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Full Length Research Paper

The immunotoxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*

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Increased use of pesticides has resulted in increased concern about the adverse effects on non-target organisms, including humans. Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides. An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential. *D. rhinocerotis* and *G. africana* exhibit antifungal properties against *Botrytis cinerea*. This paper describes the immunotoxicity of extracts of *D. rhinocerotis* and *G. africana* on mouse spleenocytes. Spleen cell cultures were prepared and exposed to varying concentrations of *D. rhinocerotis* and *G. africana*. Control cultures were exposed to the DMSO vehicle only. Results obtained showed that both *D. rhinocerotis* and *G. africana* have immunomodulatory effects. Exposure of cell cultures to both extracts resulted in a decrease in both IL-4 and IFN-γ. The cytokine inhibition was concentration dependent.

Key words: Pesticides, *Galenia africana*, *Dicerothamnus rhinocerotis*, immunotoxicity, cytokines.

INTRODUCTION

A pesticide may be defined as any substance(s) used for destroying or mitigating unwanted pests. Unwanted pests include species of plants or animals that cause harm during food production, processing, storage, transport and/or marketing (Clementi et al., 2008). The use of pesticides has increased, over recent years due to greater demand for good quality food products. Increased use of pesticides has resulted in increased concern about the adverse effects on non-target organisms, including humans.

Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides (Isman, 2006). An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential (Isman, 2006). *Galenia africana* (*G. africana*) and *Dicerothamnus rhinocerotis* (= *Elytropappus rhinocerotis*) (*D. rhinocerotis*) exhibit antifungal properties against *Botrytis cinerea* (Knowles, 2005). *B. cinerea* is a fungal pathogen that causes grey mould rot, on a large number of economically important agricultural and horticultural crops (Jarvis, 1997).

D. rhinocerotis, popularly known as rhenoster bush ("renosterbos" in vernacular) or rhinoceros bush, is a bush shrub with small grayish-green leaves and tiny flower heads (Levyns, 1935). Infusions of the young branches prepared in brandy or wine are a traditional Cape (South Africa) medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt and Breyer-Brandwijk, 1962). It is also said to be taken as tonic to improve lack of appetite, for colic and wind diarrhoea (Cillie', 1992). The medicinal properties of *D. rhinocerotis* may be due to rhinocerotinoic acid, a labdane diterpenoid with anti-inflammatory activity (Dekker et al., 1988)

G. africana is a plant that is indigenous to the Namaqualand region of South Africa (Kellerman et al., 1988). The Hottentots, an indigenous tribe, chewed the plant to relieve toothache. The plant was also used in the treatment of venereal diseases and prepared as a decoction for skin diseases and for the relief of inflammation of the eyes (Watt and Breyer-Brandwijk, 1962).

Animals are forced to graze this plant during droughts and in poor-conditions. Ingestion of the plant is associated with liver damage and severe ascites, a condition referred to as 'waterpens' or 'water belly', in sheep and goats (De Kock, 1928; Kellerman et al 1988). The mar-

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Abbreviations: IL-4, Inteluekin-4 ; **IFN-γ**, interferon-gamma.

ked liver lesions have lead researchers to believe that the plant is hepatotoxic due to the presence of a toxin (Van der Lugt et al., 1992).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Exposure of pesticides to the immune system may result in alteration of the normal immune functions. This kind of activity is known as immunotoxicity (Colossio et al., 1999). Toxicity studies are required to determine safety of the plant extracts. Toxins can have a general effect on all cells or they can attack specific organ/ physiological systems. One of the physiological systems prone to attack by toxins is the immune system (Colosio et al., 1999).

The immune system is a well-regulated organ system that involves interrelated reactions, which protect an organism from invasion by foreign substances (Ladics, 2007). The immune system fights pathogens using two methods, namely the innate immunity and the acquired/ adaptive immunity (Seely et al., 2000; Storni et al., 2005). Due to its complexity, the immune system is a target for various toxic substances, including pesticides, therefore resulting in multiple potential target sites and pathological effects (Colosio et al., 1999).

