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Title

Concentration dependant survival and motility of melanoma cell lines on treatment with a *Morinda citrifolia* extract.

Running title

Motility of cell lines on treatment with *Noni* extract.

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Abstract

Noni juice is a novel food that has been reported to have medicinal qualities. The juice is produced from the fruit of *Morinda citrifolia L.* and has been used reportedly in the Polynesian areas of the world for several centuries not only as a source of nourishment but also as a form of local medicine. Melanoma is a steadily increasing problem in the world and is particularly prevalent in the southern hemisphere. Here we investigated the motility inhibition and lethal effects of a Noni preparation and showed a significant decrease in motility of melanoma cells with an increase in the concentration of Noni used. This was accompanied by a loss in viability. The possible application of noni juice or its extracts as an adduct to traditional therapy for the prevention of metastasis and systemic cancer treatment would appear from our results as worthwhile.

Keywords: Noni, Melanoma, Motility, Toxicity.

Introduction

The world of medicinal plants is a prominent part of the pacific region with its zone of 15 million km2. Traditional intellectual property concerning medicinal plant resources has been accumulated fo use over a span of some 3000 years. *Morinda citrifolia*, commonly known as Noni or Mengkudu is one of the most widely used medicinal plants in Polynesia. More than 200 commercial entities sell and distribute Noni products worldwide as a contemporary medicine. The US food and drug administration now requires that these Noni products carry a warning label or sign (1999) indicating that such products can be the cause of serious illness in children, the elderly and persons with weakened immune system.

In fact, acute hepatitis caused by a Noni preparation has been reported [Millonig et al., 2005]. But also protective effects of Noni fruit juice against chronic liver injury induced by carbon tetrachloride in female SD rats has been reported [Wang et al., 2004a] Also cardiovascular disease prevention with Noni by improving lipoprotein profiles [Wang et al., 2005;Wang et al., 2004b]. In a heart protection study an improvement of lipoprotein profiles in current smokers was found in those receiving Noni juice [Wang et al., 2006]. Noni also inhibits the *Candida albicans* growth [Elias et al., 2003] and inhibits the angiotensin I converting enzyme [Yamaguchi et al., 2002] In addition the antioxidative activity of Noni has been well studied [Zin et al., 2007;Yang et al., 2007;Su et al., 2005].The upregulated biosynthesis of type I collagen and glycosaminoglycans by Noni has been shown in fibroblsts and is likely due to anthraquinone [Kim et al., 2005]. Most prominent is the antitumor activity of Noni [Furusawa et al., 2003;Hirazumi and Furusawa, 1999;Nowicki et al., 2005;Wang et al., 2003;Wang et al., 2002;Wang and Su, 2001]. The methanol fraction of Noni is much more effective on cancer cells with no

significant toxic activity against normal cell lines. Human laryngeal carcinoma (Hep2), breast cancer (MCF7) and neuroblastoma (LAN5) cell lines have been tested in this study [Arpornsuwan and Punjanon, 2006] Also in the lewis lung (LLC) peritonal carcinoma an immunomodulatory mechanism has been seen [Hirazumi and Furusawa, 1999]. Prevention of carcinogen-DNA adduct formation and the antioxidant activity of Noni may contribute to the cancer preventive effects [Wang and Su, 2001]. An immunomodulatory prophylactic and therapeutic potential of Noni has been found in sarcoma 180 tumour cells and when combined with a broad spectrum of chemotherapeutic drugs synergistic beneficial effects were found [Furusawa et al., 2003]. The mechanism for these anticancer effects remains unknown. We were interested to see in melanoma cells, one of the most aggressive and invasive cancer types, the influence of Noni on cancer cell motility and invasion and to see if there are any lethal effects of Noni in these cancer cells.

Materials and methods

Cell culture

The cell lines K1735-M2 (kindly supplied by Dr. I. Fiedler MD Anderson Hospital Texas) and A2058 (supplied from the European Cell Culture Collection ECACC) were cultured in DMEM with 10% FCS, L-glutamine and Antibiotics as described in [DeVaney et al., 1997]. The cells were trypsinised and removed from the flask then resuspended in DMEM (Dulbeccos modified eagles medium) containing the required amount of Noni extract and placed at a suitable cell density in the microincubator (Fig. 1) e.g. 1000 cells in 2ml medium (30 mm diameter coverslip with 25 mm diameter presented area for attachment or 4.9 cm^2) at 37°C, 95% relative humidity and 5% CO_2. The cells could be cultured for up to 8 days.

