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Apoptosis-inducing effects of *Morinda citrifolia* L. and doxorubicin on the Ehrlich ascites tumor in Balb-c mice

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Morinda citrifolia L. (Noni) is a herbal remedy with promising anti-cancer properties. However, its effects on various cancers are to be investigated to make a firm conclusion before implementing it into the clinical practice. Therefore, we investigated the cytotoxic potential of noni on Ehrlich ascites tumor grown in female Balb-c mice and also combined it with a potent anti-cancer agent, doxorubicin. One group received noni only ($n = 8$), another one doxorubicin ($n = 8$), and the other one noni + doxorubicin ($n = 8$) for 14 days after the inoculation of cells. The control group ($n = 7$) received 0.9% NaCl only. We found that short and long diameters of the tumor tissues were about 40–50% smaller, compared to those in control group. This anti-growth effect resulted from the induction of apoptosis, which was confirmed by the positive results from the *T*erminal Deoxynucleotidyl Transferase d*U*TP *N*ick *E*nd *L*abeling (TUNEL) analysis and the active caspase-3 cells in tissues. Apoptosis also confirmed by caspase-cleaved cytokeratin 18 elevation in serum of the treated groups. Further, the proliferation was decreased, which was immunohistochemically shown by the PCNA staining. We conclude that noni may be useful in the treatment of breast cancer either on its own or in combination with doxorubicin. Further studies are warranted to assess the dosage and safety of using noni fruit juice in conjuction with anti-cancer drugs against breast cancer. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS — *Morinda citrifolia* L; noni; apoptosis; proliferation; cancer; mice; *in vivo*

INTRODUCTION

It seems that there is still no satisfactory improvement in the success of cancer treatment although anti-cancer agents are constantly being introduced. Therefore, the plant-based novel treatments have started to gain importance. *Morinda citrifolia* L. (Noni) has been used in folkloric medicine for centuries against some diseases, including cancer. First studies of noni on cancer dated back to early 1990s. Hiramatsu *et al.*[1] screened tropical plant extracts for substances that induce normal morphology in K-rasts-NRK cells. As a result, they isolated an anthraquinone compound, damnacanthal, from the chloroform extract of the root of noni and found that damnacanthal was an inhibitor of Ras function. Ras signaling causes cell growth and malignant transformation. Activating mutation in Ras is found in approximately 30% of human cancer.[2] Studies showed the involvement of Ras not only in tumorigenesis but also in many developmental disorders.[3] In 1994, Hirazumi *et al.*[4] showed the anticancer activity of noni on intraperitoneally implanted Lewis lung carcinoma in syngeneic mice. In the later years, the anticancer activity was shown to be achieved by activating the host's immune system.[5] An immunomodulatory polysaccharide-rich substance is present in noni. This substance stimulated the release of several mediators from murine effector cells, including tumor necrosis factor-α, interleukin-1β, interferon-γ, while it suppressed interleukin-4 release.[5] The same immunomodulatory substance from the fruit juice of Noni has also been found to possess both prophylactic and therapeutic potentials against the immunomodulator sensitive Sarcoma 180 tumor system.[6]

After the year 2000, two novel glycosides, 6-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose and asperulosidic acid, extracted from the juice of noni fruits, were used to examine their effects on 12-O-tetradecanoylphorbol-13-acetate (TPA)- and epidermal growth factor (EGF)-induced AP-1 transactivation and cell transformation in mouse epidermal JB6 cells.[7] The results indicated that both compounds were effective in suppressing TPA- or EGF-induced cell transformation and associated AP-1 activity.

Angiogenesis is an important feature of malign tumor development and it is usually associated with poor survival in cancer patients. Noni was also found to inhibit

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angiogenesis *in vitro* by Hornick *et al*.[8] They used noni at a concentration of 10% in growth media, and observed that noni was able to induce vessel degeneration and apoptosis within a few days of its application. They also found that 10% noni juice in media was an effective inhibitor of capillary initiation in explants from human breast tumors.

