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Cyto-protective and immunomodulating properties of Amla (*Emblica officinalis*) on lymphocytes: an in-vitro study

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Abstract

The fruits extracts of *Emblica officinalis* (Amla) has been reported to have strong anti-oxidant properties. There is a paucity of studies on the immunomodulatory properties of fruit extracts of Amla in immuno-compromised states, with the emphasis on lymphocytes. Therefore, the aim of the study was to determine the anti-oxidant and immunomodulatory properties of Amla using chromium (VI) as an immunosuppressive agent. Chromium (Cr) treatment results in enhanced cytotoxicity, free radical production, lipid peroxidation and decreased glutathione peroxidase (GPx) activity and diminished glutathione (GSH) levels. There was a significant inhibition of both lipopolysaccharide and concanavalin-A-stimulated lymphocyte proliferation. Chromium also inhibited Con A stimulated interleukin-2 and γ -interferon production significantly. Further, there was enhanced apoptosis and DNA fragmentation in the presence of Cr. Amla significantly inhibited Cr-induced free radical production and restored the anti-oxidant status back to control level. Amla also inhibited apoptosis and DNA fragmentation induced by Cr. Interestingly, Amla relieved the immunosuppressive effects of Cr on lymphocyte proliferation and even restored the IL-2 and γ -IFN production considerably. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Amla; Chromium; Lymphocytes; Oxidative damage; Cytotoxicity; Interleukins; *Emblica officinalis*

1. Introduction

Emblica officinalis, commonly known as Amla, a member of a small genus *Emblica* (Family *Euphorbiaceae*), is extensively found all over India, as well as Sri Lanka, Malaya, China, Pakistan, and Bangladesh. The fruits of the plant are fleshy with sour, astringent taste and are consumed raw, cooked, or even pickled locally. The fruits have been reported to contain constituents with variable biological activity. Experiments conducted with the fruit of Amla has been shown to possess anti-oxidant (Bhattacharya et al., 1999), adaptogenic (Rege et al., 1999), and hepato-protective (Jeena et al., 1999) and anti-tumour activities (Jose et al., 2001). Leaf extracts have been shown to possess anti-inflammatory

activity (Asmawi et al., 1993; Ihtantola-Vormisto et al., 1997). Mishra et al. (1981) reported that Amla has hypocholesterolemic activity.

The fruit extracts inhibit clastogenicity and mutagenicity induced by various metals (Dhir et al., 1991; Aggarwal et al., 1992; Roy et al., 1992). The therapeutic effect has been suggested to be ascribed to its high vitamin 'C' content and possibly to tannin like structures. Ghosh et al. (1992) have reported that protection offered by Amla against CsCl—induced clastogenicity must be ascribed to vitamin 'C'. However, Dhir et al. (1991), Roy et al. (1992) reported that Amla extract provides higher protection against clastogenicity and mutagenicity induced by lead and aluminium than equivalent amounts of vitamin 'C' indicating that the combined action of different ingredients would be responsible for its biological activity.

Despite many therapeutic effects of Amla, relatively little data is available on the putative immunomodulatory effects of Amla on lymphocyte function, particularly in immuno-suppressive conditions. Therefore, an

Abbreviations: DCFDA, 2',7'-dichlorofluoresceindiacetate; GPx, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

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in-vitro study was undertaken to determine the relative effects of fruit extracts of Amla with regard to cyto-protection and immunomodulation using rat splenocytes as model system.

2. Materials and methods

2.1. Fruit extract preparation

The fruits of Amla collected from authentic source were powdered and extraction of the powder (2 g) was carried out with 200 ml of 90% ethanol in Soxhlet apparatus for 5 h. The extract was collected and dried in oven at 100 °C for 3 h. The powder obtained was then emulsified in saline containing 0.1% Tween 20 and 10% ethanol. Later on the dilutions of the extract were made in saline containing 0.02% Tween 20.

