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Research Article
Analyses of *Ramalina farinaceae* Extract Anti-proliferative Activities in Culture of Mammalian Cells and Splenocytes

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Abstract

Preliminary investigations of *Ramalina farinaceae* (RF) plant extract sourced from Southeast, Nigeria for later possible antiviral usage lead to the identification of anti-proliferative activities against some mammalian cell lines and mouse splenocytes. The extract/cell systems were incubated for 1-2 days followed by analysis of cell viability status using an MTT-based assay. Measurement of released interferon gamma cytokine was undertaken as a proliferative activation marker for immune cells derived from mouse spleen. The preliminary data showed anti-proliferative cytotoxic activities with TC_{50} = 103.1, 61.57 and 65.24 μg mL^{-1} for vero, Tsa201 and A293 cells, respectively, when tested in a concentration-dependent manner. Extract-stimulated mouse splenocytes showed no proliferative activation status contrary to INF-g release recorded for lipopolysaccharide (LPS) stimulated mouse splenocytes. *Ramalina farinaceae* extract in the present study appear to down-regulate cell proliferation indicating possible utility in counteracting cell hyperplasia. It could however, equally still be utilized in other cellular systems when adequately tailored at lower concentrations in specific given settings to meet the contingent need.

Key words: Cell viability, cell proliferation, cytokine, ELISA, immune modulation, *Ramalina farinaceae* extract

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Natural products from plants traditionally have provided the pharmaceutical industry with one of its most important sources of lead compounds and up to 40% of modern drugs are derived from natural sources, using either the natural substance or a synthesized version.

Currently, over a 100 new products are in clinical development, particularly as anti-cancer agents and anti-infectives (Gautam *et al*., 2007; Harvey, 2008; Jassim and Naji, 2003). This has influenced many of pharmaceutical companies to produce new antimicrobial formulations extracted from plants or herbs. The bioactive molecules occur in plants as secondary metabolites and as defense mechanisms against predation, herbivores, fungal attack, microbial invasion and viral infection. During the past decade, potent agents have become available against viral infections. Therefore, extracts of plants and phytochemicals are getting more important as potential sources for viral inhibitors during the recent decade. Extensive studies have shown that medicinal plants of several parts of the world contain compounds active against viruses that cause human diseases (Kott *et al*., 1999; Semple *et al*., 1998; Sindambiwe *et al*., 1999).

Lichens are known as a promising source of diverse secondary metabolites, the majority of which were polyketides, which exhibited various biological activities, such as antioxidant, antimicrobial, antiherbivore, antitumor and insecticidal activity (Gautam *et al*., 2007; Harvey, 2008). Previous chemical investigation of the lichen belonging to Ramalina genus has led to the isolation of about 50 compounds and most of them were phenolic compounds, while approximately half of them were depsides (Jassim and Naji, 2003; Kott *et al*., 1999; Semple *et al*., 1998; Sindambiwe *et al*., 1999; Molnar and Farkas, 2010; Stocker-Worgotter, 2008).

Ramalina farinacea, a lichen found in Nigeria but also available in other isolated places, has earlier been shown to possess broad anti-retro-viral and anti-adenoviral principles (Esimone *et al*., 2005, 2009; Lai *et al*., 2013).

Despite the foregoing, the wholesome investigation of *Ramalina farinaceae* intact extract for possible proliferative effects on non-immune and immune cells has not been undertaken.

However, associated with discovery of anti-infective antiviral at an early stage is an initial screening for cell viability responses towards the medicinal agent, which provide early information on its potential cytotoxic properties. Thus anti-proliferative cytotoxicity assays are widely employed both in research and industry to screen a library of compounds or extracts for desirable or undesirable cytotoxic effect (Dixon, 2001; Esimone *et al*., 2008).

