Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2010; 2(3):30-34

ISSN: 0975 4873

Research Article

Anti-Ageing Ability of *Terminalia* Species with Special Reference to Hyaluronidase, Elastase Inhibition and Collagen Synthesis *In Vitro*.

Satardekar K.V.* and Deodhar M.A.

KET's V G Vaze College of Arts, Science and Commerce, Mulund (East), Mumbai -400081, India.

ABSTRACT

Hydroalcoholic extracts prepared from bark of *Terminalia arjuna* and dried fruit rinds of *Terminalia chebula* were examined for their effects on enzyme Hyaluronidase and Elastase activity. The extracts were also studied for the effect on fibroblast proliferation and collagen synthesis *in vitro*. Both the extracts showed to inhibit enzyme Hyaluronidase and Elastase in a dose dependent manner. In addition to their antielastase and antihyaluronidase effect, the same concentrations of the extracts also stimulated fibroblast proliferation and synthesis of total soluble collagen *in vitro*. These results suggest potential of *Terminalia* extracts for facilitating delay in skin aging. These extracts not only provide an active ingredient that inhibits hyaluronidase and elastase enzymatic activities, but also to provide the necessary supplies for the cells to maintain and promote their proliferation and to produce their constituents like collagen.

Keywords: Hyaluronidase, Elastase, 3T3 Fibroblast proliferation, Collagen synthesis *in vitro*.

INTRODUCTION

Connective tissues, particularly skin, undergo significant alterations during aging. These alterations are characterized by epidermal thinning and appearance of facial lines and furrows. The reason for these alterations lies in morphological changes in extra-cellular matrix (ECM). ECM is made up of glycosaminoglycans, interwoven with fibrous matrix proteins like collagen, elastin and fibronectin forming a cross linked meshwork that gives ECM strength and resilience[1]. Collagen is the important structural component of skin that represents 70% to 80% of the dry weight. Elastin accounts for only about 1-2% of the dry weight of skin but is important for the maintenance of skin's elasticity and resilience. Hyaluronic acid is mucopolysaccharide that holds the water and keeps the body moist, lubricated and smooth.

These connective tissue proteins are constantly attacked by several enzymes like collagenases, elastases and matrix metalloproteinases, which leads to decrease in thickness of skin and it becomes dry and wrinkled. In skin, fibroblasts are the cells which synthesize collagen along with other GAGs[2]. Collagen has been shown to be produced *in vitro* by many types of cultured fibroblasts, including 3T3 mouse fibroblasts[3]. In healthy skin, the synthesis and degradation of these matrix proteins are in balance. This intricate balance is disrupted with aging, UV radiations or stress conditions and unhealthy lifestyle[4][5]. To maintain the youthful appearance of the skin, there are two methods, viz., to promote the synthesis of matrix proteins in the skin or to inhibit matrix protein degrading enzymes like hyaluronidase and elastase.

Hyaluronidases are considered to be 'spreading factors' and play a crucial role in degradation of ECM leading to bacterial invasion, envenomation of various toxins including honeybee toxins, snake toxins etc., promote tumor growth and angiogenesis. The potent hyaluronidase inhibitors are useful as contraceptives, antitumor agents and have antibacterial and antivenom properties[6]. Flavonoids like Tannic acid and quercetin are known to bring about 94% and 100% inhibition of hyaluronidase purified from Indian cobra (*Naja naja*) venom[7]. Ellagic acid and tannic acid over the range of 2μg/ml to 10μg/ml are known to have strong potency for blocking activity of boar sperm hyaluronidase and used to reduce the rate of polyspermy in *in vitro* fertilization[8].

Human neutrophil elastase (HNE) is produced by neutrophils as immune response to bacterial infection. Intracellular HNE digest the proteins of invading bacteria where as extracellular HNE, secreted by neutrophils, breakdown the skin constituents like elastin, collagen and keratin and assist the neutrophil migration at the site of inflammation. Under normal physiological conditions, endogenous inhibitors protect the damage to healthy tissue but overzealous expression of HNE leads to many inflammatory diseases like rheumatoid arthritis and cardiovascular and pulmonary disorders. Flavonoid like pentagalloyl glucose stabilizes degradation of vascular elastin in aorta in cardiovascular disorders[9]. Polyphenols like tannic acid and ellagic acid bind to newly formed tropoelastin and protect it from proteolytic damage from secreted proteolytic enzymes[4].

