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Amla (*Emblica officinalis* Gaertn.) extract promotes procollagen production and inhibits matrix metalloproteinase-1 in human skin fibroblasts

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ABSTRACT

Aim of the study: Emblica officinalis Gaertn., commonly known as amla, is a rich dietary source of vitamin C, minerals and amino acids, and also contains various phenolic compounds. Amla extract is also known to exhibits potent antioxidant properties and to provide protection for human dermal fibroblasts against oxidative stress, and therefore it is thought to be useful for natural skin care. In this study, we investigated the effects of amla extract on human skin fibroblasts, especially for production of procollagen and matrix metalloproteinases (MMPs), *in vitro*.
Materials and Methods: Mitochondrial activity of human skin fibroblasts were measured by WST-8 assay. Quantification of procollagen, MMPs, and Tissue inhibitor of metalloproteinase-1 (TIMP-1) released from human skin fibroblasts were performed by immunoassay technique.
Results and Conclusions: Amla extract stimulated proliferation of fibroblasts in a concentration-dependent manner, and also induced production of procollagen in a concentration- and time-dependent manner. Conversely, MMP-1 production from fibroblasts was dramatically decreased, but there was no evident effect on MMP-2. TIMP-1 was significantly increased by amla extract. From these results, it appears that amla extract works effectively in mitigative, therapeutic and cosmetic applications through control of collagen metabolism.

1. Introduction

Fibroblasts synthesize collagen, a fibrous protein of the extracellular matrix (ECM) and major constituent of connective tissues, such as skin, tendon, ligament, cartilage, and bone. Type I collagen accounts for 70–90% of total collagen (Nimmi, 1988), and the functional properties of skin depend on the integrity of collagen in the dermis. Moreover, collagen is tightly controlled by matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) (Scharffetter et al., 1991; Chung et al., 2001). MMPs are a family of zinc-dependent endoproteinases that play pivotal roles in dynamic remodeling of the ECM. On the basis of substrate preference and structural homology, MMPs are sub-classified into functional groups: collagenases, gelatinases, stromelysins, matrilysins, membrane type-MMPs (MT-MMPs), and other non-classified MMPs (Visse and Nagase, 2003). It is known that humans have three distinct collagenases: MMP-1, also known as collagenase I or interstitial collagenase; MMP-8, also known as collagenase II or neutrophil collagenase; and MMP-13, also known as collagenase III. All of these collagenases can cleave interstitial collagens such as type I and III collagens found in the skin (Woessner, 1991). With increasing age, collagen synthesis becomes lower and MMP-1 levels become higher in naturally aged human skin, and these alterations cause changes such as skin wrinkling and loss of elasticity (Varani et al., 2000). Therefore, control of collagen metabolism may be useful for a variety of therapeutic and cosmetic applications.

Emblica officinalis Gaertn., commonly known as amla, is a member of the small genus *Emblica* (Euphorbiaceae). It is distributed in tropical and subtropical areas of China, India, Indonesia and the Malay Peninsula. It is a rich dietary source of vitamin C, minerals and amino acids, and also contains a wide variety of phenolic compounds, such as tannins, phyllembelic acid, phyllemblin, rutin, curcuminoides and emblicol (Kim et al., 2005). The fruits of amla are widely consumed raw, cooked, or pickled, and they are also principal constituents of many preparations of Ayurved (Scartezzini and Speroni, 2000), the Indian Traditional Medicine. According to the two main classic Ayurved texts, *Charak Samhita* and *Sushrut Samhita*, amla is regarded as “the best among rejuvenative herbs”, “useful for relief of cough and skin disease”, and “the best among the sour fruits”.

Recently, amla extract has been tested for various pharmacological effects. The fruit extract has hypolipidemic (Anila and

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Vijayalakshmi, 2002), antidiabetic (Sabu and Kuttan, 2002) and anti-inflammatory activities (Asmawi et al., 1993), and works to inhibit retroviruses such as HIV-1 (Mekkawy et al., 1995), tumor development (Jose et al., 2001) and gastric ulcer (Bandyopadhyay et al., 2000). Moreover, amla extract exhibits potent antioxidant properties (Bhattacharya et al., 1999; Chaudhuri, 2002) and protects human dermal fibroblasts from oxidative stress; therefore it is thought to be useful for natural skin care (Chaudhuri, 2002). However, there are few data on the effects of amla extract in human skin fibroblasts, especially with regard to production of procollagen and MMPs.

In the present study, we carried out *in vitro* experiments using human skin fibroblasts to clarify changes in the production of procollagen and MMPs.

