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Impact of *Achyranthes aspera* L. on Protein Profile in Impaired Wound Models

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Abstract In the present study, comparative protein profile of granulation tissues of burn, diabetic and immunocompromised wounds treated with 5.0% (w/w) ointment of methanol extract of *Achyranthes aspera* was undertaken. Granulation tissue of *A. aspera* treated group showed expression of a protein with molecular weight 69.4 kD in burn wound and proteins ranging from high, medium and low molecular weight (61.0, 55.0 and 31.5kD) in diabetic and immunocompromised wounds. *A. aspera* treatment induced expression of a particular protein of molecular weight 31.5 kD in both diabetic and immunocompromised wound models, signifying that this particular protein might play a key role in *A. aspera* mediated wound healing in these two models.

Key words: *Achyranthes aspera*, Burn, Diabetic, Immunocompromised wound, SDS–PAGE.

INTRODUCTION

Wound healing is a complex phenomenon that results in the restoration of anatomic continuity and function, accomplished by several processes which involve different phases including inflammation, granulation, fibro genesis, neo-vascularization, wound contraction and epithelialization[1].

Approximately 25% of drugs in modern pharmacopoeia were derived from plants (phytomedicines) and many others were synthetic analogues built on prototype compounds isolated from plants[2]. Indian folk medicine comprises of numerous prescriptions for therapeutic purposes such as healing of wounds, inflammation, skin infections, leprosy, diarrhea, scabies, venereal disease, ulcers, snake bite, etc[3]. Considering the rich diversity of medicinal plants in North-East region, it is expected that screening and scientific evaluation of plant extracts for their diversified activity may provide new drug molecule that can combat various side effects of the commercially available synthetic drugs, moreover reducing the cost of medication. *Achyranthes aspera* Linn. locally known as Apang, is an annual, erect under shrub or rather stiff herb growing up to 0.3 to 1.0 meter in height[4]. Yunani doctors and local *kabiraj* use the stem, leaves and fruits as a remedy for piles, renal dropsy, pneumonia, cough, kidney stone, skin eruptions, snake bite, gonorrhea, dysentery etc[4]. The plant has antibacterial[4], antitumor[5], anti-inflammatory[6], abortifacient activity and produces reproductive toxicity in male rats[7]. Its antidepressant[8], anxiolytic[9] and antinociceptive[10] activity were reported by us. In spite of various scientific studies on this plant, its wound healing property is lacking. We have found promising wound healing activity in immunocompromised model[11]. It is interesting to note that there was expression of proteins of different molecular weights in different wound models, a protein with common molecular weight expressed in all the wounds. Hence the present study was projected to unravel the possible molecular mechanism leading to its wound healing activity especially in different impaired wound models in animals.

MATERIALS AND METHODS

Plant material: Leaves of the plant *Achyranthes aspera* Linn. was collected from the medicinal garden of the Department of Pharmacology & Toxicology, College of Veterinary Science, Khanapara during the month of Feb–June 2009. The plant was identified by taxonomist, NEIST, Jorhat, Assam and a voucher specimen (AAU/CVSC/PHT/ 01) was deposited in the herbarium.

Preparation of methanol extract : The leaves were washed with water, air-dried and powdered in an electric blender. About 250 g of powdered leaves was soaked in 1000 ml methanol for 72 h in beaker and the mixture was stirred at regular interval

using a sterile glass rod. Filtrate was obtained after passing through a fine muslin cloth followed by filter paper (Whatman No1), three times and concentrated in Rotary evaporator (Equitron) at 50°–60°C under reduced pressure. The dark brown residue obtained was stored in airtight container at 4°C till further use (yield was 6.89%, w/w with respect to dry powder).

Preparation of 5% ointment of the A. aspera (w/w) : Five gram of the methanol extract of *A. aspera* was mixed with 95 g of soft white petroleum jelly (S.D. Fine Chem, India) to prepare 5% ointment (w/w). Himax (Indian Herbs Research & Supply Co. Ltd. Darra Shivpuri, Saharanpur) was used as standard drug.

Experimental animals: Healthy adult Swiss Albino mice of either sex, approximately of same age, weighing between 25–30 g and adult Sprague Dawley rats of either sex weighing between 180–200 g were used for the study. They were housed under controlled conditions of temperature (25 ± 3°C), humidity (50 ± 5%) and 12 h light–dark cycles with food and water *ad lib.* Animals were housed individually in polypropylene cages containing sterile paddy husk bedding. The experiments were performed as per guidelines of the Institutional Animal Ethical Committee (770/03/ac/CPCSEA/FVScAAU/ IAEC/06/21). The animals were fasted for 14 h before tests to achieve better drug absorption through gastrointestinal tract. Animals were periodically weighed before and after experiments. All the animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study. Rats were randomly distributed into three groups for different wound *viz.* burn, diabetic and immunocompromised models. Then these groups were further divided into three sub groups consisting of six animals each.