There are very few flavonoids like, from *Areca catechu*[10], *Pomegranate* rind extract[11], which are

**Author for Correspondance: kvsatardekar@scientist.com*

Table1. Effects of various concentrations of TA and TC on inhibition of enzymes Hyaluronidase and Elastase

	Hyaluronidase % Inhibition		**Elastase** % Inhibition	
Concentrations (µg/ml)	**TA** Mean ± SD	**TC** Mean ± SD	**TA** Mean ± SD	**TC** Mean ± SD
25	47.18 ± 5.4	25.46 ± 5.9	-	-
50	49.95^{b} ± 5.2	39.07^{a} ± 4.8	63.29 ± 6.3	30.13 ± 5.9
80	-	-	70.81^{a} ± 5.9	38.80^{a} ± 6.7
100	52.97^{b} ± 4.6	48.02^{b} ± 4.9	76.77^{b} ± 5.8	47.65^{b} ± 5.5
125	62.60^{c} ± 3.9	51.75^{c} ± 5.4	-	-
150	90.38^{d} ± 6.7	64.41^{d} ± 5.6	80.26^{b} ± 6.7	50.52^{c} ± 3.2
200	-	-	85.00^{c} ± 5.4	50.95^{c} ± 5.4
250	90.40^{d} ± 5.3	83.52^{e} ± 5.3	-	-
300	84.60^{c} ± 5.3	84.80^{e} ± 4.3	-	-
400	80.65^{b} ± 4.7	88.67^{f} ± 4.8	87.04^{c} ± 6.2	66.57^{d} ± 5.9
500	67.59^{a} ± 5.8	89.65^{f} ± 3.9	-	-
600	-	-	89.95^{d} ± 5.3	78.47^{e} ± 6.6
800	-	-	91.58^{d} ± 6.8	82.42^{f} ± 5.3
1000	-	-	89.60^{d} ± 5.5	83.63^{f} ± 6.8

[Means followed by the same letter are not significantly different at $p < 0.05$ according to ANOVA test (n = 3)]

reported to contribute in fibroblast proliferation and synthesis of matrix metalloproteins.

The present communication reports the ability of hydroalcoholic extracts of dried bark of *Terminalia arjuna* and dried fruit rinds of *Terminalia chebula* to promote growth of fibroblast to synthesize matrix proteins as well as their inhibitory action against matrix proteinases.

Terminalia arjuna profusely grown in western ghats of India and throughout Indo-Subhimalayan tracts, are rich source of tannins like Catechol, Gallocatechin, epigallocatechin and Flavonoids and also phenolics like Gallic acid and Ellagic acid[12]. Since ancient times, *Terminalia arjuna* is used as a cardiotonic. Tannin containing fractions from *T.arjuna* are known to increase the rate of epithellization and hexosamine content in granulation tissue in excision wounds in rats[13]. *Terminalia chebula* acts as 'Rasayana' i.e., rejuvenating agent[14] and contains hydrolysable tannins like Chebulinic acid, Gallotannins, Phenolics like Gallic acid, Chebulic acid[15].

MATERIALS AND METHODS

Materials

All organic solvents used were of analytical grade

Table 2. Effect of various concentrations of TA and TC on 3T3 Swiss albino mice embryonic fibroblast cells in DMEM supplemented with 0.1% serum and 10% serum.

	Fibroblast Proliferation Absorbance at 570nm 0.1% FCS		**Fibroblast Proliferation** Absorbance at 570nm 10% FCS	
Concentrations (µg/ml)	**TA** Mean ± SD	**TC** Mean ± SD	**TA** Mean ± SD	**TC** Mean ± SD
Control	0.242 ± 2.0	0.242 ± 2.0	1.586 ± 1.4	1.586 ± 1.4
30	0.311^{f} ± 1.8	0.362^{e} ± 2.3	1.59^{e} ± 1.3	1.666^{d} ± 0.9
50	0.598^{g} ± 2.1	0.624^{f} ± 4.1	3.014^{f} ± 2.7	2.38^{e} ± 3.4
70	0.837^{h} ± 3.8	0.878^{g} ± 5.2	4.768^{g} ± 3.6	4.79^{f} ± 3.2
90	0.823^{g} ± 2.5	0.883^{g} ± 3.8	4.711^{g} ± 3.2	4.876^{g} ± 2.6
150	0.772^{f} ± 4.6	0.813^{f} ± 3.8	3.868^{f} ± 2.9	3.372^{f} ± 4.1
200	0.568^{e} ± 3.1	0.626^{e} ± 3.9	3.34^{e} ± 2.2	3.165^{e} ± 3.8
250	0.506^{d} ± 5.4	0.517^{d} ± 4.2	2.402^{d} ± 4.6	2.72^{d} ± 3.6
500	0.328^{c} ± 3.4	0.345^{c} ± 3.9	1.598^{c} ± 4.5	1.831^{c} ± 5.6
750	0.207^{b} ± 5.2	0.221^{b} ± 4.5	1.152^{b} ± 3.9	1.371^{b} ± 4.9
1000	0.186^{a} ± 4.8	0.194^{a} ± 3.7	1.068^{a} ± 3.8	1.049^{a} ± 5.2