Noni preparation.

An alcoholic extract from Noni juice (Noni PPT) [Hirazumi and Furusawa, 1999] was prepared using 2/3 ethanol to 1/3 Noni juice (supplied by "Good Noni" Gerald Walter Zamenhofstr. 57, A-4020 Linz). The precipitate Noni PPT was then dried in an evacuated dessicator. Aliquots of the Noni PPT were dissolved as a stock solution in DMEM (Sigma-Aldrich) 10% Foetal Calf Serum (FCS from Invtrogen EU approved South American), L-Glutamine Solution (G7513 from Sigma-Aldrich), Penicillin-Streptomycin (P4333 Sigma-Aldrich) and sterile filtered freshly before use.

Motility measurement

The motility of the cells was measured using a macro for time-lapse photography and control of the microscope (Axiovert 35, Zeiss Germany) and the digital kamera (Axiocam HRc Zeiss Germany) written in the programme KS300 (Zeiss Germany). The ensuing series of images was then analysed using imagej (Wayne Rasband (wayne@codon.nih.gov), Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) and the motility plugin (Fabrice Cordelières, Institut Curie, Orsay (France). fabrice.cordelieres at curie.u-psud.fr.)

It was necessary to calibrate the images for the determination of the distances travelled and this was done with the aid of a calibrated micrometer. Velocities and distances of the cells were calculated from the movement of the nucleus, this being considered to be the main center of the cell and the time, between the images taken. The movement distances were averaged and a sliding average calculated for a period of four hours (48 time points) at each point on the following graphs (Figs 2 and 3).

Results

As can be seen in figs. 2 and 3 after attachment of the human and mouse melanoma cells a time dependent difference in the rate of movement can be seen in a manner dependent upon the Noni PPT concentration applied. This confirms that seen in other experiments in vivo and in vitro [Hirazumi and Furusawa, 1999]. A surprising result was the increase in motility of the A2058 cells at a concentration of 0.75 mg/ml Noni PPT that then leads to a rapid decrease in motility and death. Similar results have been also seen in squamous epithelium cell cultures (unpublished results). The final lack of movement in the cells results from their loss in viability and subsequent death. A decrease in the mitochondrial activity of A2058 cells was measured making use of the XTT assay procedure [Freudlsperger et al., 2006] and is seen in fig 4.

Discussion

The use of Noni as a therapeutic agent presents itself here as the lethal effects on melanoma cells have been observed here and on many other cell lines worldwide both in vivo and vitro. The evidence here shows that the cells are responding in a concentration dependent manner. This leads to a loss in viability that eventually also leads to cell death. The form of the cells seen after treatment suggests disruption of the cellular cytoskeleton in such a manner as to induce a non-apoptotic cell death [Gilloteaux et al., 1998;Verrax et al., 2007;Verrax et al., 2005;Gilloteaux et al., 2004]. Reports of hepatotoxicity [Gulberg and Gerbes, 2006;Millonig et al., 2005;West, 2006;Yuece et al., 2006] need to be investigated on a larger scale as these reports are not conclusive that Noni plays a role in the hepatotoxicity. Anthroquinones were taken to be the cause of the hepatotoxicity whereby in noni juice no anthroquinones have been found [Westendorf et al., 2007]. Contrary to these reports it has been shown that Noni can have a liver protection effect [Jensen et al., 2006;Millonig and Vogel, 2006;Wang et al., 2004a;Wang et al., 2005] and in a variety of case studies with related cancer forms it has been reported to increase the survival of individuals . The active ingredient has still to be discovered although a variety have been suggested, none have been shown to be effective in clinical trials [West et al., 2006]. Although a trial from the National Center for Complimentary and Alternative Medicine finished in 2006 no results have been published to this date.

It is of no doubt that Noni juice, as such, results in physiological changes resulting from its ingestion. The degree and form of these changes needs to be studied so that the beneficial effects of the use of noni can be optimised. Suggested changes in chemotherapy that move away from the maximum tolerated dose method to a repeated smaller dose and continual therapy reflects that supported by users of Noni as a dietary supplement.