Immunotoxicity results in toxicant-induced injury to part of the immune system thus affecting immune functions and may result in immunostimulation, immunosuppression, hypersensitivity and autoimmunity (Descotes, 2004; Van Wijk et al., 2006). Each of these categories is associated with potential adverse effects associated with significant morbidity.

The aim of this study was to assess the toxicity of *D. rhinocerotis* and *G. africana* for the acquired immune pathway using *in vitro* assays. Acquired immunity is regulated by cytokines secreted by the Th1 and Th2 lymphocytes.

T-helper (Th) lymphocytes are divided into Th1 and Th2 subsets according to cytokine production profile which also correlate with their function (Mosmann et al., 1986). Th1 cells produce the cytokines interleukin (IL)-2 and Interferon (IFN)-γ which aid with defenses against intracellular pathogens and promote cell mediated immunity, whereas Th2 produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 which are responsible for defenses against extracellular pathogens and promote humoral immunity (Mosmann and Sad, 1996). Th1 and Th2 cytokines are mutually inhibitory for the functions of the reciprocal phenotype (Storni et al., 2005).

IFN-γ is a cytokine predominantly produced by Th1 cells (Mims et al., 2004; Seely et al., 2000; Storni et al., 2005). IFN-γ aids with defenses against intracellular pathogens and promotes cell mediated immunity ((Mims et al., 2004; Seely et al., 2000; Storni et al., 2005). IFN-γ also antagonizes IL-4 leading to inhibition of Th2 cell proliferation (Storni *et al*., 2005). On the other hand IL-4 is a cytokine that is mainly produced by Th2 cells (Mims et al., 2004; Storni et al., 2005). IL-4 stimulates proliferation and differentiation of B-cells, thus inducing antibody production (Storni et al., 2005). IL-4 also results in inhibition of Th1 cell proliferation (Fukao et al., 2000; Storni et al., 2005).

MATERIALS AND METHODS

Reagents

All reagents, solvents and biomolecules used in this study were purchased from Roche (South Africa), Merck (Germany) or Sigma Chemical Company (St Louis, USA) unless stated otherwise. All other reagents were of analytical grade.

Plant extractions

Two medicinal plant species, *G. africana* and *D. rhinocerotis,* used in traditional medicine practices in South Africa were obtained and prepared for analysis. Extracts (20%, w/v) were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2-3 mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during the overnight extraction. The extracts were then pressed, to separate the tincture and the milled leaves. Filtration was carried out to clean tincture from plant debris and the extract was stored at 20°C. For study purposes the extracts were air dried. The dried extracts were resuspended in DMSO to obtain 50% extract.

Spleen cell cultures

Male BALB/c mice were purchased from UCT animal facility (South Africa). They were sacrificed via cervical dislocation after which the spleens were dissected out. The spleens were then disrupted via passage through a sterile steel mesh. Spleen cells were suspended in full medium, which consisted of RPMI 1640 supplemented with 1% antibiotic/mycotic mix (Sigma, USA) and 1% serum replacement factor. The spleen cell suspension was washed and centrifuged at 1000 x g for 10 min, after which the cell pellets were resuspended in fresh medium to a concentration of 2.5×10^6 cells/ml.

Assays to screen toxicity of the plant extracts were conducted in 96-well culture plates (Nunc, Denmark). A serial dilution range of the extracts, in DMSO, were applied to wells of the plate. Control wells contained the DMSO vehicle only. Six replicates of each concentration were prepared. One set of wells (three replicates of each extract concentration) then received the cell suspension (unstimulated cultures). The other set of replicates received the cell suspension and 16 µg/ml phytohemmagglutinin (PHA) from *Phaseolus vulgaris* (Sigma, USA) was added. The plate was incubated for 48hours in an incubator at 37°C flushed with 5% CO_2.

After the 48hour culture period the supernatants were harvested and cytokine concentrations in the culture medium determined. IFN-γ and IL-4 were the cytokines assessed. This was done using commercially available ELISA kits in accordance with the manufacturer's instructions (eBioscience, USA).