2.2. Lymphocyte isolation and experimental setup

Sprague–Dawley rats, reared in our institute's animal house, weighing approximately 150–200 g were taken and killed by cervical dislocation. The splenocytes were isolated aseptically as described earlier (Sai Ram et al., 1997) and viability was determined by staining the cells with acridine orange and ethidium bromide (10 µg/ml each). The cell concentration was adjusted to 2×10^6 cells per ml by adding an appropriate volume of RPMI-1640 medium. The experiments were conducted using either 96- or 24-well culture plates (NUNC, The Netherlands). The cells were added to the culture plates followed by the addition of different concentrations (10 µg–1 mg/ml) of fruit extracts of Amla and chromium at 10 µg/ml (as sodium dichromate). The plates were then incubated at 37 °C in CO₂ (5%) incubator for 18 h. Since best results were obtained using 100 µg/ml of Amla, all experiments were conducted using the same concentration only.

2.3. Determination of cytotoxicity and apoptosis

Apoptosis was studied morphologically using fluorescent dyes that intercalate with DNA (Duke and Cohen, 1992). Acridine orange, a cationic dye enters only live cells and stains DNA green, allowing visualisation of the chromatin pattern. Apoptotic cells have condensed chromatin that is uniformly stained. On the contrary, Ethidium bromide stains DNA orange, but is excluded by the live cells. Dual staining allows enumeration of four populations: (1) live-nonapoptotic (LNA); (2) live-apoptotic (LA); (3) dead-nonapoptotic (DNAP); (4) dead-apoptotic (DAP). Briefly, upon incubation, the cells were stained with 4 µl of staining solution (100 µg/ml of acridine orange and 80 µg/ml ethidium bromide) and then examined by fluorescent microscopy. The

percentage of live cells and apoptosis were calculated as follows:

$$\% \text{ Live cells} = \frac{\text{LNA} + \text{LA}}{\text{Total cells}}$$

$$\% \text{ Apoptosis} = \frac{\text{LA} + \text{DAP}}{\text{Total cells}}$$

Alternatively, cytotoxicity was studied by determining lactate dehydrogenase activity (LDH) in cell-free splenocyte supernatants using RANDOX kit following the manufacturer's instructions.

2.4. Inhibition of cell proliferation

Effects on cell proliferation were studied using cell proliferation kit as per manufacturer's instructions. The assay is based on the cellular conversion of the tetrazolium salt MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt into a formazan product that is soluble in tissue culture medium and is measured at 450 nm. Lipopolysaccharide (5 µg/ml) and concanavalin A (6 µg/ml) were used for stimulating B and T lymphocytes, respectively. Briefly, the cells were incubated along with the drugs and chromium with/without ConA/LPS for 72 h. Later, 20 µl of MTS was added and further incubated for 2–5 h. The OD was then measured at 450 nm wavelength using an automated ELISA Reader.

2.5. Determination of interleukins IL-2 and γ-IFN

After culturing the cells in 96-well plates in the presence of con A for 72 h, the cells were centrifuged at 5000 rpm for 10 min and the supernatant was stored at –80 °C. IL-2 and γ-IFN levels were then assayed in the culture supernatant by using ELISA kits (Diclone, France) according to the manufacturer's instructions.

2.6. Determination of lipid peroxidation and antioxidant status

Upon incubation, treated splenocytes were lysed by lysis buffer (10 mM Tris, 20 mM EDTA, 0.25% Triton X-100, pH 8.0). The superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity were determined in cell lysates using RANDOX kits following the manufacturer's instructions. Lipid peroxidation was determined by the method of Douset et al., (1983). Reduced glutathione levels in the cells were determined fluorimetrically by the method of Burchill et al., (1978). The production of free radicals was determined by using

2',7'-Dichlorofluoresceindiacetate (DCFDA) as described earlier by Cathcart et al. (1983).

2.7. DNA-fragmentation analysis

The cells (250 μ l) were collected in 1.5 ml microfuge tubes followed by the addition of 250 μ l of lysis buffer and subsequent incubation for 30 min. The samples were then centrifuged at 15000 rpm for 30 min. The supernatants, pellets and uncentrifuged lysates were assayed for DNA content by 4,6-diamidino-2-phenylindole (DAPI) using spectrofluorometer (Brunk et al., 1979). Briefly, 25 μ l of sample was added to 2.5 ml of DAPI reagent (100 ng/ml DAPI in 10 mM Tris, pH 7.4, containing 100 mM NaCl) and the fluorescence intensity was measured at 450 nm with an excitation wavelength of 362 nm. The percentage of DNA fragmentation was calculated as the ratio of the DNA content of supernatant obtained at 15000 \times g divided by the total DNA content of the lysate (Wyllie, 1980).

