. alata* extract and kaempferol standard

The peak for kaempferol in the *C. alata* extract (Fig. 4b) was identified by comparing the relative retention time (RRT) with that of the chemical standard kaempferol (Fig. 4a). The RRT were 6.895 (Peak-1) and 6.882 (Peak-2) minutes for standard and extract, respectively.
DISCUSSION

In recent years, the targeted screening of plant material for anti-lung cancer activity has significantly increased (21−23). This has been driven significantly by the limitations of current conventional therapies which include severe unwanted effects, poor survival rates and toxicities associated with first line agents which are largely platinum based drugs such as cisplatin (24−26). In several cases, the screening of pharmacologically active plant materials for the treatment of lung cancers has represented a potentially safer and more effective therapeutic modality (27). This emphasizes the need for continued exploration of the herbal pharmacopeia for these agents and the necessity for the progression of preclinical studies to clinical trials.

In the present study, *Cassia alata* extract demonstrated cytotoxic activity in parental A549 lung cancer cells. This confirmed its ability to be potentially effective in NSCLC. Generally, the major mechanism of cytotoxicity of most exogenous agents is via apoptosis and the involvement of the key regulators of apoptosis which are caspases was examined. It was demonstrated that the knockdown of the caspase 9 gene in A549 cells did not affect the cytotoxic activity of *Cassia alata* extract, suggesting that the activation of the intrinsic apoptotic pathway was not implicated in this mechanism of the extract (28). By contrast, in caspase 3 and caspase 8 negative A549 cells, the cytotoxic activity of the extract was abolished, suggesting that activation of the extrinsic apoptotic pathway was involved in the cytotoxic activity of *Cassia alata* (29, 30). Caspase 8 recruitment is integral to the extrinsic cell death pathways (20). Upon activation of death receptors by exogenous triggers, these death receptors will recruit the death-inducing signalling complex (DISC) upon binding the specific tumour necrosis factor family (20). Following this, procaspase-8 can be recruited into this complex via the adaptor protein Fas-associated protein with

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Fig. 3a: Cytotoxic effect of *Cassia alata* extract is abolished in caspase-3 negative A549 parental cells. The data (mean ± SD of triplicate determinations) are representative of at least three independent experiments.

Fig. 3b: Cytotoxic effect of *Cassia alata* extract is abolished in caspase-8 negative A549 parental cells. The data (mean ± SD of triplicate determinations) are representative of at least three independent experiments.

Fig. 3c: Cytotoxic effect of *Cassia alata* extract is abolished in caspase-9 negative A549 cells. The data (mean ± SD of triplicate determinations) are representative of at least three independent experiments. Asterisks denote significant differences relative to control untreated cells that were exposed to media only (*p < 0.05, **p < 0.01).
Death Domain (FADD). This leads to caspase-8 activation which then initiates downstream apoptotic cascade by cleaving caspase-3, caspase-7 or Bid (31–33). The results of this study illustrate abolished cytotoxic activity in caspase 3 negative cells suggesting that caspase 3 was the downstream caspase recruited into the cytotoxic cascade initiated by the extract.

These preliminary results could be justified by the cytotoxic activity of the flavonoid kaempferol which is present in *C. alata* species (34). Similar cytotoxic activity induced by kaempferol has been reported in ovarian (35), lung (36) and A549 (37) cancer cells. Furthermore, kaempferol has been linked to caspase 8 and caspase 3 activation (38). Preliminary analysis of the *C. alata* extract using HPLC has identified kaempferol as a major constituent in the leaf extract (Fig. 4). This is the first report of *C. alata* exhibiting cytotoxic activity in cancer with an implication of caspase 8.

**CONCLUSION**

*Cassia alata* leaf extract exhibits cytotoxic activity in A549 lung cancer cells that is dependent on the activation of caspase 8. The results suggest that further analysis and investigations should be conducted on this extract in other cancer cells to validate its use for anticancer properties.

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