Micro-incubation chamber

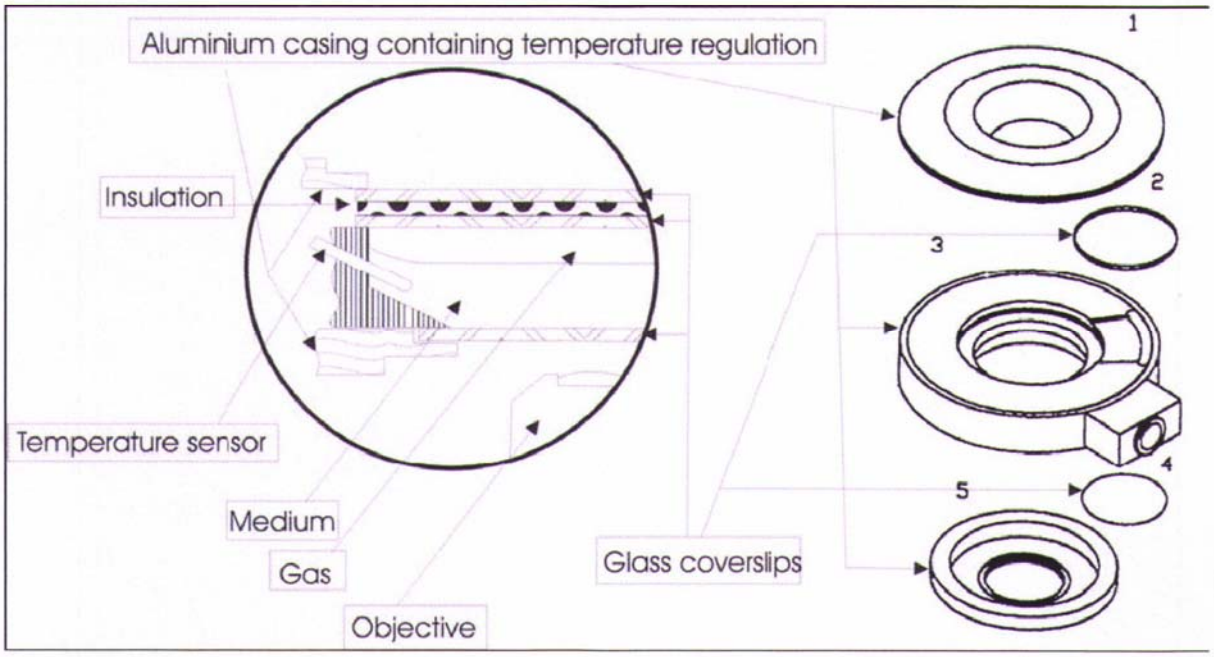

Fig 1.

The microincubation chamber has been developed at our laboratories for the incubation of viable cells on any standard research microscope and has been thoroughly described (DeVaney Ph.D thesis 2004). 1. the sealing cover 2. insulating coverslip 3. Heating element. 4. Coverslip Lower sealing element. Time lapse studies of the cells under investigation were carried out on the glass surface of a standard coverslip.