2.8. Agarose gel electrophoresis

Upon incubation splenocytes (500 μ l) were collected in 1.5 ml microfuge tubes and lysed by adding 500 μ l of lysis buffer containing 50 μ g/ml of proteinase K. The tubes were incubated at 37 °C overnight for digestion. The DNA was isolated by phenol chloroform extraction and subjected to gel electrophoresis using 1.8% agarose in TBE buffer. *Eco*RI–*Hin*dIII double digest of λ -phage DNA was used as a molecular-size standard.

All experiments were carried out in six replicates on two different occasions and the data were analysed by Student's *t*-test.

3. Results

3.1. Cytotoxicity and apoptosis

The cyto-protective effect of Amla on Cr-induced cytotoxicity and apoptosis is shown in Table 1. Chromium in the concentration tested (10 μ g/ml) has

Table 1
Effect of Amla on Cr-induced cytotoxicity and apoptosis in rat splenic lymphocytes ($n = 6$; values are mean \pm SD)

	%Survival	LDH leakage (IU/l)	%Apoptosis
Control	98 \pm 12	178 \pm 77	3 \pm 0.2
Cr (10 μ g/ml)	62 \pm 7.8 ^a	307 \pm 84 ^a	24 \pm 1.5 ^a
Amla (100 μ g/ml)	95 \pm 13.4	145 \pm 24	2 \pm 0.2
Cr + Amla	88 \pm 11.2 ^b	156 \pm 29 ^b	2 \pm 0.4 ^b

^a Vs. control.

^b Vs. Cr.

P < 0.01.

significantly induced cytotoxicity and apoptosis compared with control cells. However, Amla inhibited both cytotoxicity and apoptosis induced by chromium appreciably. Further, Cr treatment resulted in a significant leakage of cytosolic LDH compared with control cells. Interestingly Amla completely prevented the increase in LDH leakage induced by chromium.

3.2. Lymphocyte proliferation

When cells were treated with Cr, there was a significant inhibition in mitogen (LPS or Con A)-induced lymphocyte proliferation compared with the respected control cells (Fig. 1). The presence of Amla alone significantly increased the proliferation of lymphocytes in the absence of any kind of stimulant compared with control cells. However, the presence of LPS or Con A could not enhance the lymphocyte proliferation further. Interestingly, Amla inhibited the immunosuppressive effect of Cr significantly (Fig. 1).

3.3. Interleukin protection

The presence of chromium resulted in an appreciable decrease in con-A induced IL-2 and γ -IFN production by lymphocytes (Fig. 2). Interestingly, Amla alone did not affect on IL-2 production, but inhibited γ -IFN production significantly over control cells. However, Amla relieved the immuno-suppression induced by chromium and enhanced the IL-2 and γ -IFN production to a considerable extent.

3.4. Free radical production, SOD/GPx activity

Cr treatment resulted in a significant increase of free radical production and lipid peroxidation with a concurrent decrease in GSH levels and GPx activity. No change in SOD activity was observed (Table 2). The

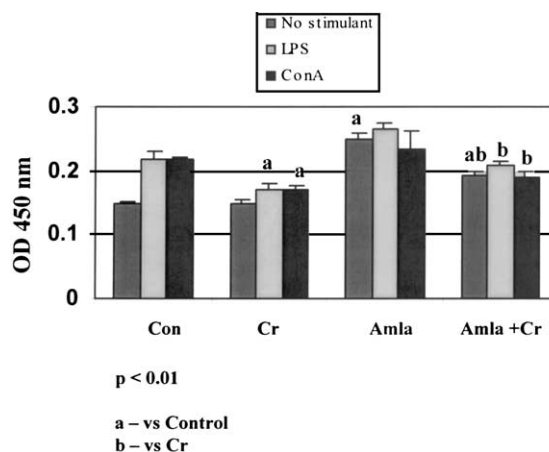


Fig. 1. Effect of Amla on lymphocyte proliferation in the presence/absence of chromium ($n = 6$; values are mean \pm S.D.).