Control group: This group of rats received topical application of vehicle twice daily for 7 days for different wound models (C_B:Control burn, C_D: Control diabetic, C_I: Control immunocompromised). *A. aspera* treated group: This group of rats received topical application of 5% ointment of *A. aspera* (w/w) twice daily for 7 days for different wound models (A_B: *A. aspera* burn, A_D: *A. aspera* diabetic, A_I: *A. aspera* immunocompromised).

Standard group: This group of rats received topical application of Himax ointment twice daily for 7 days for different wound models (S_B: Standard burn, S_D: Standard *A. aspera* diabetic, S_I: Standard immunocompromised).

Determination of LD_{50} and acute toxicity [12]*:* Acute toxicity study was carried out according to the Organization of Economic Corporation Development (OECD) guidelines No. 425. The plant extract was administered orally in doses of 100, 200, 400, 800, 1000 and 2000 mg/kg to the group of mice (n=3) and the percentage mortality was recorded for a period of 24 h. During the first 1 h after the drug administration, the mice were observed for any gross behavioral change and the parameters observed were hyperactivity, grooming, convulsions, sedation and loss of righting reflex, respiration, salivation, urination and defecation.

Burn wound model:

The animals were anaesthetized by intra-peritoneal injection of thiopentone (25 mg kg^{-1}). The dorsal surface of the rat was shaved and the underlying skin was cleaned with 70% ethanol. Full-thickness burn wound was created by using an aluminum metal rod (diameter 1.8 cm, area 250 mm square, melting point 660°C) heated to 85°C. The temperature of the metal rod was monitored with a fabricated digital computerized multi-meter. The hot rod was exposed on the shaved area of the rat for 20 s, resting on its own weight of 30 g. No additional pressure was applied on the hand-leaded metal rod. Single burn wound was created on dorsal part of each rat. Animals were allowed to recover from anesthesia and housed individually in sterile cages. After 24 h, dead tissues were excised using sterile surgical blade[13]. The granulation tissue was excised on eighth post wounding day for SDS-PAGE study.

Diabetic wound model:

The basal blood glucose levels of rats were measured using glucometer (Ames, Bayer Diagnostic, India). Next day, after overnight fasting, the animals were injected with single dose of streptozotocin 40 mg kg^{-1} in 0.1 M citrate buffer (pH 4.5), intra-peritoneally to produce diabetes. The following day, the blood glucose levels of all the animals were monitored and animals showing blood glucose level nearly three fold higher than initial or basal value were considered diabetic and selected for the study. Wounds were created on day 7 after induction of diabetes and the granulation tissue was collected on day 8 of creation of wound from control, *A. aspera* and standard drug treated groups for SDS-PAGE study [14].

Immunocompromised wound model:

The immunocompromised state was induced by administration of hydrocortisone (Effcorlin) 40 mg kg^{-1}, by intra-muscular injection in rats until the end of the experiment. Hydrocortisone (HC) was prepared fresh in sterile, pyrogen-free distilled water. The animals were primed daily with HC treatment for 7 days, followed by wounding on the 8^{th} day. The granulation tissue was collected on day 8 of wounding from control, *A. aspera* and standard drug treated groups[15].

Protein profile by SDS-PAGE:

Wound tissues were chopped into small pieces and collected in Tris buffer (50 mM, pH 6.8) containing protease inhibitor phenyl methyl sulfonyl fluoride (PMSF, AMRESCO, Pro Pure Proteomic Grade) at 10 µg ml^{-1}. Tissues were homogenised in Polytron homogeniser (Kinematica AG, Switzerland Model: PT 1200E) with 4 strokes of 15 sec each in ice bath. After homogenisation, the samples were spun at 3000 × g at 4°C.