The cell viability was spectrophotometrically measured by MTT assay interpreted as relative absorbance at 570nm. [Means followed by the same letter are not significantly different at $p < 0.05$ according to ANOVA test (n = 3)].

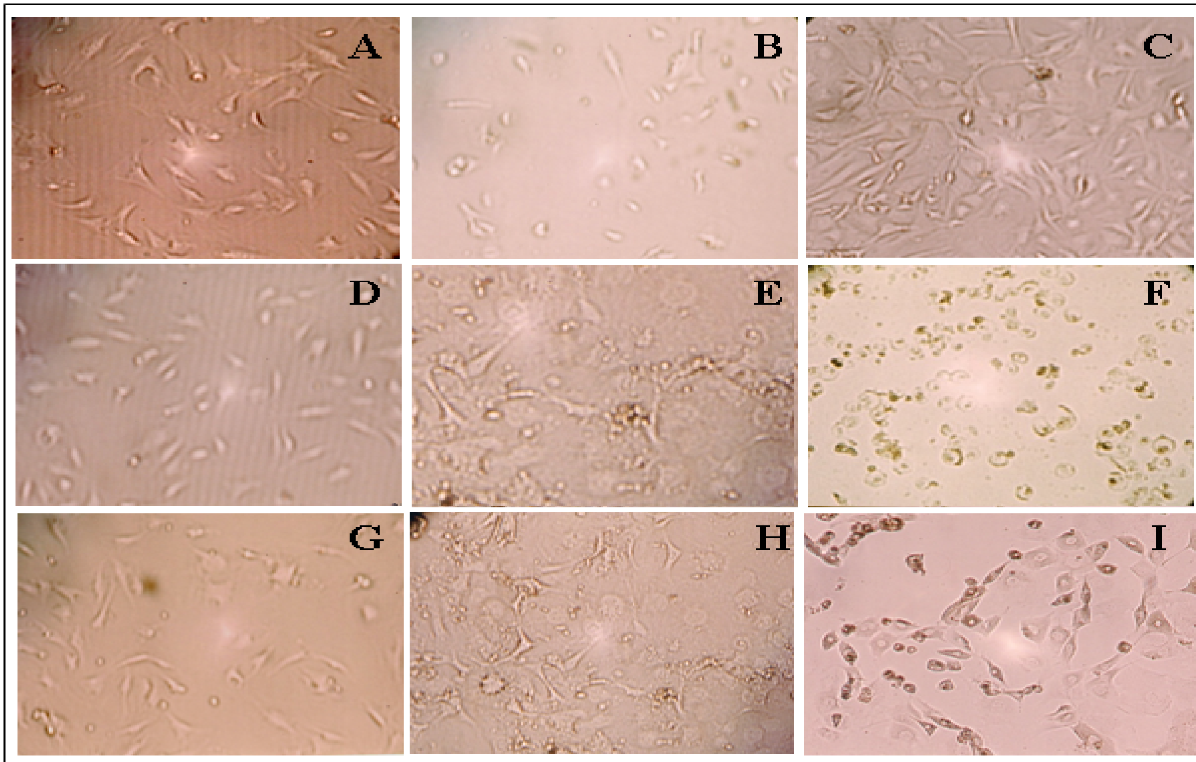

Figure 1. Growth of 3T3 Swiss albino embryonic fibroblast cells in monolayer observed under inverted microscope. **(A)** Control cells in DMEM supplemented with 10% FCS, **(B)** Control cells in DMEM supplemented with 0.1% FCS, **(C)** Cells treated with TA at 70µg/ml of TA in DMEM supplemented with 0.1% FCS, **(D)** Cells treated with TA at 250µg/ml in DMEM supplemented with 0.1% FCS, **(E)** Cells treated with TA at 500µg/ml in DMEM supplemented with 10% FCS, **(F)** Cells treated with TA at 500µg/ml in DMEM supplemented with 0.1% FCS, **(G)** Cells treated with TC at 250µg/ml in DMEM supplemented with 0.1% FCS, **(H)** Cells treated with TC at 500µg/ml in DMEM supplemented with 10% FCS and **(I)** Cells treated with 500µg/ml in DMEM supplemented with 0.1% FCS.

