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An Immunomodulatory Polysaccharide-Rich Substance from the Fruit Juice of *Morinda citrifolia* (Noni) with Antitumour Activity

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The fruit juice of *Morinda citrifolia* (noni) contains a polysaccharide-rich substance (noni-ppt) with antitumour activity in the Lewis lung (LLC) peritoneal carcinomatosis model. Therapeutic administration of noni-ppt significantly enhanced the duration of survival of inbred syngeneic LLC tumour bearing mice. It did not exert significant cytotoxic effects in an adapted culture of LLC cells, LLC1, but could activate peritoneal exudate cells (PEC) to impart profound toxicity when co-cultured with the tumour cells. This suggested the possibility that noni-ppt may suppress tumour growth through activation of the host immune system. Concomitant treatment with the immunosuppressive agent, 2-chloroadenosine (C1-Ade) or cyclosporin (cys-A) diminished its activity, thereby substantiating an immunomodulatory mechanism. Noni-ppt was also capable of stimulating the release of several mediators from murine effector cells, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-10, IL-12 p70, interferon-γ (IFN-γ) and nitric oxide (NO), but had no effect on IL-2 and suppressed IL-4 release. Improved survival time and curative effects occurred when noni-ppt was combined with sub-optimal doses of the standard chemotherapeutic agents, adriamycin (Adria), cisplatin (CDDP), 5-fluorouracil (5-FU), and vincristine (VCR), suggesting important clinical applications of noni-ppt as a supplemental agent in cancer treatment.

Keywords: antitumour; immunomodulator; polysaccharide; Lewis lung carcinoma; fruit juice; *Morinda citrifolia*.

INTRODUCTION

Immunomodulatory polysaccharides are rapidly emerging as promising immunotherapeutic agents in the treatment of cancer (Wong *et al.*, 1994). Preclinical studies of several polysaccharides isolated from higher plants, mushrooms and seaweeds have demonstrated antitumour activity against transplantable tumours in mice (Sakagami *et al.*, 1987; Yamada *et al.*, 1990; Müller *et al.*, 1989; Tsukagoshi *et al.*, 1984; Chihara, 1991; Furusawa and Furusawa, 1985; Furusawa *et al.*, 1992; Furusawa *et al.*, 1995; Yamamoto *et al.*, 1974). Unlike conventional chemotherapeutics, many are relatively nontoxic and stimulate the immune system. The exact mechanism of antitumour action has not been clearly elucidated. Lentinan, a β-glucan, from the edible mushroom *Lentinus edodes* (shiitake mushroom), is probably the best characterized of the immunomodulatory polysaccharides. Activated macrophages, NK cells and cytotoxic T lymphocytes (CTLs) are generally involved with its antitumour activity (Chihara, 1991). Macrophages may play a role in antitumour activity in part due to the production of effector molecules such as NO, TNF-α and IL-1β. These macrophage-derived mediators have been recognized for their cytostasis and/or cytotoxic properties against tumour cells (Stuehr and Nathan, 1989; Keller *et al.*, 1990; Lovette *et al.*, 1986). NK cells and CTLs may provide effective antitumour responses via lytic mechanisms (Atkinson and Bleackley, 1995). The cytokines, IL-12 and IL-2, have been shown to enhance the lytic responses of NK and CTL cells. They also stimulate the production of IFN-γ, which may augment macrophage activation (Trinchieri and Scott, 1995; Smith, 1993).

Most antitumour polysaccharides investigated have been obtained from traditional Chinese medicinal herbs. Hawaii also has a rich heritage of medicinal herbs (Chun, 1994). *Morinda citrifolia* L., known as 'noni' in Hawaii, was one of the most commonly used herbal medicines by the ancient Hawaiians (Abbott and Shimazu, 1985). In particular, the fruit of the noni plant had been applied alone or in combination with other plant products as a remedy for numerous ailments (Chun, 1994; Degener, 1973; Gutmanis, 1994; Krauss, 1993). There is little scientific evidence that actually ascertains the biological activity of the fruits (Bushnell *et al.*, 1950; Levand, 1963; Sim, 1993; Locher *et al.*, 1996). In this paper, we report the antitumour and immunomodulatory activity of a water-soluble, ethanol-precipitable, polysaccharide-rich substance isolated from the fruit juice of the noni.