2. Materials and methods

2.1. Sample preparation

Dried amla powder from India was supplied by Nippon Shinyaku Co., Ltd. (Kyoto, Japan). It was treated with ethanol and water (1:1) for 1 h at room temperature and centrifuged at 600 × *g* for 10 min. The supernatant was collected, and the sediments was then re-extracted with acetone and water (7:3) and centrifuged again at 600 × *g* for 10 min. The resultant solution was mixed with the previous one, and evaporated to dryness under reduced pressure. 1.92 g of amla extract were obtained from 6.0 g of dried amla powder.

2.2. Cell and cell culture

NB1RGB human skin fibroblasts (Riken Cell Bank) were maintained in Eagle's MEM-α (GIBCO) with 10% heat-inactivated fetal calf serum (FCS) and 1% antibiotic–antimycotic (GIBCO) at 37 °C in a humidified 5% CO_2 atmosphere, and all experiments were conducted in Eagle's MEM (GIBCO) with 0.5% heat-inactivated FCS and 1% antibiotic–antimycotic (GIBCO).

2.3. WST-8 assay

The effects of amla extract on *mitochondrial activity* to evaluate cell proliferation were determined by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) assay using a commercial kit (Tetra Color ONE, Seikagaku Corporation, Japan). Briefly, 1×10^4 cells in 100 μL medium were plated on a 96-well microplate. After 24 h of incubation, 100 μL medium with several concentrations of amla extract dissolved in a final concentration of 0.25% dimethyl sulfoxide (DMSO) was added. After incubation for 48 h, it was replaced with new medium with 10% WST-8. After 1 h of incubation, the formation of formazan was determined photometrically at 450 nm with a microplate reader. All analyses were performed on sets of 4 wells. The results are expressed as the ratio to the untreated control value.

2.4. Immunoassay

The assay was performed using commercial kit as suggested by the instructions. Briefly, 1×10^4 cells in 100 μL medium were plated on a 96-well microplate. After 24 h of incubation, 100 μL medium with several concentrations of amla extract dissolved in a final concentration of 0.25% DMSO was added. After incubation for 24 h, medium was replaced with 100 μL new medium and incubated again for 24 and/or 48 h, after which the concentrations of procollagen type I C-peptide (PIP), MMP-1, MMP-2, and TIMP-1 in the medium were measured using PIP EIA Kit (TAKARA BIO), Human pro-MMP-1 Immunoassay (R&D systems), Matrix Metalloproteinase-2 Human Biotrak ELISA System (GE Healthcare), and Human TIMP-1 ELISA systems (GE Healthcare), respectively. Values were standardized to total cell protein concentrations measured by the Bradford Protein Assay, and all analyses were performed on sets of 3 wells.

2.5. Statistical analysis

Significant difference from the control group was analysed by Student's *t*-test.

3. Results

3.1. Effects of amla extract on mitochondrial activity

When fibroblasts were incubated with various concentrations (0–40 μg/mL) of amla extract for 48 h, we found that amla extract elevated the *mitochondrial activity* in a concentration-dependent manner (Fig. 1). Amla extract at a dose range of 5–20 μg/mL increased the activity by 16–27% ($p < 0.01$), as compared with the non-treated cells. However, the highest concentration at 40 μg/mL did not elevate the activity.

3.2. Effects of amla extract on procollagen production

The concentration of PIP was determined by an enzyme-immunoassay technique. This assay uses an antibody to the PIP that is part of the collagen molecule as it is synthesized and secreted until being proteolytically cleaved. Amla extract was added to fibroblast culture media at varying concentrations (0–40 μg/mL) and subjected to incubation for 48 h or to time course test (0, 24 and 48 h) at 20 μg/mL. The result summarized in Fig. 2A shows that amla extract induced production of PIP in the fibroblasts in a concentration-dependent manner. PIP significantly increased by 36% at 20 μg/mL ($p < 0.01$) and by 17% at 40 μg/mL ($p < 0.05$), as compared with the non-treated control cells. The time-dependent production of PIP summarized in Fig. 2B shows that PIP significantly increased ($p < 0.01$ vs control) at 48 h although not significant at 24 h.