(Merck and Qauligen). Hyaluronidase type-1S from Bovine testes (999 units/mg solid) and Hyaluronic acid sodium salt from human umbilical cord, Elastase type IV from porcine pancreas, *N*-Succinyl-Ala-Ala-Ala-*p* nitroanilide (SANA) were purchased from Sigma Aldrich, USA. For Hyaluronidase and Elastase inhibition activity, Spectrophotometric measurements were performed on UV-Visible Spectrophotometer (Varian, Cary50).

Flasks (25 cm^2) for culture of cells were procured from Tarsons. Dulbecco's Modified Eagle's Medium [Himedia ATO 68], Foetal Calf Serum (FCS) [Biowest], Trypsin-versene mixture [Himedia], Gentamycin [Nicholas Piramal]. All the chemicals used for MTT assay for determining cellular proliferation were of analytical grade (Merck and Qauligen). Chemicals for collagen assay including collagen standards were used from Sircol™ Soluble Collagen Assay Kit [Bicolor, Ireland, UK]. Colorimetric measurements for fibroblast proliferation assay and collagen content assay were performed on Elisa plate reader [Multiscan SKNENS, Thermoelectron corp.].

Methods

Preparation of Plant extracts

Hydro-alcoholic extracts of dried bark of *T. arjuna* (TA) and dried fruit rinds of *T. chebula* (TC) were prepared by extracting 30 grams of the powdered dried plant materials in 50% ethyl alcohol (V/V) for 6 hours. The alcoholic extracts were air dried and the residues of TA (yield: 34% w/w) and TC (yield: 41% w/w) were used for various assays.

Hyaluronidase inhibition assay

Hyaluronidase inhibition activity was spectrophotometrically determined by measuring amount of N-acetyl glucosamine splited from pottassium hyaluronate as described by **Lee *et al.*, 1999[16].** 50µl bovine hyaluronidase (7900 units/ml dissolved in 0.1M acetate buffer, pH 3.5) was mixed with 50µl of various concentrations of TA and TC ranging from 25µg/ml to 500µg/ml dissolved in 5% Dimethyl Sulfoxide (DMSO). It was incubated at 37°C for 20 minutes. The control group was treated with 50µl of DMSO instead of plant extract. Hyaluronidase was activated by adding 50µl of 12.5mM calcium chloride in reaction mixture and incubated at 37°C for 20 mins. The Ca^{+2}activated hyaluronidase was subjected to 250µl of sodium

Table 3. Effect of various concentrations of TA and TC on Collagen released by the cells *in vitro* measured using Sircol^{TM} Soluble Collagen Assay Kit.

Concentrations (µg/ml)	Collagen synthesis *in vitro* µg/ml	
	TA Mean ± SD	TC Mean ± SD
Control	150 ± 0.0	150 ± 0.0
30	154[a] ± 0.2	170[a] ± 0.2
70	245[b] ± 0.4	230[b] ± 0.3
90	240[c] ± 0.2	235[c] ± 0.4
150	231[b] ± 0.2	233[b] ± 0.2
250	220[a] ± 0.2	230[a] ± 0.3

Means followed by the same letter are not significantly different at $p < 0.05$ according to ANOVA test (n = 3)

hyaluronate (1.2 mg/ml dissolved in 0.1 M acetate buffer, pH 3.5) and then incubated in waterbath at 100°C, exactly for 3 minutes. Reaction mixture was allowed to cool to room temperature, then 1.5 ml of p-Dimethyl amino benzaldehyde (4gms PDMAB dissolved in 350 ml of Glacial acetic acid and 50 ml of 10N HCl) was added to the reaction mixture and it was then incubated in waterbath at 37°C for 20 mins. The absorbance was measured at 585 nm.

Elastase assay

Porcine pancreatic Elastase (PPE, Sigma, Type IV) was assayed spectrophotometrically by the method of **Lee *et al.*, 1999 *loc Cit*.[16]** using N-Succinyl-Ala-Ala-Ala-nitroanilide (SANA) as the substrate and monitoring the release of p-nitroanilide for 20 mins at 25°C. The reaction mixture contained 800µl of 0.2M Tris-HCl buffer (pH 8.0), 100µg/ml elastase, various concentrations of TA and TC ranging from 50µg to 1000µg dissolved in Tris-HCl buffer and 0.8 mM succ-$(Ala)^3$- nitroanilide as substrate. Extracts were preincubated with enzyme for 20 min at 25°C and the reaction was started with the addition of substrate. In control, only Tris HCl buffer was used instead of the extracts. The absorbance was monitored for 20 mins at 410nm using UV spectrophotometer.