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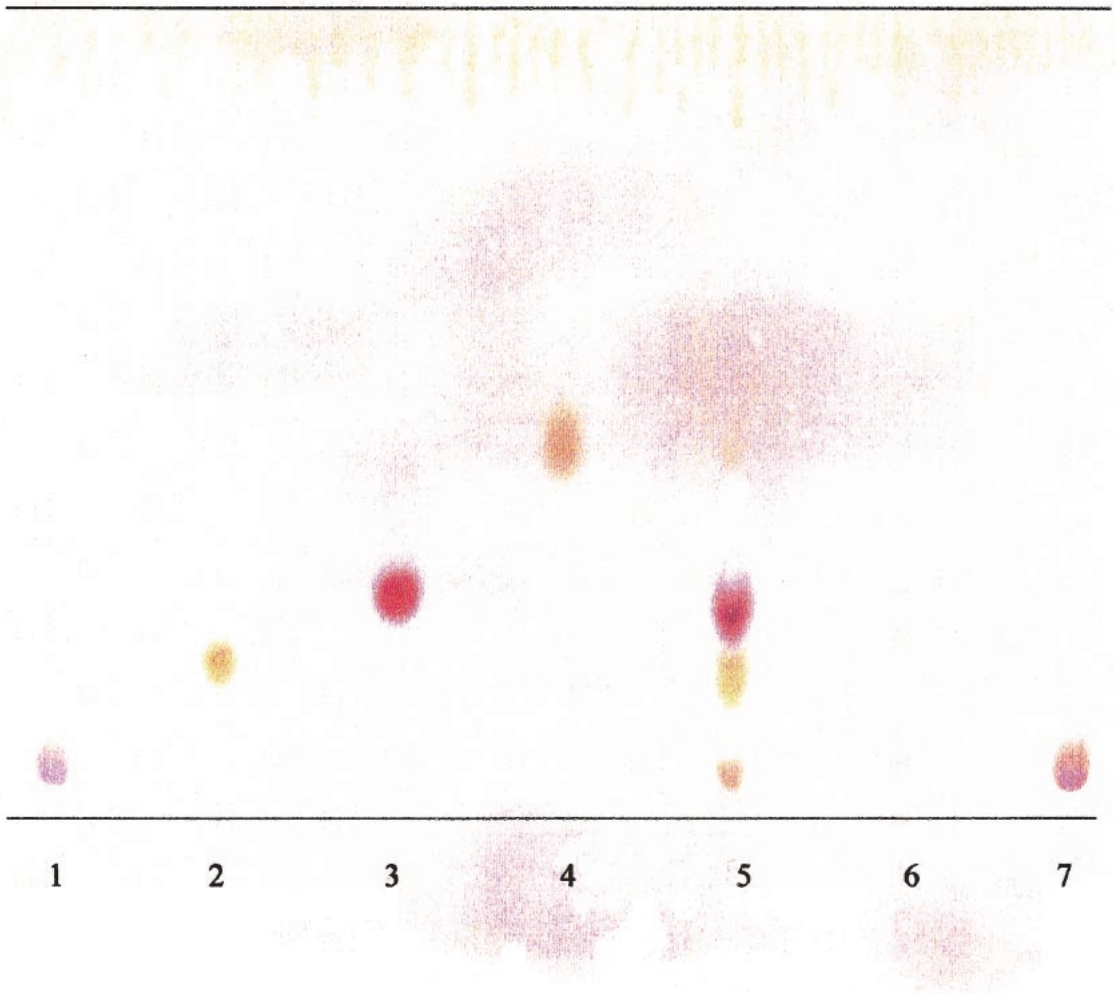

Figure 1. A cellulose thin-layer chromatogram was used to separate the sugar components. (1) glucuronic acid; (2) galactose; (3) arabinose; (4) rhamnose; (5) hydrolysed noni-ppt; (6) unhydrolysed noni-ppt; (7) glucuronic acid. Detection was made with a *p*-anisidine–phthalic acid spray reagent.

MATERIALS AND METHODS

Preparation of agents. Yellowish-white noni fruits were collected from the islands of Kauai and Hawaii. The fruits were allowed to ripen to a soft consistency and rinsed in a food sanitizer. They were then placed in a sterile covered glass jar out in the sunlight for 1–3 days to allow the juice to seep out. Filtration or centrifugation removed the insoluble products from the juice and flash evaporation concentrated the juice. The addition of copious amounts of 95% ethanol partitioned the juice into a soluble fraction (noni-sol) and precipitable fraction (noni-ppt). Noni-ppt was collected by centrifugation, rinsed in ethanol and dried. Flash evaporation removed the ethanol from noni-sol. The fruit juice contained approximately 13% w/w yield of noni-ppt, which was further purified by repeated precipitation for the *in vitro* and characterization assays. Noni samples were freshly prepared by dissolving in RPMI-1640 medium supplemented with 10% FCS and antibiotics (complete medium) for *in vitro* studies and either dH_2O or phosphate buffered saline (PBS, pH 7.4) for *in vivo* studies. Samples were neutralized with sodium bicarbonate and filtered through a 0.2 μm cellulose acetate membrane filter.

Adria, CDDP, 5-FU, methotrexate (MTX), VCR, Cl-Ade, cys-A, concavalin A (con A), lipopolysaccharide (LPS, E. coli 026:B6), polymyxin B (poly B) and IFN-γ were purchased from commercial sources. Con A, LPS, poly B and IFN-γ were dissolved in complete medium. Con A and LPS were stored frozen at −20 °C. IFN-γ was stored frozen at −70 °C. All other agents were dissolved in dH_2O and stored frozen at −20 °C.

Carbohydrate analysis. The phenol–sulphuric acid (PSA) test was used to quantitiate the carbohydrate content of noni-ppt. The methods were followed as described by Keleti and Lederer (1974) with slight modifications. Briefly, 200 μL of 5% v/v of liquid phenol in dH_2O was added to 200 μL of the standard, dextran sulphate (50–250 μg/mL in dH_2O) or noni-ppt (100 μg/mL in dH_2O). Concentrated H_2SO_4 (1 mL) was rapidly added to the phenol mixture and incubated for 10 min at room temperature, then 15 min at 37 °C. Absorbances were measured at 490 nm using a UV-VIS spectrophotometer.