Even though these studies have examined various effects of noni on cancer and have elucidated some of the mechanisms involved, there is still little information of the usefulness of noni in the field of cancer, especially in terms of its effect on various tumor types. Therefore, we studied the cytotoxic and apoptosis-inducing effects of noni on Ehrlich ascites tumor *in vivo*. In addition, we combined noni with doxorubicin to understand if there is a synergistic interaction between them. We found that noni inhibited the growth of cancer cells by inducing apoptosis and suppressing proliferation *in vivo*.

MATERIALS AND METHODS

Animals

The protocol used in this study was approved by the Istanbul University's Ethic Board (No: 09/11.02.2008). Female Balb-c mice weighing 30 g in average, bred at the Department of Veterinary Pre-science, were used in this study. The animals were housed in polypropylene cages in a controlled environment (12 h dark/light cycle), fed with standard laboratory chow, and given tap water *ad libitum*.[9]

Experimental in vivo

Mice were divided into 4 groups: control ($n = 7$, 0.5 ml/bw gavage 0.9% NaCl); noni (Alnoni®, Hanoju Europe Ltd, Dinxperlo, The Netherlands) ($n = 8$, 0.5 ml/bw gavage); doxorubicin ($n = 8$, 3×3 mg/kg i.p.); noni + doxorubicin ($n = 8$, 0.5 ml/bw gavage + 3×3 mg/kg i.p.). 2×10^6 EAC cells diluted in 0.5 ml of 0.9% NaCl were subcutaneously injected in the napes of all animals to induce a solid carcinoma. Oral treatments with noni or 0.9% NaCl (control group) started on the first day of tumor inoculation and continued for 14 days. The dose of noni fruit juice was arbitrarily decided. Doxorubicin was administrated on the second day after tumor inoculation. The dose and duration of doxorubicin treatment was slightly modified from a previously published study.[10] After the first application, it was repeated twice at 5 day intervals. Noni + doxorubicin group received both noni and doxorubicin in the same way as they were seperately applied to the other relevant groups. The tumor development was evaluated by palpation method.[9] On day 14, tumor growth was evident in all animals. At this day, after sacrificing animals with servical dislocation, tumor tissues were removed from the noni, doxorubicin, and control groups. Extirpated tumor tissues were measured short-long diameters with the help of a caliper. The tissues were fixed in 10% formaldehyde, paraffin embedded (Thermo Shandon Inc, Pittsburg, USA) and sectioned at 4 μm thickness (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and collected on Poly-L-Lysine coated glasses.

Immunohistochemical detection of active-caspase-3 and PCNA

The tissue slices were air-dried overnight at room temperature and the tissue sections were then deparaffinized. Specimens were washed in 0.01 M phosphate-buffered saline (PBS). After three washes, tissues sections were incubated in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 min at 100°C in a microwave oven, (additionally, after this step %0.3 TritonX-100 used for only active caspase-3 staining for 10 min at room temperature) and endogenous peroxidase were blocked by 3% hydrogen peroxide in distilled water for 10 min at room temperature. Subsequently, the section were incubated with anti-h/m active caspase-3 (R&D Systems, Minneapolis, USA) at 1:100 dilution and PCNA (Santa Cruz, USA) at 1:100 dilution at room temperature for 30 and 50 min, respectively. The sections were washed three times with PBS, and then incubated with biotinylated secondary antibody at room temperature for 30 min. After that, the avidin-biotin-peroxidase complex was used for visualization, followed by the application of DAB for active caspase-3 and AEC (3-amino, 9 ethyl-carbazole) for PCNA staining. Finally, the sections were counterstained with Mayer's haematoxylin. The negative control sections were incubated with PBS instead of the primer antibody.

Detection of DNA fragmentation in tissues

The TUNEL (*T*erminal Deoxynucleotidyl Transferase d*U*TP *N*ick *E*nd *L*abeling) assay was performed by using a commercially available kit (ApopTag Plus *in situ* apoptosis detection kit, Chemicon International, Germany) in order to detect apoptosis in tissues. 5 μM sections of paraffin blocks prepared from tumor tissues were incubated overnight at 37°C. In brief, paraffin sections were de-waxed, rehydrated through a graded alcohol series and washed with PBS. Subsequently, tissues were digested with 20 μg/ml proteinase K at room temperature for 15 min. For quenching of endogenous peroxidase, sections were incubated at room temperature for 5 min in 2–3% H_2O_2 prepared in PBS. They were incubated with equilibration buffer at room temperature for 30 min, followed by the application of enzyme for 1 h in 37°C. Then they were washed 3 changes of PBS for 1 min and anti-digoxigenine-peroxidase was applied 30 min at room temperature. For color development, sections were covered with DAB substrate (diaminobenzidine) for 3–6 min. Methyl green was used for counterstaining. For negative controls, distilled water was used instead of TdT.