Maintenance of cell line:

3T3 Swiss albino embryonic fibroblast cell line was procured from National Centre for Cells Sciences (NCCS), Pune, Maharashtra, India. Cells were maintained at 37^{0}C in CO_2 incubator in humid conditions in 5%CO_2/95% air in CO_2 incubator in DMEM supplemented with 10% FCS and 50 IU/ml Gentamycin. Cells were subcultured on every 3rd day using 0.25% Trypsin-Versene mixture for harvesting cells.

Fibroblast Proliferation assay:

3T3 fibroblast cells were harvested from the flasks using trypsin-versene mixture and seeded in 96-well plate at the density of 1X10^{3}cells/well grown in DMEM medium with 10% FCS. For attaining serum-starved condition, cells were grown in DMEM supplemented with 0.1% FCS. The cells were incubated in 5% CO2/95% air in CO_2 incubator. It was allowed to get attached overnight. After 24 hours, 10µl of TA and TC in concentrations ranging from 30µg/ml to 1000µg/ml were added to the respective wells. The effect of extracts on cell viability/proliferation was studied by MTT assay. The MTT Assay was performed on 2nd, 4th and 6th day as described by **Thang *et al.*, 1998[17].**

Collagen Estimation Assay:

Effect of TA and TC on Collagen synthesis *in vitro* by the fibroblast cells was measured by Sircol^{TM} Soluble Collagen Assay Kit (BIOCOLOR). The cells were cultured in 96 well plate at a density of 1X10^3 cells/well in DMEM supplemented with 0.1% serum. 10µl of various concentrations of TA and TC in the range of 30µg/ml to 1000µg/ml were added to the wells.

Assay was performed according to the assay manual and amount of soluble collagen produced by fibroblasts was determined by measuring the absorbance at 540nm. A standard graph was plotted by using 5µg to 50µg of standard collagen provided in the kit and was used to determine the collagen content in the test samples.

Statistical analysis

Three or more separate experimental sets were performed. Difference between groups in experiments was analyzed for statistical significance by ANOVA ($P<0.05$).

RESULTS AND DISCUSSIONS

Effect of TA and TC on enzyme Hyaluronidase

Table 1 gives the effect of various concentrations of TA and TC on inhibition of enzyme Hyaluronidase and enzyme Elastase. In case of TA, the most significant inhibition of enzyme hyaluronidase (more than 90%) was observed at 150µg/ml to 250µg/ml of TA. At concentrations higher than 250µg/ml, there was little decrease in the activity of TA. At 300µgs/ml, it significantly decreased to 84.60% and at 500µg/ml, it was observed to be only 67.59%. At lower concentrations of 25µg/ml to125µg/ml of TA, the inhibition was observed to be in the range of 47.18% to 62.60%.

With TC, the highest inhibition (89.65%) was observed comparatively at higher concentrations Viz. 500µg/ml. At lower concentrations (25µg/ml to 100µg/ml), the inhibition was negligible, i.e. 25.46% to 48.02%. At 150µg/ml TC was responsible for bringing about 64.41% inhibitions. At higher concentrations of 250µg/ml to 400µg/ml, there was a significant increase in enzyme inhibition, which ranged from 83.52% to 88.67%, highest being at 500 µg/ml with 89.65% inhibition. **Lee *et al.*, 2001[18]** have reported more than 57% and 82% inhibition of enzyme hyaluronidase at concentrations of 250 and 500µg/ml of purified *Areca catechu* extracts.