The sugar composition of noni-ppt was characterized by thin-layer chromatography (TLC) (Keleti and Lederer, 1974; Churms *et al.*, 1982). 250 μL of noni-ppt (20 mg/mL in dH_2O) was placed in a glass ampule with 250 μL of 2 N H_2SO_4 and sealed with a flame torch. The ampule was heated in a dry oven (100 °C) for 4 h. The hydrosylate was centrifuged at 2000 rpm for 10 min at 4 °C and the supernatant collected. The supernatant was diluted with 1.2 mL dH_2O and neutralized with activated Amberlite IRA-410 (HCO_3^-) resin. The Amberlite was allowed to settle by gravity and the supernatant was removed and lyophilized. The lyophilized hydrosylate was redissolved in dH_2O (50 mg/mL) and 1 μL was applied to a cellulose thin-layer paper chromatogram, Cellulose 300 (Fisher Scientific, Santa Clara, CA). 0.8 μL of glucuronic acid, galactose, arabinose and rhamnose (0.1 M) were applied as references and 1 μL of unhydrolysed noni-ppt (50 mg/mL) was applied as a negative control. The chromatogram was eluted for 3 h in a saturated (24 h) TLC tank with the following solvent system: *n*-butanol–glacial acetic acid–water (6:1:2). The chromatogram was air-dried overnight and then sprayed with a *p*-anisidine–phthalic acid detecting reagent (0.1 M *p*-anisidine and phthalic acid in 95% ethanol). The chromatogram was heated at 100 °C for 3 min. On the chromatogram, hexose and deoxyhexose stained yellow to green, pentose red and uronic acid brown.

Protein analysis. The Bio-Rad Protein Assay kit (Bio-Rad, Cambridge, MA) was used to determine the presence of protein in noni-ppt. The methodology from the Bio-Rad instruction manual was followed with slight modifications. Briefly, 200 μL of the protein assay dye reagent concentrate (Coomaisse Brilliant Blue G-250 dye) was added to 100 μL of the standard, bovine serum albumin (BSA, 10–160 μg/mL in dH_2O), or noni-ppt (10 mg/mL in dH_2O). Subsequently, 1 mL of dH_2O was added to the dye-sample solution and vortexed. The solution was incubated at room temperature for 5 min. Aliquots (200 μL) of the solution were removed and placed into the wells of a microtitre plate. Absorbances were measured at 595 nm using a Bio-Tek microplate reader.

Animal-tumour system. LLC, originally obtained from the National Cancer Institute (NCI), has been maintained in this laboratory by serial passages subcutaneously (s.c.) in inbred C57BL/6 mice since 1980. The tumour mass (2–3 g, 3–4 weeks old) was minced in 10 mL Hank's solution and filtered through an 80-mesh screen with a 21-gauge needle. An aliquot (0.2 mL) of the tumour homogenate containing $2–4 \times 10^5$ live tumour cells was inoculated intraperitoneally (i.p.) into young adult (18–22 g) male and female inbred C57BL/6 mice. Treatment began 24 h after tumour inoculation. The survival of each mouse was monitored for 50 days. Improvement in survival was evaluated as follows: $\text{ILS } \% = [(T/C) - 1] \times 100$, where ILS is the increase in life span, C is the mean survival days of the control mice and T is that of the treated mice.

Collection of effector cells. Peritoneal exudate cells (PEC), thymocytes and splenocytes were aseptically collected and pooled from 3–5 young adult (6–8 weeks) male C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice inoculated 4 d prior with 3 mL thioglycollate medium. PEC were obtained by sterile lavage of the peritoneal cavity with 5 mL cold RPMI-1640 medium (incomplete medium). Cells were washed three times and resuspended in complete medium. Adherent cells were prepared by incubating PEC for 2 h. Non-adherent cells were removed by three washings with incomplete medium. PEC were also elicited by five daily (QD × 5) i.p. injections of 0.1 mL phosphate buffered saline (PBS) or noni-ppt dissolved in PBS (0.5 mg/mouse). PEC were harvested 1 day after the last injection.

Thymus or spleens were pressed between two sterile glass microscope slides to release cells. Thymocytes were washed three times and resuspended in complete medium. Splenocytes were layered over an equal volume of Histopaque-1077 and separated from red blood cells by centrifugation. The buffy coat layer was washed three times and resuspended in complete medium.

All tissue culture experiments were performed at 37 °C in a humidified 5% CO_2 incubator.

Table 1. Antitumour effect of noni fractions on LLC peritoneal carcinomatosis

Agent	Dose (mg)/mouse	MST ± SEM (days)	No. mice survived/total	ILS (%)
Test 1				
Control		15.9 ± 0.8	0/55	
Crude juice	3	27.5 ± 5.0	1/10	73
	6	32.7 ± 3.2[a]	4/18[b]	106
	12	28.0 ± 3.6[b]	4/17[b]	76
	15	34.7 ± 3.3[a]	9/22[a]	119
	20	21.0 ± 4.5	2/11	32
Test 2				
Control		14.8 ± 0.9	0/58	
Noni-ppt	0.8	32.2 ± 2.5[a]	15/39[a]	118
	1.6	29.0 ± 3.1[a]	5/22[b]	96
Noni-sol	5.2	19.7 ± 2.2	0/12	33
	10.4	14.6 ± 1.1	0/19	0

Inbred C57BL/6 mice were inoculated i.p. with LLC ($2–4 \times 10^5$ cells/mouse) on day 0. 0.1 mL of vehicle or noni samples was administered i.p. at the indicated doses QD or QOD × 4–5 injections beginning on day 1. Survival of mice were recorded up to 50 days. Mice surviving 50 days were considered cured. [a] $p < 0.001$, [b] $p < 0.01$ compared with control. MST, mean survival time; ILS, increase in life span.

Table 2. R_f values of reference sugars and hydrolysed and unhydrolysed noni-ppt

Compound	$R_f \times 100$	Colour
Glucuronic acid	8	br
Galactose	20	y-gr
Arabinose	29	r
Rhamnose	47	y-gr
Hydrolysed noni-ppt	7, 18, 27, 46	br, y-gr, r, y-gr
Unhydrolysed noni-ppt		

A cellulose thin-layer chromatogram was used to separate the sugar components. Detection was made with a *p*-anisidine–phthalic acid spray reagent, which detects hexose and deoxyhexose sugars as yellow to green, pentose as red and uronic acid as brown. br: brown; y-gr: yellow-green; r: red.