Thus, cytotoxicity can be monitored by different approaches including the use of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay. In this study, this method was employed which measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTT reagent to a colored formazan product which can easily be read off in a colorimeter. In this study, *Ramalina farinaceae* extract was analysed on some mammalian cell lines and immune cells utilized in routine biological screening as preliminary assays for possible subsequent utility in the human system. The goal was to define the up or down-regulatory status of the extract on routine cells so as to establish very important criteria for continual use and to create a useful practical baseline for the further utilization of *Ramalina farinaceae* extract in cell systems for other likely antiviral and immune-modulatory screening studies.

MATERIALS AND METHODS

Medicinal plant collection: The lichen was collected in Nigeria and identified by Mr. A.O Ozioko of International Centre for Ethnomedicine and Drug Discovery, Nsukka (InterCEDD). A voucher specimen (No. InterCEDD/1567) was deposited at the herbarium of InterCEDD. Plant collection and study duration was between January, 2012 and December, 2013.

Extraction and isolation: The air-dried lichen of *Ramalina* sp., (100 g) was powdered and extracted with methanol (400 mL) under cold maceration overnight. After filtration, the methanol in the filtrate was removed by air-drying and the marc re-dissolved in DMSO to yield the working stock solution.

Cells: All tissue culture materials were obtained from Invitrogen (Karlsruhe, Germany). Monolayers cultures of vero cells (monkey kidney cell origin), human TsA201 cells (a derivative of human embryonic kidney cell line 293), 293A cell line (a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA) were grown in D-5 consisting of Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 2 mM L-glutamine (Glu) and supplemented with 5% v/v heat-inactivated Foetal Calf Serum (FCS) and a mixture of penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) (Pen-Strep) as previously described (Ternette *et al*., 2007; Esimone *et al*., 2008).

Cytotoxicity assay: The cytotoxicity of the compounds was evaluated using the MTT assay (Esimone *et al*., 2008). Vero, TsA201 or A293 cells were seeded onto a 96-well plate at a concentration of 6000 cells per well and a volume of 200 µL of D-5 (5% FCS DMEM medium) per well. The extracts each were solubilized in 0% FCS DMEM medium containing DMSO to give 1000, 500, 250 and 125 µg mL^{-1} compound such that the final DMSO concentration in cell culture did not exceed 0.6%. The D-5 containing 0.6% DMSO was used as the no drug control. After incubation at 37°C under 5% CO_2 for 2 days, a solution of MTT (5 mg mL^{-1}, 50 µL per well) was added to each well and further incubated at 37°C+5% CO_2 for 1 h to allow for formazan production. After this time, medium were removed and 200 µL of 20% SDS solution in water/dimethylformamide (1:1) (pH 4.7) was used to dissolve the resulting blue formazan crystals in living cells overnight. The optical density was determined at 550 nm using a multi-well microtiter plate reader (Tecan, Austria). Each single value of the triplicates were expressed as percent of the mean of triplicates of the control (no drug) cultures and the mean and standard error of the percent values were calculated for each triplicate. The concentration of 50% cellular toxicity (TC_{50}) of the test compounds were calculated by simple regression analysis.

Immune modulatory studies: Splenocytes were isolated from animal spleens in Hank's Buffered Saline Solution (HBSS) using a cell strainer (BD Biosciences). Red blood cells were lysed in 2 mL ACK lysis buffer for 2 min and the reaction was stopped in 8 mL of HBSS. Cells were collected in R10 medium (RPMI with 10% fetal calf serum and 0.1% ß-mercaptoethanol) and 1.5×10^6 cells per well were plated in 96-well round-bottom plates. Splenocytes were stimulated by compounds for 24 h with varying concentrations 25, 12.5 and 6.25 µg mL^{-1}, respectively per well at 37°C (Jung *et al*., 1993). Splenocytes stimulated with lipopolysaacharides (LP) served as a positive control. As a negative control, cells were cultured in R10 medium alone containing DMSO. After incubation the plate containing cells were diluted 1:1 v/v in blocking buffer/tween and incubated for 15 min at room temperature in order to allow a complete lysis of cells. After lysis plate containing cells was ultra-centrifuged for 10 min at 250×g. Supernatant was collected, freezed (-20°C) and kept for further cytokine ELISA.