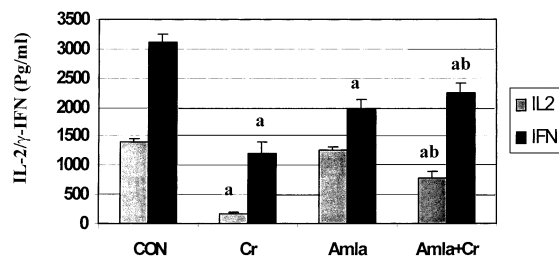


Fig. 2. Effect of Amla on IL-2 and γ -IFN production by lymphocytes in the presence/absence of chromium ($n = 6$; values are mean \pm S.D.).

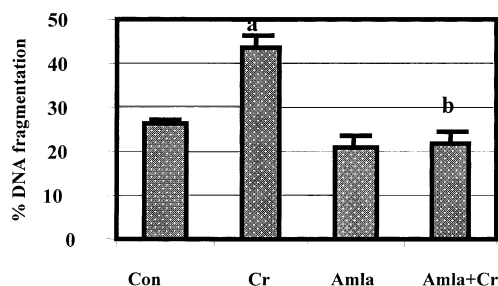
presence of Amla appreciably inhibited the increase in free-radical production and maintained anti-oxidant status similar to that of control cells.

3.5. DNA fragmentation analysis

We observed a significant increase in DNA fragmentation when the cells were exposed to Cr (Fig. 3). Agarose gel electrophoresis of DNA isolated from cells also showed the presence of typical DNA ladder (hall mark of apoptosis) in the presence of Cr (Fig. 4). However, the fruit extracts of Amla inhibited the DNA damage significantly (Figs. 3 and 4).

4. Discussion

Free radical-induced oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficiency of natural antioxidant defences. Potential antioxidant therapy should therefore include either natural free radical-scavenging antioxidant enzymes or agents which are capable of augmenting the activity of these enzymes, which include SOD, CAT and GPx. Studies have shown that the fruit extract of Amla was found to increase the cortical and striatal concentration of the anti-oxidant enzymes SOD, Catalase, GPx significantly and reduced lipid peroxidation in the rat brain (Bhattacharya et al., 1999). Fruits of *E. officinalis* have been used for thousand of years in traditional Indian medicine for the treatment of various diseases. The fruit extract



$p < 0.001$

a - vs Control
b - vs chromium

Fig. 3. Effect of Amla on DNA fragmentation induced by chromium ($n = 6$; values are mean \pm S.D.).

inhibited metal-induced clastogenicity and mutagenicity (Dhir et al., 1991; Ghosh et al., 1992; Dhir et al., 1993) and protects cells from radiation (Yadav, 1987). The extracts of fruits were also reported to inhibit retroviruses such as HIV-1 (El-Mekkawy et al., 1995) and to enhance immune parameters (Suresh and Vasudevan, 1994). The fruit extracts were also reported to have hypolipidemic effect (Thakur, 1985).

Amla fruits have long been postulated to be a rich source of vitamin C and the prophylactic, curative, and restorative effects of the fruits were thought to be mainly due to this factor. Bhattacharya et al. (1999) reported that the fresh juice and fruit extracts of Amla does not contain vitamin C, but that the vitamin C like activity of Amla was due to the presence of hydrolysable tannins emblicanin A, emblicanin B, puningluconin, and pedunculagin. Recently, Zhang et al. (2001) have characterised novel sesquiterpenoids having cyto-toxic properties from the roots of *Phyllanthus emblica*.

Although many studies have been performed on the biological activities of Amla, little is known about its ability to modulate immune responses especially under immunosuppressive conditions. In the present study, we studied the efficacy of fruit extracts of Amla on lymphocyte cytotoxicity and apoptosis using chromium (VI) as immunosuppressing agent. In the presence of Cr (10 μ g/ml), significant cytotoxicity and apoptosis was

Table 2

Effect of Amla on free radical production and antioxidant status in chromium-induced oxidative stress in rat lymphocytes ($n = 6$; values are mean \pm S.D.)