Cytokine ELISAs

Nunc 96 well microtiter ELISA plates (Serving life science, Denmark) were used for all ELISA protocols. Cytokine (IL-4 and IFN-γ) concentrations were determined using ELISAs. The ELISAs were carried out according to the manufacturer's instructions. The ELISA plates were coated with capturing antibody (purified anti-mouse IL-4 or IFN-γ) diluted appropriately in coating buffer and incubated over-

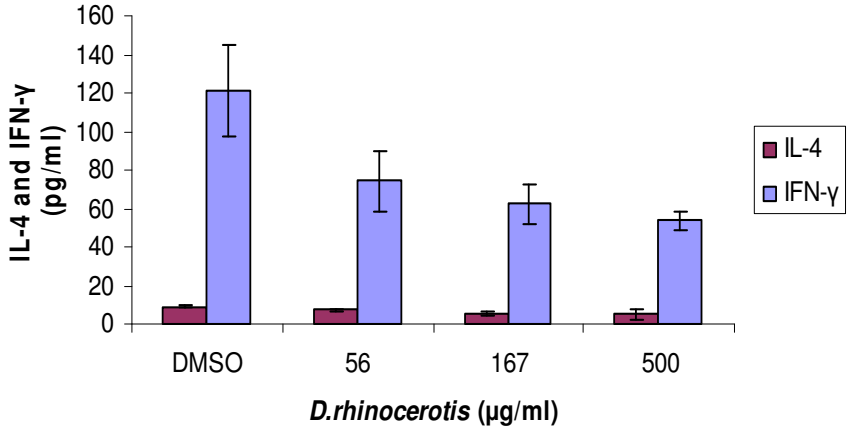

Figure 1. IL-4 and IFN-γ produced by spleenocytes, under stimulated conditions, in the presence of *D. rhinocerotis*.

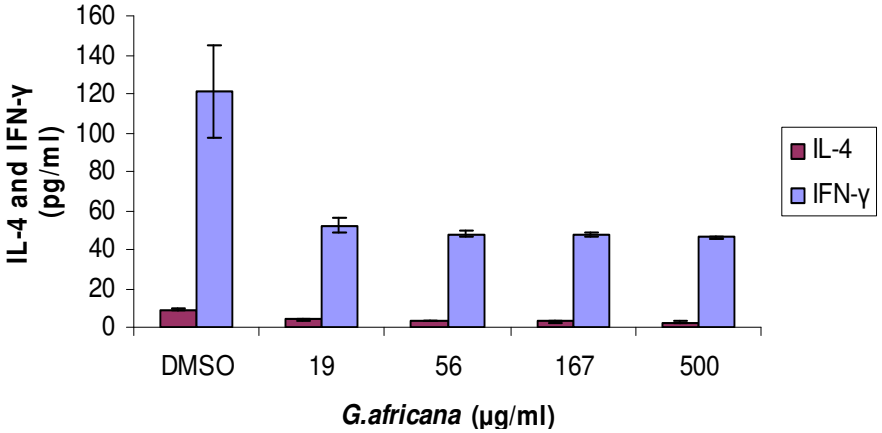

Figure 2. IL-4 and IFN-γ produced by spleenocytes, under stimulated conditions, in the presence of *G. Africana*.

night at 37°C. The plates were washed 5 times with wash buffer (autoclaved PBS, 0.05% Tween-20 and distilled water), after which the non-specific binding sites were blocked with assay diluent. Cell free supernatants were then added to the plate. The assay was standardized using 2-fold serial dilutions of recombinant mouse IL-4 (500pg/ml) and IFN-γ (2000 pg/ml) respectively. The plates were then sealed and incubated for 2 h at room temperature. After 5 washings, with wash buffer, detection antibody (Biotin-conjugate anti-mouse IL-4 and IFN-γ) was added to each well and the plate was incubated for 1 h at room temperature. Avidin- Horseredish peroxidase (HRP) was added to all wells in order to detect the bound cytokine. This was incubated for 30 min after which the plate was washed 7 times. Substrate solution (1X TMB) was then added to every well and incubated for approximately 15 min. The reaction was then stopped with stop solution. Absorbances were read on a plate reader at 450 nm.

Statistical analysis

All experiments were performed three times in triplicate and data was statistically analysed via one-way ANOVA (P<0.001) and regression analysis.