3.3. Effects of amla extract on MMPs production

We incubated fibroblasts with various concentrations (0–40 μg/mL) of amla extract for 48 h, and determined MMP-1 concentrations using ELISA technique, since MMP-1 is secreted extracellularly. As shown in Fig. 3, amla extract in a dose range

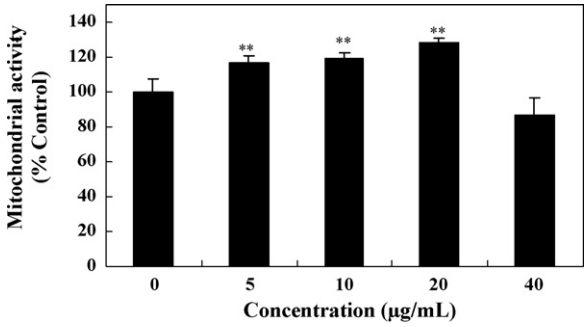

Fig. 1. Effects of amla extract on *mitochondrial activity* of human skin fibroblasts in culture. Fibroblasts were treated with amla extract at concentrations indicated in the figure and incubated for 48 h. *Mitochondrial activities* were determined as described in Section 2. Each value is presented as mean ± S.D. for each set of four wells. $^{**}p < 0.01$, significantly different when compared with the control value (Student's *t*-test).

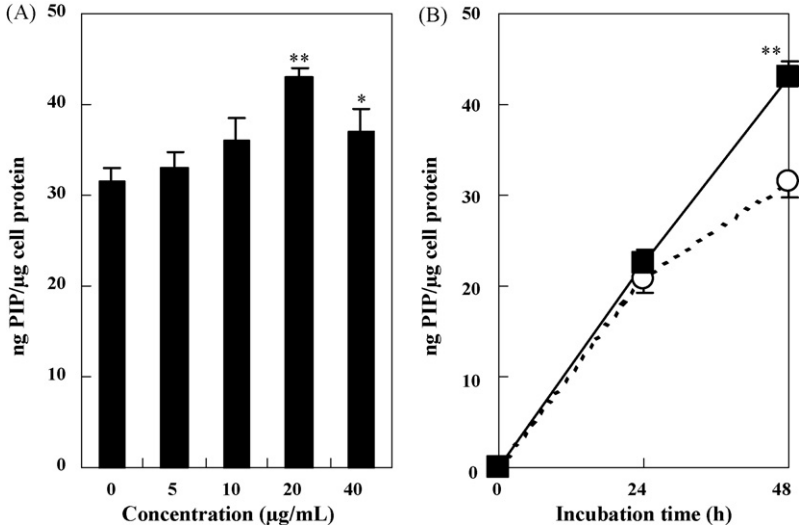

Fig. 2. Effects of amla extract on PIP production by human skin fibroblasts in culture. (A) Fibroblasts were treated with amla extract at concentrations indicated in the figure and incubated for 48 h. The concentrations of PIP were determined as described in Section 2. Each value is presented as mean ± S.D. for each set of three wells. $^{*}p < 0.05$; $^{**}p < 0.01$, significantly different when compared with the control value (Student's *t*-test). (B) Fibroblasts were incubated with DMSO (vehicle; open circles) or 20 μg/mL of amla extract (closed squares) for times indicated in the figure. Each value is presented as mean ± S.D. for each set of three wells. $^{**}p < 0.01$, significantly different when compared with the control value (Student's *t*-test).

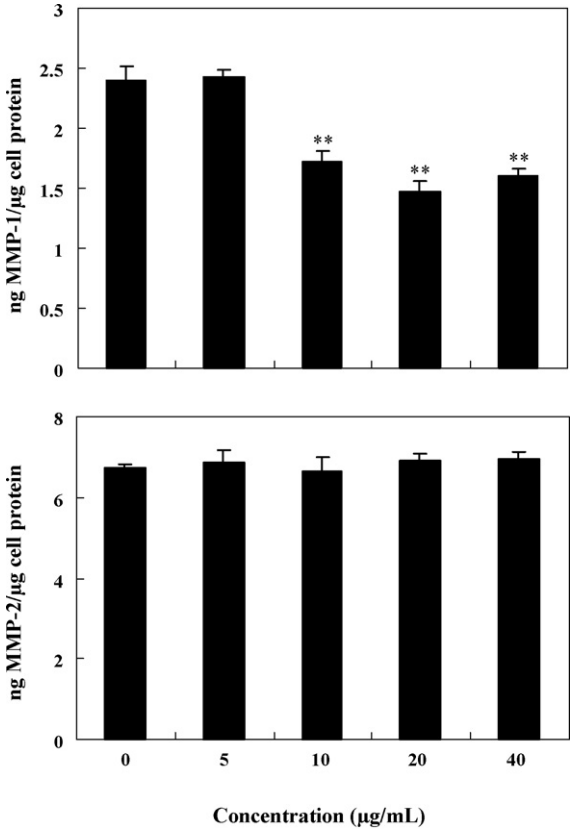

Fig. 3. Effects of amla extract on MMPs production by human skin fibroblasts in culture. Fibroblasts were treated with amla extract at concentrations indicated in the figure and incubated for 48 h. The concentrations of MMP-1 and MMP-2 were determined as described in Section 2. Each value is presented as mean ± S.D. for each set of three wells. $^{**}p < 0.01$, significantly different when compared with the control value (Student's *t*-test).