Biochemical determination of caspase-cleaved cytokeratin-18 (M30 antigen) in serum

To measure apoptosis in serum, an ELISA assay (a solid phase, two-site immunosorbent assay) was used. This assay

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was also performed to confirm apoptosis observed in tissues. The kit (M30-Apoptosense ELISA kit, Peviva, Sweden) for the assay was obtained commercially. Briefly, samples were placed into wells coated with a mouse monoclonal antibody used as catcher. A horseradish peroxidase conjugated monoclonal antibody (M30) was used as detecting antibody (conjugate). Following the sandwich formation, excess unbound conjugate was removed and then TMB substrate was added. The absorbance was finally measured in a microplate reader at 450 nm. By plotting a standard curve from known concentrations versus measured absorbancies, the amount of M30 antigen in the sample was estimated.

Proliferation index, apoptotic index, and statistical analyses

Sections were examined under Image Pro-Plus. PCNA-positive cells were used to quantify the proliferation index (percentage of PCNA-positive cells in 1000 cells. Similarly, TUNEL-positive cells were used to quantify the apoptotic index (percentage of TUNEL-positive cells in 1000 cells). One way ANOVA test (SPSS 8.0, SPSS for Windows Advanced Statistics Release 8.0, 1997) was used in the comparison of groups. The value of $p < 0.05$ was accepted as statistically significant.

RESULTS

The anti-growth effects of noni were investigated by application of a number of immunohistochemical and biochemical methods. Twenty four animals in total were used in the treatment groups and none died during the experiment. After the treatment with noni, doxorubicin, and their combination, it was found that short and long diameters of the tumor tissues in each treated group were statistically significantly smaller, compared to those in control group ($p < 0.001$) (Table 1).

Proliferation of tumor cells was assessed by PCNA staining. Figure 1 shows the proliferation index of control and treated groups. The proliferation index was about 68% in control group while it significantly decreased to 43, 34, and 24% in noni-treated, doxorubicin-treated and noni + doxorubicin-treated group, respectively.

To investigate if apoptosis is also involved in the growth-inhibition by the treatments, the active caspase-3 staining and the TUNEL assay were performed on tissues. Figure 2 presents the apoptotic cells which are shown by active caspase-3 positivity. Noni or its combination with doxorubicin resulted in higher number of apoptotic cells, compared to controls. In accordance with this finding, the rate of DNA fragmentation (apoptotic index) was increased by noni or its combination with doxorubicin (Figure 3). In addition, apoptosis in tissues was also confirmed by apoptosis in serum biochemically. Figure 4 shows the level of M30 antigen in treated and control groups. M30 antigen level was 231 U/L in control group while it statistically significantly increased to 370 U/L in noni group and further increased to 704 U/L in noni + doxorubicin group ($p < 0.05$).

Table 1. Tumor tissue diameters after the application of the agents

	Short diameter (mm)	Long diameter (mm)
Control	21.83 ± 1.85^{a}	30.30 ± 2.31^{a}
Noni	10.48 ± 1.45^{c}	16.41 ± 2.38^{b}
Doxorubicin	14.42 ± 0.67^{b}	20.29 ± 1.18^{b}
Noni + doxorubicin	$12.90 \pm 0.91^{b,c}$	17.00 ± 1.25^{b}

[a,b,c]: Means with different superscripts are significantly different ($p < 0.001$).