Cytotoxicity assays. LLC1 cells, purchased from ATCC (Rockville, MD), were maintained by serial passages in complete medium. Cellular viability of LLC1 cells (5×10^4 cells/mL) incubated in a 96-well microtitre plate (200 μL) for 70 h was determined with a solution of the tetrazolium salt, sodium 3-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulphonic acid hydrate (XTT), dissolved in dH_2O (1 mg/mL) mixed with an electron coupler, phenazine methosulphate (PMS), dissolved in PBS (30.6 μg/mL). 50 μL of the XTT/PMS solution was added to each well and incubated for an additional 2 h. Absorbances were measured at 450 nm.

Cellular viability of LLC1 (2×10^5 cells/mL) co-incubated with PBS or noni-ppt elicited PEC (5×10^5 cells/mL) in a 96-well microtitre plate (200 μL) for 46 h was determined with the tetrazolium salt, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT), dissolved in PBS (2 mg/mL). 50 μL of MTT was added to each well and incubated for an additional 2 h. The supernatants were decanted and 200 μL of acidified isopropanol (40 mM HCl) was added to each well to solubilize the MTT reaction product. Absorbances were measured at 550 nm. PEC-mediated cytotoxicity was calculated as follows: % cytotoxicity = $1 - [(OD_{550}$ tumour + PEC) − OD_{550} PEC]/OD_{550} tumour.

Nitrite determination. The Griess reagent (Sotomayor *et al.*, 1995) was used to measure nitrite production from adherent thioglycollate-elicited PEC ($<1 \times 10^6$ cells/mL) incubated in a 96-well microtitre plate (200 μL) for 24 h. The Griess reagent was prepared by mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride in dH_2O and 1% suphanilamide in 5% concentrated phosphoric acid. 50 μL of the Griess reagent was added to each well and incubated for 10 min at room temperature. Absorbances were measured at 550 nm.

Cytokine determination. Cytokine (IL-1β, IL-2, IL-4, IL-10, IL-12 p70, IFN$_{-\gamma}$ and TNF$_{-\alpha}$) release by murine effector cells were measured with ELISA kits commercially available from Amersham (Arlington Heights, IL) or Genzyme (Cambridge, MA). Cytokine levels were determined from the cell-free supernatants of adherent thioglycollate-elicited PEC ($<1 \times 10^6$ cells/mL) incubated in either a 24- or 96-well microtitre plate (1 mL or 200 μL, respectively) for 16 or 24 h and thymocytes (5×10^6 cells/mL) or splenocytes (2.5×10^6 cells/ml) incubated in a 96-well microtitre plate (200 μL) for 72 h. Each ELISA kit was a solid-phase enzyme immunoassay employing the 'sandwich' principle. Briefly, antibodies specific for each individual cytokine were coated on the microtitre plate provided in the kit. Supernatants were pipetted into each well and incubated either along or subsequently following washings (depending on the methodology recommended by the vendor) with a biotinylated antibody reagent. After repeated washings, a streptavidin HRP conjugate was added. Following a final wash, a substrate solution was added and a coloured product developed in proportion to the amount of cytokine present in the samples. Absorbances were measured at 450 nm.

Statistical analysis. Experimental results were analysed for their significance by the Student's two tailed *t*-test or Fisher's exact probability test.

RESULTS

Effect of the noni fruit juice against LLC peritoneal carcinomatosis

Graded doses of the crude noni fruit juice were administered i.p. QD or every other day (QOD) for a total of 4–5 injections commencing from day 1 after i.p. LLC tumour inoculation in syngeneic inbred C57BL/6 mice. The results are shown in Table 1. The juice demonstrated curative effects from 3 to 20 mg/mouse and statistically significant antitumour activity between 6–15 mg/mouse. Noni juice at doses of 6–15 mg/mouse prolonged the life span by more than 75%. Ethanol fractionation of the juice concentrated the antitumour activity in the ethanol-insoluble fraction (noni-ppt). Noni-ppt prolonged the life span of tumour-bearing mice by more than 75%, whereas noni-sol was unable to elicit significant beneficial antitumour activity. The dose of

Table 3. Cytotoxicity of noni-ppt against LLC1 cells

Agent	Dose (mg/mL)	Abs (450 nm)	Cytotoxicity (%)
Control		2.049 ± 0.196	
Noni-ppt	6.25	1.590 ± 0.105[a]	22.4
	2.08	2.136 ± 0.151[b]	+4.3
	0.69	2.054 ± 0.220	+0.2
CDDP	0.005	0.530 ± 0.060[a]	74.1

Serial dilutions of noni-ppt were added to LLC1 cell cultures (5×10^4 cells/mL) in a 96-well microtitre plate. Cultures were incubated at 37 °C for 72 h. The XTT assay was used to determine cellular viability. The results are shown as mean ± SD of 8 replicates from one of three similar experiments. CDDP was used as a positive control. [a] $p < 0.001$, [b] $p < 0.05$ compared with control.

noni-ppt and noni-sol tested corresponded to the proportion produced upon ethanol fractionation.