RESULTS

From the IL-4 and IFN- γ ELISAs performed, standard curves were plotted using the optical density readings obtained for the serial-dilution of the standards. The IL-4 and IFN-γ concentrations of the spleen-cell cultures after incubation with *D. rhinocerotis* and *G. africana* were extrapolated using the standard curves and are shown in Figures 1 and 2. The results are expressed as the effects of the individual extracts vs. the control, untreated cells.

The results showed that *D. rhinocerotis* had no significant (P<0.001) effect on both IL-4 and IFN-γ production under unstimulated conditions. However under stimulated conditions *D. rhinocerotis*, at concentrations above 56 µg/ml, significantly decreases IL-4 production (Figure 1). *D. rhinocerotis also* significantly (P<0.001) decreased IFN-γ production, at all concentrations, under stimulated conditions (Figure 1).

G. africana, at concentrations above 19 µg/ml, significantly (P<0.001) decreases IL-4 production under stimu-

lated conditions (Figure 2). It also significantly ($P< 0.001$) decreased IFN-γ production, at all concentrations, under stimulated conditions (Figure 2). However under unstimulated conditions *D. rhinocerotis* had no significant effect on IFN-γ production.

DISCUSSION

Exposure to pesticides may have various effects on the immune system, ranging from slight modulation of immune functions to development of clinical immune diseases (Colosio et al., 1999). For example exposure to the organophosphorous compound pentachlorophenol results in decreased lymphocyte proliferative responses to mitogens (Colosio et al., 1993). It is therefore a necessity to evaluate the immunotoxic effects of pesticides before putting them to use.

The results in this study showed that *D. rhinocerotis* had no significant effect on IL-4 production under unstimulated conditions. However under stimulated conditions *D. rhinocerotis*, decreases IL-4 production. IL-4 is a cytokine that is mainly produced by Th2 cells (Storni et al., 2005). This therefore implies that exposure to *D. rhinocerotis* may result in impairment of the Th2 response. Th2 cells are required to mount effective humoral and cell-mediated responses that are required to fight extracellular microbes and parasites. An impairment of these responses may thus result in increased susceptibility to extracellular microbes (O'Garra and Arai 2000).

This study also shows that both *D. rhinocerotis* and *G. africana* significantly decreased IFN-γ production. IFN-γ is a cytokine that is predominantly produced by the Th1 cells (Storni et al., 2005). Th1 cells are required to mount an effective cell-mediated response that is required to fight intracellular pathogens, viruses and cancers. An impairment of these responses may thus result in increased susceptibility to intracellular pathogens, viruses and cancers (Storni et al., 2005, Seely et al., 2000). This therefore implies that exposure to *D. rhinocerotis* or *G. africana* may result in impairment of the Th1 response, which may in turn lead to increased susceptibility of intracellular pathogens. Previous studies, done by us, showed that *D. rhinocerotis* and *G. africana* are not cytotoxic at the low concentrations used in this current study, indicating that specific immune functions are impaired at concentrations that do not necessarily cause cell death.

Exposure to *G. africana* also results in lowered IL-4 levels. This implies that exposure to *G. africana* may result in impairment of the Th2 response, which inturn may lead to increased susceptibility to extracellular microbes (O'Garra and Arai 2000).

The properties of whole plant extracts can either be from a single chemical compound or from a combination of compounds. The active chemical compounds present in the plants are known as secondary metabolites (Lewinsohn and Gijzen, 2008). It may thus be useful to study the composition of the plant extracts to identify the compound(s) with pesticidal activity. The compound(s) may then be isolated be and evaluated for use as pesticides.

Studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2007). Complete inhibition of the fungi *B. cinerea* was observed for *D. rhinocerotis and G. africana* concentrations greater than at 0.95 mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005; Vries, 2007). Immunotoxicity studies can therefore be carried out for the combination of kresoxim-methyl and the botanical extracts at these low concentrations.

A major consideration in approving pesticides for commercial use is whether they pose an unreasonable risk to humans and to the environment. In order to evaluate safe levels of chemicals toxicological data is required. The aim of this study was to determine the immunological risks associated with using extracts of *D. rhinocerotis* and G*. africana*, and toxicological data was acquired.

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