Cytokine ELISA: Interferon gamma (INF-g) was measured by ELISA using the capture Ab JES6-1A12 and the biotinylated detection Ab JES6-5H4 (BD Pharmingen™, Belgium). Briefly, Secreted INF-g upon activation of splenocytes was measured in an ELISA using the capture antibody JES6-1A12 and the biotinylated detection antibody JES6-5H4 (BD PharmigenTM, Erembodegem, Belgium). The capture antibody was diluted in ELISA binding buffer at a 1 µg mL^{-1} concentration and 50 µL per well were added to the ELISA plate (Maxisorp, NUNC) and incubated overnight at 4°C. Next day, the plate was equilibrated at room temperature and blocked with 100 µL per well of ELISA blocking buffer for at least 1 h at room temperature. The ELISA plate was washed 3 times with 100 µL per well of PBS/tween. The plate was washed 3 times with PBS/tween and the detection antibody diluted to 1 µg mL^{-1} in blocking buffer/tween was added (50 µL) for 1-2 h incubation at room temperature. The plate was washed 3 times with PBS/tween and the streptavidin-peroxidase diluted 1:1.000 in Blocking buffer/tween 50 µL per well was added. The plate was incubated 30 min at RT and then washed 5 times with PBS/tween. The TMB substrate solution (BD Sciences) was added 50 µL per well and run for 15-30 min to develop. A blue color should appear. The reaction was stopped in the same order that the substrate solution was added and measured at 450 reference wavelength 620 nm with an ELISA plate reader (Tecan®).

Statistical analysis: For the cell viability assays each single value of the triplicate experiments was expressed as percent of the mean of triplicates of control experiments and the mean and standard error of the percent values were calculated for each triplicate. Using the results obtained the 50% values Toxic Concentration (TC_{50}) were then determined by a simple regression analysis. All statistical analysis were done using the Graph pad prism® software.

RESULTS AND DISCUSSION

Figure 1-3 show the outcome of cell toxicity profile of methanol extract of *Ramalina farinaceae* extract after exposure to various human cell types. Three cell lines were tested and recorded toxicity profiles TC_{50} = 103.1, 61.57 and 65.24 µg mL^{-1} for vero, Tsa201 and A293 cells, respectively when tested in a concentration-dependent manner. All 3 recorded moderate toxicities. Of these three, vero cell type recorded toxicity around the 50% marginal line. The remaining two Tsa201 and A293 cell types were above this level.

Vero cells were incubated with various concentrations of *R. farinaceae* extract (RF extract). After corresponding 48 h of infection, MTT was added for 1 h and formazan crystal dissolved overnight and absorbance measured at 550 nm. The results are expressed as percentage of the activity (cell