	DCFDA (cpm/ml)	MDA (nmol per million cells)	SOD (IU/l)	GPx (IU/l)	GSH (μ g per million cells)
Control	48 000 \pm 1000	16.1 \pm 1.0	1.47 \pm 0.12	553 \pm 63	12.7 \pm 0.8
Chromium	59 600 \pm 1600 ^a	24.5 \pm 2.3 ^a	1.35 \pm 0.10	370 \pm 90 ^a	10.1 \pm 0.4 ^a
Amla	19 333 \pm 1258 ^a	12.9 \pm 1.1	1.73 \pm 0.09	464 \pm 22	11.3 \pm 1.4
Amla + chromium	20 833 \pm 1755 ^{a,b}	16.6 \pm 1.3 ^b	1.66 \pm 0.33	548 \pm 64 ^a	12.9 \pm 1.0 ^b

^a Versus control.

^b Versus Cr.

$P < 0.01$.

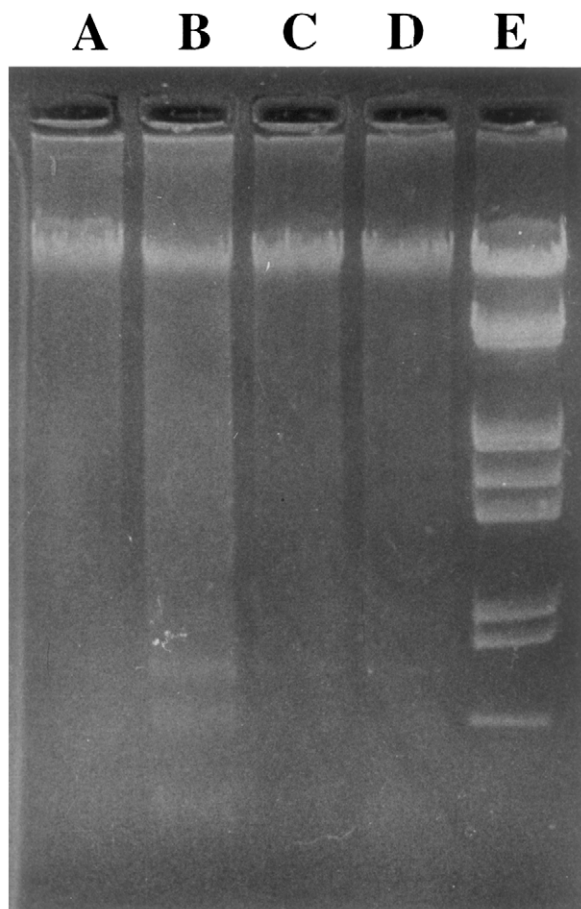


Fig. 4. Agarose gel electrophoresis depicting the DNA damage induced by chromium in the presence/absence of Amla. Arrows indicate the typical DNA ladder, a hall mark of apoptosis. (A) Control; (B) Cr; (C) Amla; (D) Amla+Cr; (E) molecular weight marker.

observed compared with control cells. Chromium treatment enhanced DNA fragmentation and produced a typical DNA ladder which is a hallmark of apoptosis. This was attributed to the enhanced free-radical production as revealed by increased fluorescence of DCFDA and MDA levels in the cells exposed to chromium. However, there was no change in cytochrome c reduction (data not shown) and SOD activity in cells treated with chromium indicating that Cr is essentially involved in the production of H_2O_2 , nitric oxide, and other oxygen free-radicals, but not superoxide. In this regard, our studies confirm with earlier studies by Bagchi et al. (1995), Sai Ram et al. (2000). Amla resulted in enhanced cyto-protection, decreased apoptosis and DNA fragmentation which could be attributed to decreased free-radical production. These findings are in line with the reported potent anti-oxidant activity of Amla (Bhattacharya et al., 1999; Bandopadhyay et al., 2000).

Lymphocyte proliferation is a very sensitive test and is being used as a potential biomarker for toxic exposures (Snyder and Valle, 1991). In the present study, it was

found that Cr inhibited the stimulation of B & T Lymphocyte (by LPS and ConA, respectively) significantly over control treatment. Further, we observed a significant inhibition of ConA-induced IL-2 and γ -IFN production by chromium. Earlier, Snyder and Valle (1991) reported that Cr inhibited both T & B lymphocyte proliferation significantly. The presence of Amla, did not only increase lymphocyte proliferation, but even restored the interleukin production considerably.

In conclusion, the fruit extracts of Amla have cyto-protective and immunomodulatory properties which could fully be attributed to its anti-oxidant activity.

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