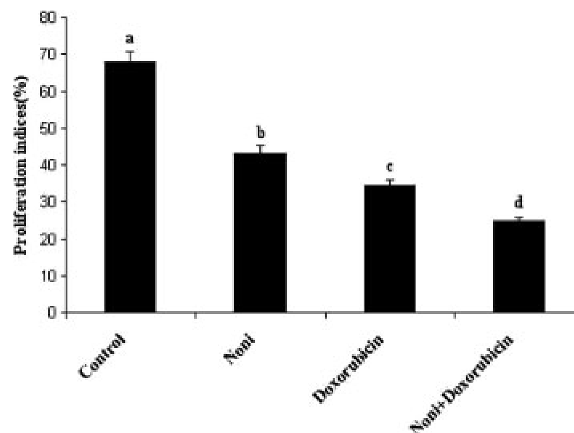

Figure 1. Proliferation indices in control and treated groups. Proliferation was immunohistochemically assayed by PCNA staining and the evaluation was made as explained in material and methods. [a, b, c, d]: Means with different superscripts are significantly different ($p < 0.001$)

DISCUSSION

In study presented, the possible cytotoxic and apoptosis-inducing effects of noni on Ehrlich ascites tumor in Balb-c mice were investigated. Noni resulted in a significant anti-growth effect on Ehrlich ascites tumor cells grown in Balb-c mice in our study.

Assuming this anti-growth effect could be achieved by either suppression of proliferation or activation of cell death, or both, we first stained cells in terms of PCNA reactivity for proliferation. Noni or its combination with doxorubicin significantly decreased the proliferation rate. This can explain why noni reduced the size of tumor diameters in treated groups. However, this effect might also be resulted from the activation of cell death (apoptosis or necrosis, or another type of cell death). The other cause might be the inhibition of angiogenesis but we did not investigate this issue. In fact, Arpornsuwan and Punjanon reported that a concentration of 0.1 mg/ml of crude extract exhibited cytotoxic activity against breast cancer (MCF7) and neuroblastoma (LAN5) cell lines at 29 and 36%, respectively.[11] Therefore, we analyzed if apoptosis takes place after in the treated groups. Apoptosis was shown by the TUNEL assay and the active caspase-3 staining. The results of these two methods were in concordance, which indicates that

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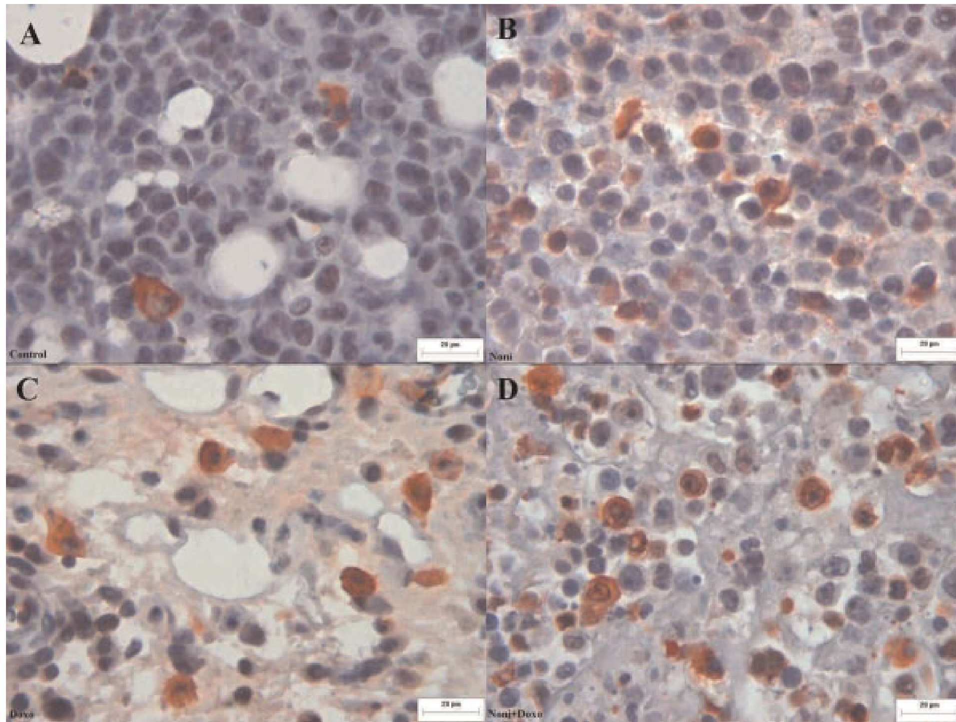