Effect of TA and TC on enzyme Elastase

TA was also effective in bringing about inhibition of enzyme elastase. The results are summarised in **Table 1.** It exhibited about 80.26% inhibition of enzyme elastase at 150µg/ml. At lower concentrations of 50µg/ml to 100µg/ml the inhibition was in the range of 63.29% to 76.77%. At 150µg/ml, it increased to 80.26%. At higher concentrations of 400µg/ml and 600µg/ml, it significantly increased to 87.04% and 89.95%

respectively. The highest inhibition (91.58%) was observed at 800µgs/ml. At 1000µg/ml it declined to 89.60%.
TC showed an increase in elastase inhibition with increase in concentration. TC also showed a dose dependent increase in increase in inhibition of enzyme elastase. TC exhibited the highest inhibition (83.63%) at 1000µg/ml. It exhibited only 30.13% inhibition at 50µg/ml, but it significantly increased to 50.52% inhibition at 150µg/ml. At 600µg/ml and 800µg/ml, TC exhibited about 78.47% and 83.42% inhibition respectively. In *Areca catechu*, **Lee and Choi, 1999 *loc cit*,**[10] have reported 37% inhibition at 10µg/ml and about 80% inhibition at 100µg/ml. At 250µg/ml of *Areca catechu* extract, they reported about 90% inhibition of enzyme elastase.

Effect of various concentrations of TA and TC on fibroblast cell proliferation:

Our earlier study on effect of various serum concentrations on fibroblast cell growth indicated that, at 0.05% serum concentrations, most of the cells did not attach to the substratum and remained in the suspension[19]. 0.1% serum concentration was just sufficient for the growth and adherence of the cells. At 0.1% serum concentration, the fibroblast proliferated at slow rate which was depicted by little increase in O.D. in MTT assay. On 2nd day, the fibroblasts supplemented with 0.1% serum exhibited 0.143 O.D. which increased to 0.261 on 6th day. But, the fibroblast supplemented with 10% serum on 2nd day showed O.D. of 0.312, which increased to 1.586 on 6th day.
Figure 1 (A) represents a monolayer of fibroblast supplemented with 10% serum i.e., optimal concentration. As seen in **Figure 1 (B),** though 0.1% FCS concentration showed very slow rate of proliferation, the cells showed good adherence with no remarkable change in cell morphology. The cells grown in 0.1% FCS were only less well-spread and were smaller in size than cells grown in 10% FCS concentration. Hence, this concentration was chosen to attain serum starved condition for our studies.
As seen in **Table 2**, serum deprived fibroblasts, when supplemented with TA and TC exhibited a significant increase in fibroblast proliferation at concentration as low as 30µg/ml. The most significant increase was observed with 70µg/ml of TA, which exhibited 0.837 O.D. against 0.242 O.D. in control fibroblasts supplemented with 0.1% FCS **(Figure 1 C**). This increase was 245.87% over control. Similarly, serum deprived fibroblasts when treated with 90µg/ml of TC on 6thday produced 0.883 O.D. against 0.242 O.D. in control, which was 264.88% increase. Whereas, in control fibroblasts treated with 10% serum, O.D. was 1.586. These results indicate that the lack of growth factors for cellular proliferation in serum deprived condition was compensated by addition of TA and TC extracts to cells.
In 150µg/ml to 250µg/ml of TA and TC in serum deprived fibroblasts, though there was a little decline in rate of proliferation, there were no much visible morphological alterations in the cells **(Figure1, D and G**). At 500µg/ml of TA and TC, granulation was first observed in the cells with significant alterations in the cells with cell shrinkage **(Figure1, E, F, H and I**).
The most striking feature is in serum deprived conditions. TA and TC are not cytotoxic at 500µg/ml concentration. Fibroblasts grown in 10% serum when supplemented with 500µg/ml of TA showed negligible proliferation i.e., only 0.76%, but in serum deprived condition when supplemented with 500µg/ml of TA shows 35.54% proliferation. It shows that, under stressful conditions, these extract supplementations are more effective.
Similar observation is made by **Phillips et al. in 1994**[20]**.** They examined the effect of ascorbic acid on dermal fibroblast from different aged donors. In absence of ascorbate, at the end of 13 days, the fibroblasts derived from newborn donor increased from $1.0X10^5$cells/100mm culture dish to 1.0 to $2.4X10^6$cells/100mm culture dish, showing increase in proliferative capacity by 100-200%. The fibroblasts cultured from elderly donors, increased from $1.0X10^5$cells/100mm culture dish to 2.0 to $6X10^5$cells/100mm culture dish, which was just 2-6 fold increase. When the cells were treated with ascorbic acid, the newborn cells increased by 167 to 303%, whereas, increase in elderly cells was 208 to 315%.
Aslam *et al*., 2005; *loc cit*.,[11] in their work on Pomegranate (*Punica granatum*) peel extracts, studied the effect on fibroblast proliferation and collagen synthesis. They incubated human dermal fibroblasts in low Ca^{+2} and serum-free basal medium and treated with various concentrations of pomegranate extracts. In absence of extracts, there was no growth in serum-free media and showed less than 50% viability over 3 days of incubation. Pomegranate peel extract stimulated fibroblast proliferation significantly at 0.1µl/ml. Type-I procollagen synthesis was significantly stimulated at 0.05µl/ml. At the same concentration of 0.01µl/ml to 0.05µl/ml, it inhibited MMP-I (interstitial collagenase) production by dermal fibroblast. Thus, Pomegranate peel extracts prevented cell death due to low serum concentration and allowed proliferation of human dermal fibroblasts to occur. These results suggest potential of Pomegranate fractions for facilitating skin repair.
Recently, **Pacheco-Palencia *et al*., 2008**[21], investigated effect of Pomegranate (*Punica granatum* L.) peel extract on UV-A and UV-B induced damage in SKU-1064 human skin fibroblasts. Both, UV-A and UV-B induced a significant decrease in total number of cells. Pomegranate peel extracts inhibited UV-B induced cell death at concentration as low as 20 mg/l. Inhibition of cell death induced by UV-A was achieved at 60mg/l.