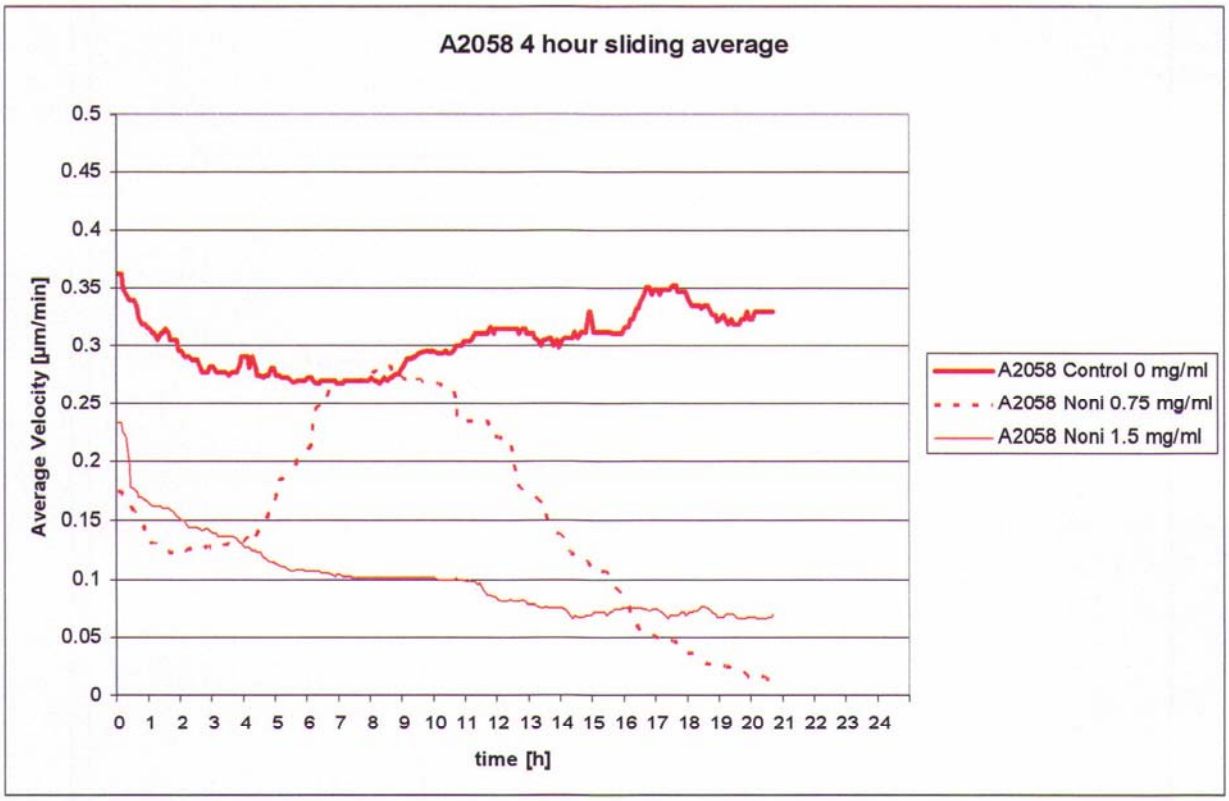

Fig 2.

Motility studies of a human melanoma cell line A2058 over a 24 hour period. Various concentrations of Noni PPT were applied at time 4 hours before time 0 and the cells placed in a micro incubation chamber and their subsequent movement measured. Each spot represents at least 20 cells averaged over a 4 hour period.