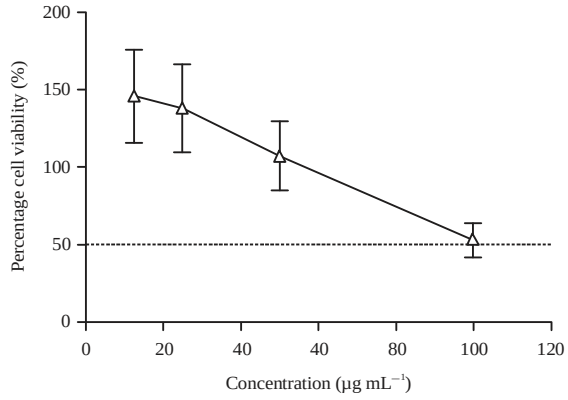

Fig. 1: Viability of vero cells after treatment with RF extract

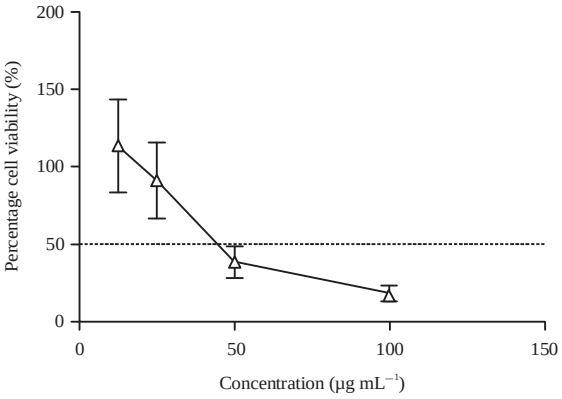

Fig. 2: Viability of TsA201 cells after treatment with RF extract

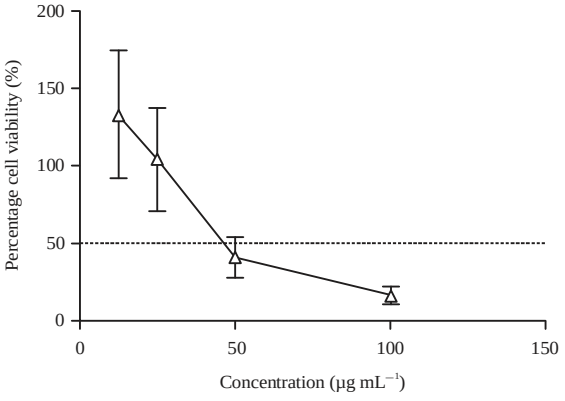

Fig. 3: Viability of A293 cells after treatment with RF extract

viability) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given (Fig. 1).

The TsA201 cells were incubated with various concentrations of *R. farinaceae* extract (RF extract). After corresponding 48 h of infection, MTT was added for 1 h and formazan crystal dissolved overnight and absorbance measured at 550 nm. The results are expressed as percentage of the activity (cell viability) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given (Fig. 2).

The A293 cells were incubated with various concentrations of *R. farinaceae* extract (RF extract). After corresponding 48 h of infection, MTT was added for 1 h and formazan crystal dissolved overnight and absorbance measured at 550 nm. The results are expressed as percentage of the activity (cell viability) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given (Fig. 3).

Moreover, careful observation of the plots generated could show a good reflection of the dose-dependency of the recorded results. Here, again vero cells displayed a better robustness, while A293 cells remained most fragile to the extract treatments.

Cytotoxicity assays are widely employed both in research and industry to screen a library of compounds or extracts for desirable or undesirable cytotoxic effect. Hence, the approach to look out for cytotoxic compounds, if the goal is in developing a anti-neoplastic therapeutic that targets rapidly dividing cancer cells or as in the usual approach to screen "Hits" from initial high-throughput compound/extract screens for unwanted cytotoxic effects sequel to further developmental studies. Thus cytotoxicity can be monitored by different approaches including, the use of 3-(4, 5-Dimethyl-2-thiazolyl)- 2, 5-diphenyl-2H-tetrazolium bromide (MTT) (Lai *et al*., 2013; Esimone *et al*., 2008). In this study, this method was employed, which measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTT reagent to a colored formazan product which can easily be read off in a colorimeter (Lai *et al*., 2013; Esimone *et al*., 2008).

Our findings could show that of cell lines tested in all 3 were found to be moderately susceptible to the deleterious effects of the *Ramalina farinaceae* extract. This seeming status is seen for the extract in vero cell type and a nearly replicated in TsA201 and A293 cells where it was rather of slightly higher toxicity. This scenario may not be unconnected to the understanding that TsA201 and A293 cells are slightly less robust than vero cells. With the exception of vero cells the other two cell types represent cells that have been subjected to some procedure of transformation to suit some cell culture and molecular biology manipulations. These values may be considered favorable on their own merit, especially when juxtaposed with a corresponding analysis for therapeutic