Total Soluble Collagen assay

Sircol™ Soluble Collagen Assay is a quantitative dye binding method, which measures the collagen released in the culture medium by cells during in vitro culture. The control fibroblast cells growing in DMEM with 0.1% serum produced 150µg of collagen at the end of the 6th day. The fibroblast treated with TA or TC exhibited a

significant increase in production of collagen in vitro. The most significant increase in collagen production (245µg) was observed in cells treated with 70µg/ml of TA, which was 63% increase. Similarly, when cells were treated with 90µg/ml of TC, collagen produced was 235µg, which was 56%, increase **(Table 3).** These were the concentrations, which promoted highest proliferation of fibroblast. **Lee *et al.*, 2001 *loc cit***., reported about 40% increase in collagen content at 100µg/ml of Ethanolic extract of *Areca catechu* which they designated as CC-516 at the end of 24 hours.

For TA, 150µg/ml appears to be the best concentration as it brings about 90.38% and 80.26% inhibition of enzyme Hyaluronidase and Elastase respectively. At the same time, this concentration is also supportive for the growth of fibroblast. At this concentration, there was 219% increase in the growth of the fibroblast at the end of the 6th day. Increase in the amount of the collagen was 54%. In case of TC, Hyaluronidase & Elastase inhibition was brought about at comparatively higher concentration (500µg/ml to 1000µg/ml).

In case of TC, the effective inhibition of hyaluronidase and elastase was brought about at comparatively higher concentration of 500µg/ml to 1000µg/ml. But, these concentrations are not supportive for cell proliferation. The highest concentration at which there is no toxicity is 250µg/ml. At this concentration, it brings about 83.52% inhibition of hyaluronidase and about 50% inhibition of elastase.

Recently, **Fujii *et al.*, 2008[22]** studied effect of *Emblica officinalis* extracts on procollagen production and inhibition of matrix metalloproteinases in human skin fibroblasts. They studied the effect of amla extract on fibroblast proliferation by WST assay. Amla extract was supportive to the cell proliferation at concentration range of 5-20µg/ml. At this concentration, proliferation was 16-27%. However, at concentrations beyond 40µg/ml, amla extracts did not support growth of fibroblasts. At 20µg/ml of amla, there was 36% increase in procollagen production and same concentration was responsible for 33% decrease in MMP-2 levels

Efforts are underway to incorporate *Terminalia* extracts in antiaging formulations and to evaluate its effect on chronological aging and photoaging.

ACKNOWLEDGEMENT

Authors thank Mr. G D Kelkar, Chairman, KET's Scientific Research Centre, Dr. G T Paratkar, Principal, KET's V G Vaze College for providing research facilities and Dr. B P Hardikar for her valuable suggestions in cell cultures.