Figure 2. Active caspase-3 staining in control and treated groups: (A) control group, (B) noni group, (C) doxorubicin group, and (D) noni + doxorubicin group. The staining is cytoplasmic. The apoptotic cells are shown in red-brown

apoptosis was also another factor which contributes to the tumor anti-growth effect of noni. After the induction of apoptosis, the cytoskeleton is damaged. Because the cytokeratins are abundant in the cytoskeleton, they are released into the extracellular environment with an unknown mechanism after the activation of apoptosis-specific proteases (caspases).[12,13] Recently, it has been shown that the determination of cell death mode (e.g., apoptosis) in serum is possible by measuring the soluble cytokeratin 18 fragments (CK-18 Asp396, also called M30 antigen) that are formed by caspase activation.[14] It was even reported that it could be used to predict the response to chemotherapy in lung cancer.[15]

However, the determination of apoptosis by M30 antigen assessment in serum may be considered as an indirect method, comparing to those (the TUNEL assay and the active caspase-3 staining) performed in tissues. Maybe, that is why we did not find an enhancing effect of noni on the efficacy of doxorubicin by measuring serum M30 antigen level (Figure 4). In contrast, this enhancing effect was evident when measured using the TUNEL assay that is a tissue parameter (Figure 3).

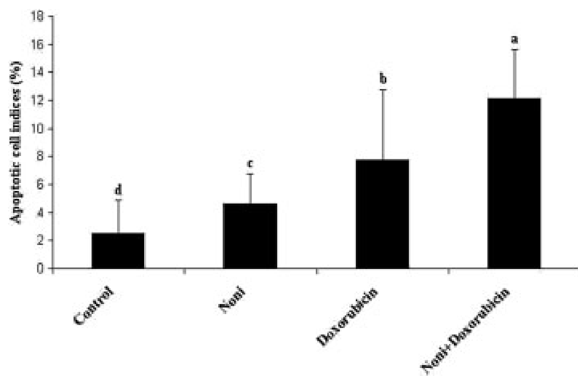

Figure 3. Apoptotic index in control and treated groups. Apoptosis was detected by the TUNEL assay and the evaluation was made as explained in material and methods. [a,b,c,d]: Means with different superscripts are significantly different ($p < 0.001$)

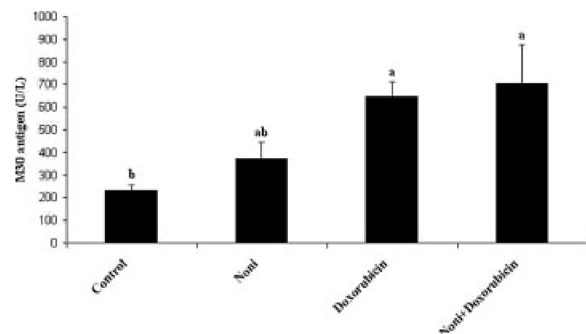

Figure 4. Caspase-cleaved cytokeratin 18 (M30 antigen) in serum of control and treated groups. It was performed by ELISA. [a,b]: Means with different superscripts are significantly different ($p < 0.05$)

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We also combined noni with a well-known anti-cancer agent doxorubicin. Noni augmented the cytotoxic effect of doxorubicin against the tumor cells. Similarly, it was reported that it increased the potency and efficacy of adriamycin, cisplatin, 5-fluorouracil, and vincristine, suggesting important clinical applications of noni as a supplemental agent in cancer treatment.[5] However, it did not show any synergistic or additive beneficial effects when combined with paclitaxel, cytosine arabinoside, or immunosuppressive anticancer drugs such as cyclophosphamide, methotrexate or 6-thioguanine.[6] When it comes to the mechanism by which noni kills the tumor cells, it was reported that noni ethanol precipitate (noni-ppt) suppressed tumor growth through activation of the host immune system,[5] suggesting that noni might not directly induce the cell death.

Taken together, noni resulted in an anti-growth effect on Ehrlich ascites tumors via both the suppression of proliferation and activation of apoptosis. In conclusion, although more studies are required, noni seems to be useful medicinal plant which may be considered as a supplemental agent in the breast cancer treatment with doxorubicin.

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