of 10–40 μg/mL markedly reduced MMP-1 production by 28% at 10 μg/mL ($p < 0.01$), 38% at 20 μg/mL ($p < 0.01$), and 33% at 40 μg/mL ($p < 0.01$), as compared with the non-treated control cells. In contrast, MMP-2 levels did not change significantly in all the concentrations of amla extract.

3.4. Effects of amla extract on TIMP-1 production

Amla extract was added to fibroblast culture media, at varying concentrations (0–40 μg/mL) and subjected to incubation for 48 h or to time course test (0, 24 and 48 h) at 20 μg/mL. The result summarized in Fig. 4A shows that TIMP-1 significantly increased by 27% at 20 μg/mL ($p < 0.01$), as compared with the non-treated control cells. The time-dependent production of TIMP-1 summarized in Fig. 4B shows that TIMP-1 significantly increased at 24 h ($p < 0.01$) and also at 48 h ($p < 0.01$), as compared with the non-treated control cells.

4. Discussion

We demonstrated that amla extract increased PIP and TIMP-1 production, and decreased MMP-1 production, concomitant with elevated *mitochondrial activity* in the fibroblast, in a concentration-dependent manner.

It has been reported that water extract from dried amla powder contains 2 wt% ascorbic acid and 29.4 wt% polyphenols such as gallic acid and elaeocarpusin by HPLC analysis (Yokozawa et al., 2007). As amla extract used in our present study was prepared from 50% aqueous EtOH solution of dried amla powder, it is assumed that our amla extract also contains such compounds. It is widely known that ascorbic acid enhance the proliferation and collagen production in fibroblasts, and thus, ascorbic acid may contribute to these activities even in our amla extract. Concerning polyphenols, however, there are little data available so far.

In 40 μg/mL of amla extract, the activity of mitochondria in fibroblast was slightly decreased compared with that of 20 μg/mL. Similar results were also observed in the PIP and TIMP-1. It was reported that juice, peel, and seed cake extracts from pomegranate inhibited keratinocyte proliferation in a high concentration (Aslam

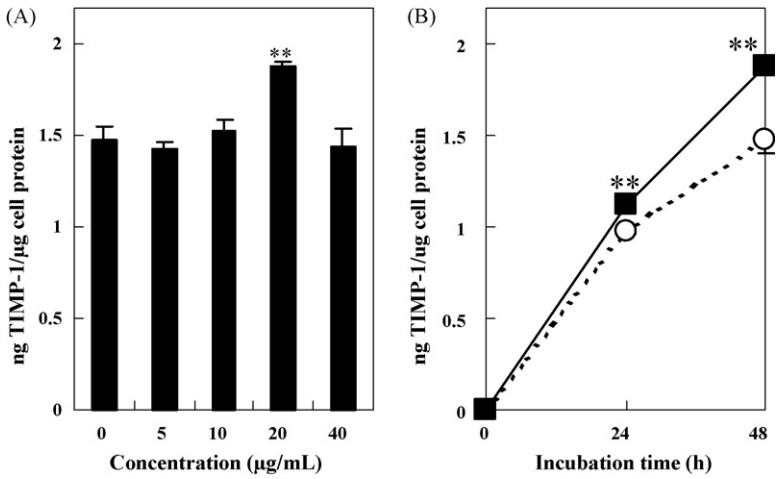

Fig. 4. Effects of amla extract on TIMP-1 production by human skin fibroblasts in culture. (A) Fibroblasts were treated with amla extract at concentrations indicated in the figure and incubated for 48 h. The concentrations of TIMP-1 were determined as described in Section 2. Each value is presented as mean ± S.D. for each set of three wells. $^{**}p < 0.01$, significantly different when compared with the control value (Student's *t*-test). (B) Fibroblasts were incubated with DMSO (vehicle; open circles) or 20 μg/mL of amla extract (closed squares) for times indicated in the figure. Each value is presented as mean ± S.D. for each set of three wells. $^{**}p < 0.01$, significantly different when compared with the control value (Student's *t*-test).