Partial characterization of noni-ppt

The PSA assay indicated that noni-ppt was almost entirely composed of carbohydrates. Noni-ppt was found to contain approximately 1.39 ± 0.05 mg of carbohydrate per mg compared with a dextran sulphate standard ($n = 3$). A TLC of noni-ppt revealed the existence of a gum arabic heteropolysaccharide (Fig. 1, Table 2). Glucuronic acid, galactose, arabinose and rhamnose were identified from the chromatogram of the hydrolysed noni-ppt based on their R_f values and staining colour in comparison with known sugars. In Fig. 1, the hue of the sugars and the visibility of rhamnose from the hydrolysed sample of noni-ppt were affected in the reproduction of the original chromatogram. Also, in the original chromatogram, sugar residues from the unhydrolysed sample of noni-ppt were only faintly detected, indicating that the sugars, glucuronic acid, galactose, arabinose and rhamnose were principally part of the polymer rather than free monosaccharides. The glucuronic acid reference was applied on both ends of the chromatogram to demonstrate the elution pattern, which was linear since their R_f values were identical. Because the sensitivity of the *p*-anisidine phthalic acid reagent is between 0.1–0.5 μg (Randerath, 1966) and detects only hexoses, deoxyhexoses, pentoses and uronic acids (Churms *et al.*, 1982), other sugars may be present in the noni-ppt, but may not be apparent using this staining method.

The presence of protein in noni-ppt was determined colorimetrically with the Bio-Rad Protein Assay. Noni-ppt was determined to contain 18.56 ± 3.98 μg protein per mg (approximately 2%) compared with a BSA standard ($n = 2$ experiments in triplicates).

Cytotoxicity test of noni-ppt in cell cultures

Serial dilutions of noni-ppt were incubated with LLC1 cells for 72 h in a 96-well microtitre plate to determine the degree of cytotoxicity (Table 3). The XTT assay was employed to assess the cellular viability of the cells. Although very high doses (6.25 mg/mL) of noni-ppt exerted cytotoxic effects, the extent of the observed cytotoxicity was substantially less than the positive cytotoxic control agent, CDDP (22% vs 74% cytotoxicity). Noni-ppt was not cytotoxic below 2 mg/mL. Routinely, extracts of crude natural product origin are designated as nontoxic when it is non-cytotoxic at concentrations of 1–2 mg/mL (Furusawa *et al.*, 1995).

Effect of noni-ppt with concomitant treatment with immunosuppressive agents

Toxicity screening indicated that noni-ppt was essentially nontoxic, yet *in vivo* studies demonstrated antitumour activity against LLC. Therefore, concurrent administration of immunosuppressive agents along with the noni-ppt was investigated to see if the antitumour activity involved the host immune system. Cl-Ade (a macrophage inhibitor) or cys-A (a T-lymphocyte inhibitor) and the noni-ppt were administered i.p. 1 day after tumour inoculation and continued every other day for a total of five injections or given as a single *sc* injection, in the case of cys-A. The results shown in Table 4 indicate that the Cl-Ade completely abolished the antitumour activity of noni-ppt, whereas cys-A moderately attenuated the antitumour activity. The combination of cys-A and noni-ppt was significantly higher than the control, but significantly less than noni-ppt treatment alone.

Effect of chemoimmunotherapy of noni-ppt with standard chemotherapeutic agents

Sub-optimal doses of standard cytotoxic agents for combination therapy in the LLC tumour system have been established (Furusawa and Furusawa, 1990). The effect of noni-ppt as a supplementary agent in combination with chemotherapy was assessed in view of its

Table 4. Combination of immunosuppressive agents with noni-ppt against LLC peritoneal carcinomatosis

Agent	Dose/mouse, schedule (route)	MST ± SEM	No. mice survived/total	ILS (%)
Control		15.0 ± 1.7	0/10	
Noni-ppt	0.8 mg, QOD × 5 (i.p.)	35.9 ± 5.6[a]	5/9[b]	139
Cl-Ade	50 μg, QOD × 5 (i.p.)	12.2 ± 0.9	0/9	−19
Cl-Ade + Noni-ppt	same as above	12.8 ± 0.8[c]	0/9	−15
Cys-A	2 mg, day 1 (s.c)	15.4 ± 1.4	0/9	3
Cys-A + Noni-ppt	same as above	19.2 ± 1.6[b,d]	0/9	28

Inbred C57BL/6 mice were inoculated with LLC ($2–4 \times 10^5$ cells/mouse) on day 0. 0.1 mL of vehicle or agents were administered i.p. at the indicated doses beginning on day 1. Survival of mice was recorded up to 50 days. Mice surviving 50 days were considered cured. [a] $p < 0.005$, [b] $p < 0.01$ compared with control. [c] $p < 0.01$, [d] $p < 0.05$ compared with noni-ppt. MST, mean survival time; ILS, increase in life span.

Table 5. Combination of suboptimal doses of chemotherapy with noni-ppt against LLC peritoneal carcinomatosis

Agent	Dose/mouse, schedule (route)	MST ± SEM	No. mice survived/total	ILS (%)
Exp. 1				
Control		13.9 ± 0.9	0/9	
Noni-ppt	0.8 mg, QOD × 5 (i.p.)	28.0 ± 4.9[b]	2/9	101
Adria	2 μg, day 1 (i.p.)	24.2 ± 3.9[b]	1/9	74
Adria + Noni-ppt	same as above	42.3 ± 4.0[c]	6/9[a]	204
Exp. 2				
Control		14.9 ± 1.4	0/10	
Noni-ppt	0.8 mg, QOD × 5 (i.p.)	28.4 ± 5.3[b]	2/8	91
CDDP	10 μg, day 1 (i.p.)	25.2 ± 5.0[b]	1/8	69
CDDP + Noni-ppt	same as above	41.4 ± 4.5[c]	5/8[a]	178
Exp. 3				
Control		16.9 ± 2.4	0/7	
Noni-ppt	0.8 mg, QOD × 5 (i.p.)	33.8 ± 6.6[b]	4/8[b]	100
5-FU	300 μg, day 1 (i.p.)	33.4 ± 6.3[b]	3/8	98
5-FU + Noni-ppt	same as above	45.6 ± 4.4	7/8[a]	170
Exp. 4				
Control		16.7 ± 1.4	0/10	
Noni-ppt	0.8 mg, QOD × 5 (i.p.)	35.5 ± 4.6[b]	3/8[b]	113
VCR	1 μg, day 1 (i.p.)	30.5 ± 4.8[b]	2/8	83
VCR + Noni-ppt	same as above	44.4 ± 4.4[c]	6/8[a]	166