activity where a derived ratio of cell viability value to therapeutic potency value leading to activity index (therapeutic window) in a setting where, therapeutic effect, is sought. Nonetheless, *Ramalina farinaceae* extract may find application in human or mammalian host as therapeutic agents especially as possible anti-neoplastic therapy in accurately targeted neoplastic cells. Of particular interest in this study is the observed concentration dependent nature of this finding in all cell types tested meaning that this property can be taken into advantage to further harness to applicability of the extract. The further implication therefore, is apart from satisfying necessary safety rating leading to its further development into any defined therapeutics, it present an enormous potential of being harnessed for use to neoplastic cells in a carefully targeted manner. This property could well render it a good tolerable cellular depressor agent that could be very valuable in cancer disease management and treatment (Gautam *et al*., 2007; Dixon, 2001; Cragg and Newman, 2005; Okouneva *et al*., 2003). All the other cell lines have displayed varying very moderate to slightly high cytotoxic responses to the extract. Thus, they could then possibly be employed for other possible therapeutic screening exercises and applications (Dixon and Ferreira, 2000).

Given all the foregoing, the *in vitro* activity of *Ramalina farinaceae* extract in cell culture of mammalian cells show that its possible utility can be tailored in a given setting to meet the contingent need either as anti-biologics in tolerable cellular systems biological systems or as antineoplastic where itinerant cells behavior are evident (Cragg and Newman, 2005; Okouneva *et al*., 2003).

Additionally investigations of our extract on immune cells of splenic origin could show that there was an observed up-regulation of specific activated mouse splenocytes that were largely interferon-gamma-releasing only when lipopolysacchrides (LPS) (a non-specific immune activator) is used, however, in the RF-treated groups and DMSO control no effect is seen on INF-g-immune-specific activation. This trend is seen even at all concentrations up to 25 μg mL^{-1} concentration of RF.

Vero cells were incubated with various concentrations of *R. farinaceae* extract (RF extract). After corresponding 48 h of infection, MTT was added for 1 h and formazan crystal dissolved overnight and absorbance measured at 550 nm. The results are expressed as percentage of the activity (cell viability) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given (Fig. 4).

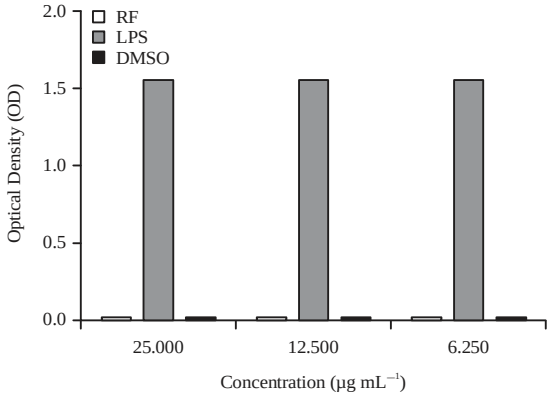

Fig. 4: The INFgamma-dependent immune activation of mouse naïve splenocytes after treatment with *Ramalina farinaceae*

The up or down-regulatory effect of interferon-gamma from some cells of the immune system on other cells of the immune system as typified in the sub-populations of immune cells making up the mouse splenocytes may be expected to positive or negatively regulate the functionality of other cells of the innate or adaptive immunity due to their modulatory functions. This in turn would enable the host mount a better and more avid immune response in the fate of infections or other immune-driven conditions.

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is twofold in the development of new drugs: First, they may become the base for the development of a medicine, a natural blueprint for the development of new drugs or/and second, A phytomedicine to be used for the treatment of disease. There are numerous illustrations of plant derived drugs. It is estimated that today, plant materials are present in or have provided the models for at least 50% Western drugs (Schuster, 2001). Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

CONCLUSION

An initial screening for cell viability responses towards the medicinal agent, methanol extract of *Ramalina farinaceae* to

provide early information on its potential effect on cell expansion and proliferation has yielded promising results. *Ramalina farinaceae* extract in the present study appear to down-regulate cell proliferation indicating possible utility in counteracting cell hyperplasia. It could however, equally still be utilized in other cellular systems when adequately tailored at lower concentrations in specific given settings to meet the contingent need.

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