et al., 2006). Moreover, higher concentrations of flavonoids, which act in general as antioxidant, exerted cytotoxicity via increase in intracellular reactive oxygen species in human normal lung embryonic fibroblasts and umbilical vein endothelial cells (Matsuo et al., 2005). Amla extract was also reported to contain antioxidants such as ascorbic acid and polyphenols (Kim et al., 2005; Yokozawa et al., 2007). Therefore, amla extract may exert cytotoxicity through a mechanism similar to these and thus, the toxicity caused by 40 μg/mL *in vitro* may not be specific to amla extract.

Transforming growth factor-β (TGF-β) has been shown to act on human dermal fibroblasts at the pretranslational level by stimulating the accumulation of fibronectin and type I procollagen mRNAs (Roberts et al., 1986; Varga and Jimenez, 1986; Raghow et al., 1987; Ohji et al., 1993). In addition, TGF-β is able to decrease the synthesis of MMPs and increase the synthesis of TIMP (Reynolds et al., 1994). Thus, in the present study, amla extract may have exerted an effect similar to that of TGF-β.

To help minimize the development of skin wrinkles, not only promotion of collagen production but also restoration of dermal collagen-fiber-bundle ultrastructure is necessary (Rakic et al., 2000). Thus, proteolytic degradation in addition to synthesis of the ECM is also required during dermal remodeling. To verify this mechanism, we examined the production of MMPs involved in matrix degradation, and demonstrated that MMP-1 production by fibroblasts was decreased by amla extract in a concentration-dependent manner. Epigallocatechin-3-gallate (EGCG), a tannin component of green tea, modulates MMP expression and production through AP-1 and NF-κB activation (Kim et al., 2001; Trautinger, 2001; Fisher et al., 2002). Amla extract contains a large amount of functional tannins such as emblicanin, pedunclagin, and puniglucoin (Chaudhuri, 2002), and therefore may exert its activities through a mechanism similar to that of EGCG.

Although MMP-1 production from fibroblasts was decreased, no effect on MMP-2 production was observed. It has been established that UV irradiation to cultured human skin fibroblasts induces upregulation of MMP-1, but has no effect on MMP-2 (Moon et al., 2005). However, most synthetic MMP inhibitors have broad-spectrum activity against MMPs, and this lack of specificity restricts their use in clinical trials (Coussens et al., 2002). Recently, it has been widely accepted that natural products from medicinal plants are a potential source of selective MMP inhibitors. Moon et al. have recently reported that compounds isolated from natural plants may prevent or mitigate the UV-induced skin aging process through inhibition of MMP-1 expression (Kim et al., 2004; Moon et al., 2004). We have found that amla extract inhibits MMP-1 activity even in a cell-free system (data not shown). MMPs are inhibited by a decrease of free zinc concentration. Since polyphenols have already been shown to be very effective for chelating metal ions (Souza and Giovani, 2005; Satterfield and Brodbelt, 2000), it is possible that amla extract also acts as collagenase inhibitor through metal chelation. These results indicate that amla extract may inhibit the activity of MMP-1 as well as its production.

TIMPs are synthesized in dermal fibroblasts. They impede the actions of MMPs in the dermis, which comprise one of the two axes important for ECM metabolism (Kahari and Saarialho-Kere, 1997). Human TIMPs comprise four groups, among which TIMP-1 is the most important for ECM metabolism, and can suppress the action of all MMPs except for MT-MMP (Bertaux and Hornebeck, 1993). However, studies of ECM metabolism have focused only on the proteases, i.e. MMPs, and collagen synthesis. The precise regulatory mechanisms and functions of TIMPs are still unclear. TIMPs can also function as growth factors for keratinocytes and fibroblasts (Hornebeck, 2003). This suggests that proliferation of fibroblasts induced by amla extract may involve the action of TIMP-1 in the culture medium. Previous studies have demonstrated that various antioxidants such as vitamins and flavonoids decrease MMPs expression but have little effect on TIMP expression (Nusgens et al., 2001; Hantke et al., 2002). In this study, amla extract upregulated TIMP-1 production by fibroblasts, suggesting that it is due to a particularly unusual plant-derived material.

In summary, the present study has shown that amla extract elevates the *mitochondrial activity* of human skin fibroblasts and promotes production of procollagen. These results suggest that amla extract has a number of potential mitigative, therapeutic and cosmetic applications.

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