Inbred C57BL/6 mice were inoculated i.p. with LLC (2–4 × 10^5 cells/mouse) on day 0. 0.1 mL of vehicle or agents was administered i.p. at the indicated doses beginning on day 1. Survival of mice was recorded up to 50 days. Mice surviving 50 days were considered cured. [a] $p < 0.01$, [b] $p < 0.05$ compared with control. [c] $p < 0.05$ compared with chemotherapeutic agent or noni-ppt. MST, mean survival time; ILS, increase in life span.

clinical application. Administration of the chemotherapeutic agents (Adria, CDDP, 5-FU, MTX or VCR) as a single dose on day 1 was combined with noni-ppt treatment beginning also on day 1 QOD × 5 (Table 5). Significant beneficial effects occurred with the combined regimen of noni-ppt with Adria, CDDP, 5-FU or VCR compared with the chemotherapy alone. The lifespan of tumour bearing mice was dramatically increased to more than 150% in all cases and improved cure rates were also observed. The combined regimen with MTX was not effective.

PEC-mediated cytotoxicity asssay

PEC elicited by PBS or noni-ppt were co-incubated with LLC1 cells for 48 h at a ratio of (2.5:1). The MTT assay was used to determine the cellular viability of the cells (Table 6). Noni-ppt-elicited PEC exerted significant cytotoxic effects to LLC1 cells compared with the PBS-elicited PEC. Noni-ppt-elicited PEC reduced the viability of LLC1 by 37% in contrast to 4% by PBS-elicited PEC.

Table 6. Cytotoxicity test of PBS-elicited vs noni-ppt-elicited PEC against LLC1 cells

PEC	Cytotoxicity (%)
PBS-elicited	4.03 ± 2.39
Noni-ppt-elicted	37.4 ± 2.80[a]

PEC were elicited by i.p. injections of PBS (0.1 mL, QD × 5) or noni-ppt (0.1 mL of 5 mg/mL, QD × 5). PEC (5 × 10^5 cells/mL) were coincubated with LLC1 (2 × 10^5 cells/mL) for 48 h. The MTT assay was used to determine cellular viability. The results are shown as mean ± SEM of 3 experiments in triplicate. [a] $p < 0.001$ compared with PBS-elicited PEC.

NO and cytokine production by noni-ppt

The effect of noni-ppt on macrophage function was assessed by measuring the amount of NO, TNF-α, IL-1β and IL-12 p70 produced from thioglycollate-elicited adherent PEC. The Griess reagent was used to measure nitrite levels, the stable endproduct of NO metabolism. Noni-ppt at 1.25 mg/mL slightly elevated nitrite levels (Fig. 2). Medium alone elicited 0.56 nmol nitrite per 10^6 cells compared to 1.85 nmol from noni-ppt. When combined with IFN-γ (25 U/mL), noni-ppt enhanced the production of nitrite (8.03 nmol). Similar results were obtained with the positive control, LPS. LPS alone (0.5 mg/mL) stimulated an increased amount of nitrite

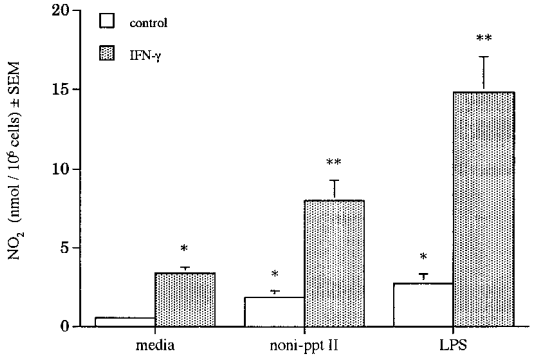

Figure 2. Production of nitrite by adherent thioglycollate-elicited PEC (<1 × 10^6 cells/mL) treated 24 h with medium alone, noni-ppt (1.25 mg/mL), LPS (0.5 μg/mL), or combinations with IFN-γ (25 U/mL). The Griess reagent was used to detect the presence of nitrite. The results are shown as mean ± SEM of 5 experiments in triplicates. Error bars not seen fall within the columns. *$p < 0.01$ compared with media alone. **$p < 0.001$ compared with IFN-γ.

Table 7. Cytokine production from adherent thioglycollate-elicted PEC

		Treatment		
Cytokine	Incubation time (h)	Media	Noni-ppt (1.25 mg/mL) *pg/mL*	LPS (0.5 μg/mL)
IL-1β	24	0.68 ± 0.58	49.24 ± 8.93[a]	127.33 ± 12.12[a]
IL-12 p70	16	1.23 ± 1.23	10.26 ± 1.16[b]	9.10 ± 3.35
TNF-α	24	14.77 ± 1.46	757.37 ± 400.62	1213.92 ± 442.97[b]

IL-1β, IL-12 p70 and TNF-α release from adherent thioglycollate-elicited PEC ($<1 \times 10^6$ cells/mL) incubated in a 24-well plate. ELISA kits were used to detect the presence of these macrophage-derived cytokines from the cell-free supernatants. The results are shown as mean ± SEM of at least two experiments in duplicate. [a] $p < 0.01$, [b] $p < 0.05$ compared with media.