REFERENCES

1. Oikarinen A. Connective tissue and ageing. *Int J Cosmet Sci* 2004; 26(2): 107-108.
2. Stipcevic T, Piljac J, Berghe DV. Effect of different flavonoids on collagen synthesis in human fibroblasts. *Plant Food Hum Nutr* 2006; 1-11.
3. Goldberg B and Green H, Todaro GJ. An analysis of collagen secretion by established mouse fibroblast lines. *Exp Cell Res* 1963; 31: 444-447.
4. Jimenez F, Mitts F, Liu K, Wang Y, Hinek A. Elagic acid and tannic acids protect newly synthesized elastic fibers from premature enzymatic degradation in dermal fibroblast cultures. *J Invest Dermatol* 2006; 126: 1272-1279.
5. Varani J, Dame MK, Rittie L, Fligiel SEG, Kang S, Fischer GJ, Voorhees JJ. Decreased collagen production in chronologically aged skin (Roles of age dependant alteration in fibroblast function and defective mechanical stimulation. *Am J Pathol* 2006; 168(6): 1861-1868.
6. Isoyama T, Thwaites D, Selzer MG, Carey RI, Barbucci R, Lokeshwar VB. Differential selectivity of hyaluronidase inhibitors toward acidic and basic hyaluronidases. Glycobiol 2006; 16:11-21.
7. Girish K, Kemparaju K. Inhibition of Naja naja Venom Hyaluronidase by plant derived bioactive components and polysaccharides. *Biochemistry* 2005; 70: 1145-1150.
8. Tatemoto H, Tokeshi I, Nakamura S, Muto N, Nakada T. Inhibition of boar sperm hyaluronidase activity by tannic acid reduces polyspermy during *invitro* fertilization of porcine oocytes. *Zygote* 2006; 14: 275-285.
9. Isenburg JC, Simionescu DT, Starcher BC, Vyachare NR. Elastin stabilization for treatment of abdominal arotic aneurysms. *Circulation* 2007; 115: 1729-1737.
10. Lee KK, Choi J. The effects of *Areca catechu* L extracts on anti ageing. *Int J Cosmet Sci* 1999; 21: 285-294.
11. Aslam MN, Lansky EP, Varani J. Pomegranate as a cosmeceutical source: Pomegranate fractions promote proliferation and procollagen synthesis and inhibit matrix metallo proteinases-1 production in human skin cells. *J Ethnopharmacol* 2005; 103(3): 311-318.
12. Rahman Z, Kohli K, Khar RK, Lamba HS, Rathour A, Pahwa R. An overview of *Terminalia arjuna*: chemistry and pharmacological profile. *Indian Drugs* 2004; 41(11): 641-649.
13. Chaudhari M, Mengi S. Evaluation of phytoconstituents of *Terminalia arjuna* for wound healing activity in rats. *Phytother Res* 2006; 20(9): 799-805.
14. Sumantran V, Kulkarni A, Harsulkar A, Wele A, Koppikar S, Chandwaskar R, Gaire V, Dalvi M, Wagh U. Hyaluronidase and collagenase inhibitory activities of the herbal formulation *Triphala guggulu*. *J Bioscience* 2007; 32(4): 1-7.
15. Juang LJ, Sheu SJ, Lin TC. Determination of

hydrolyzable tannins in the fruit of *Terminalia chebula* Retz. by high-performance liquid chromatography and capillary electrophoresis. *J Sep Sci* 2004; 27(9): 718-724.

16. Lee KK, Kim JH, Cho JJ, Choi JD. Inhibitory effects of 150 plant extracts on anti-elastase activity and their anti-inflammatory effects. *Int J Cosmet Sci* 1999; 21: 71-82.
17. Thang TP, Margaret AH, George WC. Enhanced proliferation of fibroblasts and endothelial cells treated with an extract of the leaves of *Chromolaena odorata* (Eupolin), an herbal remedy for treating wounds. *Plastic Reconstr Surg* 1998; 101(3): 756-765.
18. Lee KK, Cho JJ, Park EJ, Choi JD. Anti-elastase and anti- hyaluronidase activity of phenolic substance from *Areca catechu* as a new anti-ageing agent, *Int J Cosmet Sci* 2001; 23(6): 341-346.
19. Satardekar KV. Studies on Anti-ageing ability of *Terminalia* Species. *Ph.D. Thesis*, 2009; *Uni Mum*.
20. Phillips CL, Combs SB, Pinnel SR. Effects of ascorbic acid on proliferation and collagen synthesis in relation to the donor age of human dermal fibroblasts. *J Invest Dermatol* 1994; 103(2): 228-232.
21. Pacheco-Palencia LA, Noratto G, Hingorani L, Talcott ST, Mertens-Talcott SU. Protective effects of standardised Pomegranate (Punica granatum L.) polyphenolic extract in Ultraviolet-irradiated human skin fibroblasts. *J Agri Food Chem* 2008; 56(18): 8434-8441.
22. Fujii T, Wakaizumi M, Ikami T, Saito M. Amla (Emblica officinalis Gaertn.) extract promotes procollagen production and inhibits matrix metalloproteinase-1 in human skin fibroblasts. *J Ethnophamacol* 2008; 119:53-57.