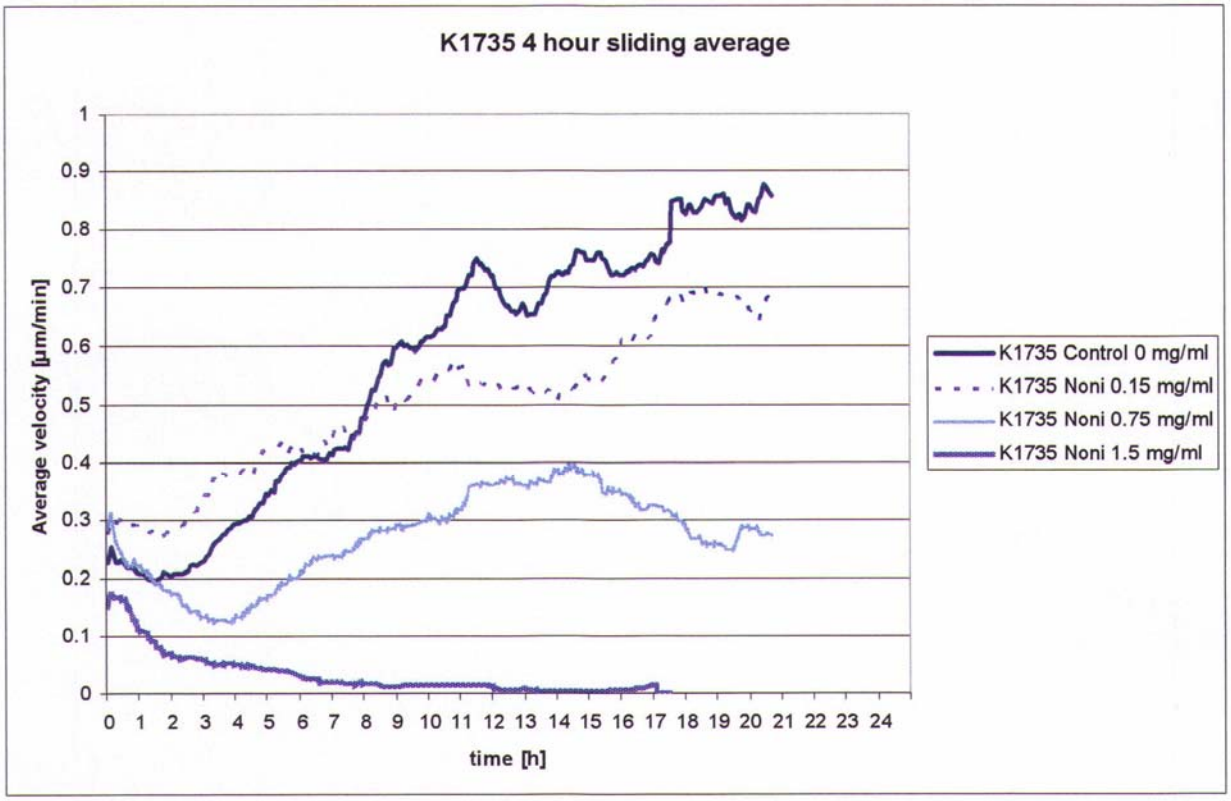

Fig. 3.

Motility studies of a mouse melanoma cell line K1735 M2 over a 24 hour period. Various concentrations of a Noni preparation were applied a time 0 and the cells placed in a micro incubation chamber and their subsequent movement measured. Each spot represents at least 20 cells averaged over a 4 hour period.

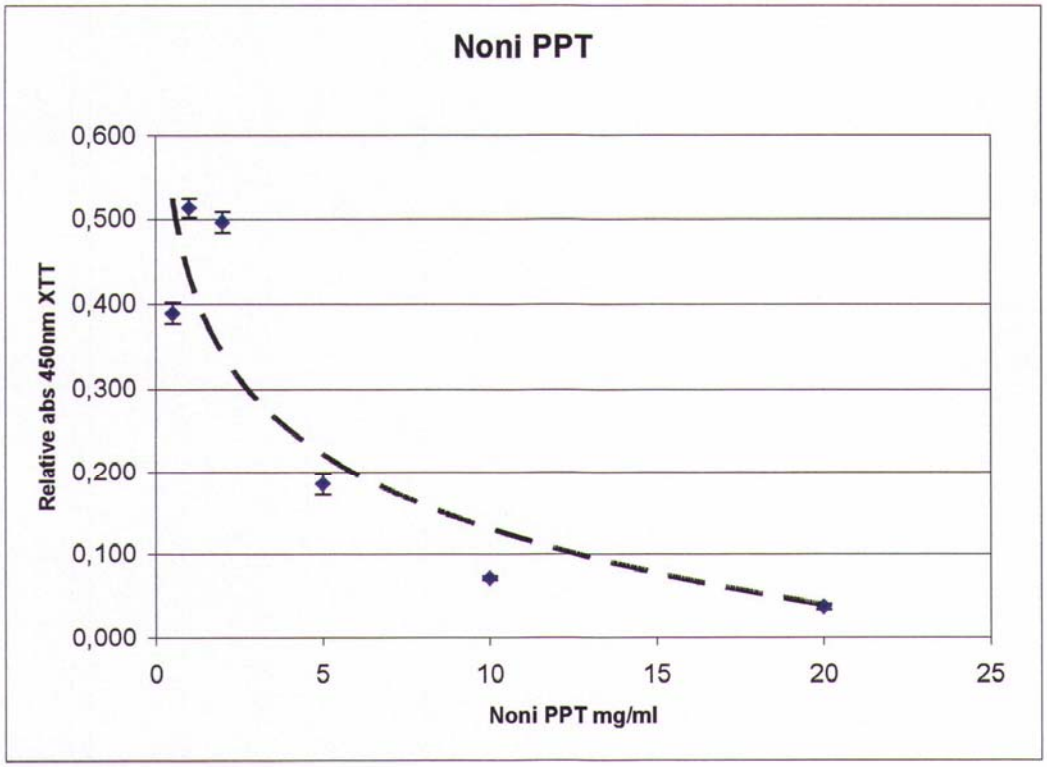

Fig 4

Mitochondrial activity of A2058 cells expressed as the relative absorption measured at 450nm of XTT after 20 hours incubation at 37°C after 24 hours exposure to various concentrations of Noni PPT. The bars are a single standard deviation of the quadruplicate results.

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