production (2.75 nmol) and induced a 5-fold increase when combined with IFN-γ (14.84 nmol). IL-1β and IL-12 p70 release were also significantly enhanced by noni-ppt. The production of IL-1β by noni-ppt was substantially less than LPS, although the production of IL-12 p70 was comparable to LPS (Table 7). The IL-12 p70 levels detected were below the assay range (20–1620 pg/mL), but greater than the ELISA kit's (Genzyme) sensitivity (5 pg/mL). Noni-ppt also stimulated TNF-α production, and its level, similar to IL-1β, was less than that produced by LPS (Table 7). The effect of poly B on noni-ppt was assessed by measuring TNF-α production from thioglycollate-elicited murine PEC with an ELISA kit. Figure 3 illustrates that poly B exerted little effect on noni-ppt. Poly B inhibited the activity of noni-ppt by only 0.67%, whereas it inhibited the activity of LPS by 42.04% ($n = 3$ experiments in duplicates).

The effect of noni-ppt on splenocytes and thymocytes was evaluated by measuring the levels of the cytokines, IL-2, IL-4, IL-10 and IFN-γ (Table 8). Noni-ppt had no effect on IL-2 release by splenocytes, even when combined with con A, whereas LPS alone induced a suppressive effect on IL-2 and acted synergistically with a con A concentration of 3 mg/mL. Noni-ppt exerted a significant suppressive effect on IL-4 production when combined with con A concentrations of 3 and 6 mg/mL. LPS synergistically enhanced IL-4 release with 3 mg/mL of con A and had no effect on IL-4 release with 6 mg/mL of con A. IL-10 release by splenocytes was significantly increased by the presence of noni-ppt. Additive effects occurred when noni-ppt was combined with increasing concentrations of con A. Levels of IL-10 stimulated with LPS were high regardless of the concentration of con A. IFN-γ production from thymocytes was not elicited by noni-ppt alone, but synergistic effects occurred when noni-ppt was combined with con A. LPS alone stimulated a significant increase of IFN-γ and acted synergistically with con A.

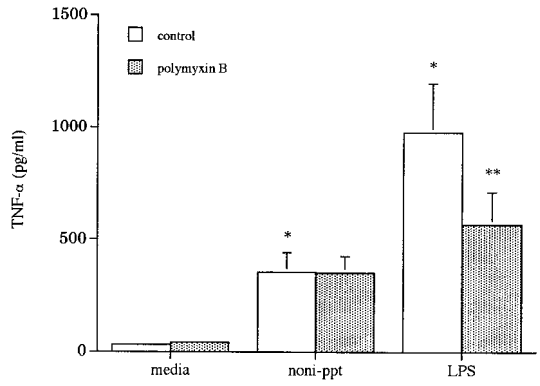

Figure 3. TNF-α production from adherent thioglycollate-elicited PEC ($\sim 1 \times 10^6$ cells/mL) incubated with cell culture media alone, polymyxin B (100 U/mL), noni-ppt (1.25 mg/mL), LPS (0.5 μg/mL) or combinations of polymyxin B and noni-ppt or LPS for 24 h. An ELISA kit was used to detect the presence of TNF-α from the cell-free supernatants. The results are shown as mean ± SEM of 3 experiments in duplicates. Error bars not seen fall within the columns. *$p < 0.01$ compared with media alone. **$p < 0.01$ compared with LPS.

DISCUSSION

The fruit juice of *Morinda citrofolia* L., a traditional Hawaiian medicinal plant known as noni, has been found to possess significant antitumour activity against LLC peritoneal carcinomatosis. The antitumour principle(s) was isolated in the ethanol-precipitable fraction, noni-ppt. The ethanol-soluble fraction, noni-sol, did not demonstrate any significant antitumour activity. Generally, the primary purification step of naturally occurring polysaccharides entails acetone/alcohol precipitation (Wong *et al.,* 1994). We have characterized noni-ppt as containing a gum arabic heteropolysaccharide, composed of the sugars glucuronic acid, galactose, arabinose and rhamnose, by phenol-sulphuric acid staining and TLC.

Cytotoxicity testing of noni-ppt against LLC1, a cell line adapted for culture from the primary LLC (Bettram and Janik, 1980), indicated that it was not directly cytotoxic, even at a high concentration of 2 mg/mL. However, it could indirectly exert significant cytotoxic effects against LLC1 cell by eliciting the tumoricidal activity of PEC. The immunosuppressive agents, Cl-Ade (macrophage inhibitor) and cys-A (T cell inhibitor) also attenuated the activity of noni-ppt, signifying a possible immunomodulatory role of noni-ppt involving macrophage and T cell activation.

A series of cytokine assays and NO determination corroborated the speculation that noni-ppt can induce macrophage activation. Noni-ppt effectively enhanced the production of NO, TNF-α, IL-1β and IL-12 p70 from thioglycollate-elicited adherent PEC, which are mainly composed of macrophages. These macrophage-derived products are considered important mediators of tumour cytostasis and/or cytotoxicity. NO may produce cellular toxicity through the inhibition of DNA synthesis and mitochondrial respiration (Stuehr and Nathan, 1989). TNF-α may be able to directly cause haemorrhagic necrosis of tumours (Männel *et al.*, 1996). IL-1β has been shown to be a mediator of tumour cytostasis (Lovett *et al.*, 1986) and IL-12 p70, the biologically active

Table 8. Cytokine production from splenocytes or thymocytes incubated in the presence of increasing concentrations of con A

			Treatment		
				Noni-ppt (1.25 mg/mL)	
Cytokine	Cellular source	Con A (μg/mL)	Media	*pg/mL*	LPS (0.5 μg/mL)
IL-2	splenocytes	0	51.73 ± 3.94	56.05 ± 18.11	25.9 ± 3.04[b]
		0.3	73.76 ± 7.72	77.00 ± 6.78	58.6 ± 1.97
		3.0	1405.97 ± 120.90	1388.13 ± 36.69	2614.09 ± 320.96[b]
		6.0	2653.94 ± 104.89	2456.87 ± 479.67	2517.98 ± 326.63
IL-4	splenocytes	0	17.09 ± 3.83	13.74 ± 5.43	20.38 ± 3.49
		0.3	13.64 ± 3.02	17.09 ± 1.73	19.03 ± 4.10
		3.0	181.89 ± 16.97	68.43 ± 5.38[a]	323.72 ± 49.89
		6.0	920.03 ± 72.80	295.68 ± 129.47[a]	818.97 ± 200.52
IL-10	splenocytes	0	165.69 ± 25.30	1132.27 ± 183.82[b]	2548.70 ± 19.59[a]
		0.3	630.21 ± 14.41	1717.66 ± 101.68[a]	2616.24 ± 30.49[a]
		3.0	1423.70 ± 61.13	2392.34 ± 49.50[a]	2541.15 ± 39.32[b]
		6.0	2154.24 ± 15.02	2568.48 ± 57.48[a]	2495.36 ± 58.57[b]
IFN-γ	thymocytes	0	1.64 ± 1.58	0.36 ± 0.36	24.84 ± 8.12[b]
		0.3	3.24 ± 1.20	8.56 ± 4.15	35.87 ± 5.03
		3.0	62.94 ± 4.76	313.20 ± 59.90[a]	1303.82 ± 139.50[a]
		6.0	105.37 ± 10.56	692.07 ± 216.31[b]	1817.62 ± 400.08[a]

IL-2, IL-4, IL-10 and IFN-γ release from splenocytes (2.5×10^6 cells/mL) or thymocytes (5×10^6 cells/mL) incubated in a 96-well microtitre plate for 72 h. ELISA kits from were used to detect the presence of these cytokines from the cell-free supernatants. The results are shown as mean ± SEM of at least 2 experiments in duplicate. [a] $p < 0.01$, [b] $p < 0.05$ compared with control concentration of con A.

heterodimer of IL-12, may induce toxic effects indirectly by enhancing the Th1 cellular immunity response (Trinchieri and Scott, 1995). Therefore, noni-ppt may mediate its antitumour activity by stimulating macrophages to release an arsenal of tumoricidal mediators.

Noni-ppt also enhanced the release of IFN-γ from con A stimulated thymocytes. IFN-γ, a potent macrophage activator (Young and Hardy, 1995), may serve as a positive feedback mechanism to promote further activation of macrophages, initially stimulated by noni-ppt. On the other hand, noni-ppt also stimulated the release of high levels of IL-10, a cytokine synthesis inhibitor and macrophage deactivator (Bogdan *et al.*, 1991), from splenocytes. Conceivably, IL-10 may act in a negative feedback mechanism to prevent potential detrimental effects from excessive macrophage activation.

The influence of noni-ppt on the cell-mediated cytotoxicity of NK and T cells may be inferred from its suppressive effect on IL-4 production from con A stimulated splenocytes, as well as its stimulatory effect on IL-12 p70 production. IL-4 and IL-12 generally have opposing effects on cell-mediated immunity (Trinchieri, 1995). IL-4 acts in most instances opposite to a Th1 cellular immunity response, whereas IL-12 promotes a Th1 response. The contrary effects of noni-ppt on IL-4 and IL-12 production may augment the Th1 response and enhance the cytotoxicity of CTL and NK cells.

The antitumour activity of noni-ppt may not involve IL-2, a T-cell growth factor, since noni-ppt did not demonstrate any significant effect on the production of IL-2 from splenocytes. However, cys-A, an inhibitor of IL-2 expression (Wiederrecht *et al.*, 1993), diminished the antitumour activity of noni-ppt in tumour-bearing mice, possibly indicating that following *in vivo* administration of noni-ppt, IL-2 may be indirectly involved in the antitumour response of noni-ppt. Otherwise, the immunosuppressive effect of cys-A on the other components of the immune system may be contributing to its inhibitory effect on noni-ppt.

The immunomodulatory effect of noni-ppt on NO, IL-1β and TNF-α production was similar to LPS. Both agents stimulated a comparable pattern of release, with the quantity produced by noni-ppt consistently lower than LPS. Due to the ubiquitous nature of LPS, the possibility of LPS contamination in the noni-ppt does exist. However, polymyxin B was unable to inhibit the stimulatory effect of noni-ppt on TNF-α production, while inhibiting the positive LPS control. Also, several differences in the cytokine profile produced by noni-ppt suggest that its effects are unique. For example, noni-ppt significantly suppressed IL-4 production in the presence of 3–6 mg/mL of con A while LPS did not. The amount of IL-12 p70 stimulated by noni-ppt, although relatively low, was approximately the same as the amount produced by LPS, implying that noni-ppt may have a selective bias for IL-12 p70 production. Routinely, the crude noni juice and noni-ppt was cultured fastidiously in media to confirm that there was no microbial contamination.

Chemoimmunotherapy of the noni-ppt with conventional chemotherapeutic agents (Adria, CDDP, 5-FU and VCR) revealed a significant beneficial effect compared with the chemotherapy alone. The combination regimen, using sub-optimal doses of chemotherapy, increased the life span as well as cure rates in tumour-bearing animals. Anticancer chemotherapy is generally accompanied by severe toxic effects and immunosuppression. Thus, the possibility of combining noni-ppt with sub-optimal doses of chemotherapy may not only reduce the toxic side effects of the chemotherapy by lowering its dose, but may improve the curative probability.

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