Electrophoresis was carried out with 14.5% polyacrylamide seperating gel. Thirty five (35) µl protein extract was treated with 5 µl sample buffer comprising of Tris 100 mM ; SDS 5% ; Sucrose 5% and β- mercaptoethanol 2.5% (v/v). The proteins were denatured in boiling water bath for 5 minutes prior to loading. After the completion of the electrophoretic run, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 for 6 h. Subsequently, the gel was de-stained with destainer till clear

blue band became visible. A standard protein molecular weight marker (PMW-M, Bangalore Genei) was also co-electrophored to determine the molecular weight of individual protein in kD (kilo dalton) from standard curve prepared out of the size marker.[16]

RESULTS

Burn wound model:

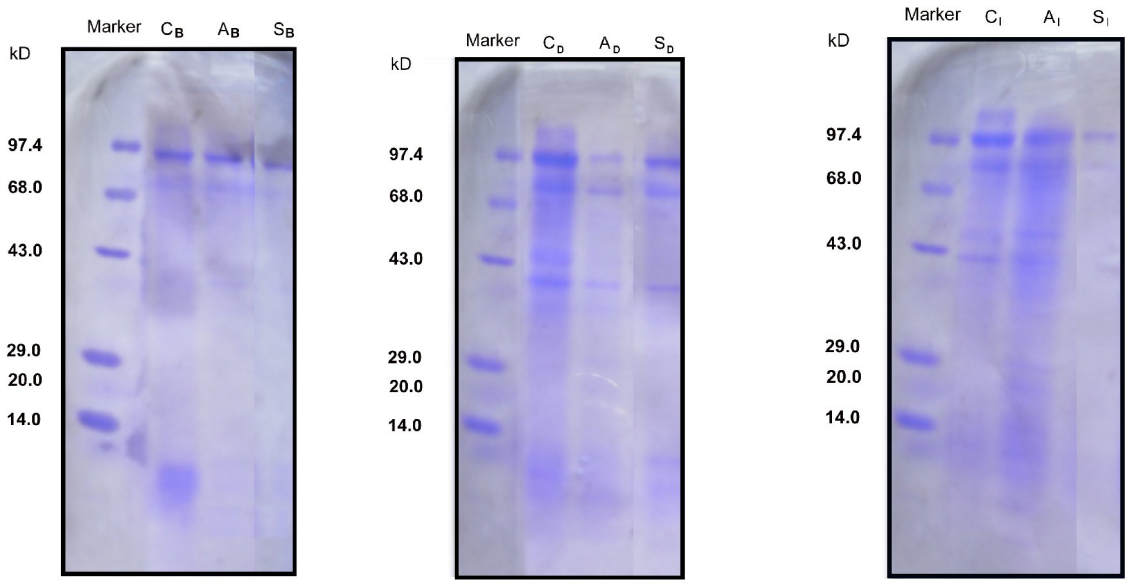
In the burn wound model, 6 proteins were invariably present in control (C_B), *A. aspera* (A_B) as well as standard drug (S_B) treated groups. In A_B as well as S_B treated groups, the protein profile was identical and a particular protein of molecular weight 69.4 kD was expressed in both the groups, which was supressed in C_B group. Appeareance of two proteins of molecular weight 17.2 and 15.2 kD were observed only in C_B group, which were absent in A_B and S_B groups(Table 1, Figure 1 A).

Table 1. ***Protein profile in the granulation tissues of control, A. aspera and standard group in burn wound model.***

Protein profile (Molecular weight in kilo-dalton)	**Control (C_B)**	***A. aspera* (A_B)**	**Standard (S_B)**
	>97.4	>97.4	>97.4
	97.4	97.4	97.4
	85.0	85.0	85.0
	81.7	81.7	81.7
	78.0	78.0	78.0
	-	69.4	69.4
	17.2	-	-
	16.5	16.5	16.5
	15.2	-	-

Diabetic wound model:

In diabetic wound model, the control (C_D) group exhibited highest number of 13 proteins followed by *A. aspera* (A_D) treated group with 9 proteins and standard (S_D) group with 6 proteins. Four proteins (81.7, 78.0, 19.5 and <14.3 kD) were uniformly present in all C_D, A_D and S_D groups (Table 2, Figure 1 B). Three unique proteins of molecular weight 61.0, 55.0 and 31.5 kD were present only in A_D treated group.



1(A) 1(B) 1(C)

Figure1. ***Photographs showing protein profile of the granulation tissues of different wound models: (A) Burn wound; (B) Diabetic wound and (C) Immunocompromised wound (Resolved in 14% Polyacrylamide Gel and visualised with Coomassic brilliant blue R-250 and Marker: PMW-M Bangalore Genei).***

Table 2. ***Protein profile in the granulation tissues of control, A. aspera and standard group in diabetic wound model***

Protein profile (Molecular weight in kilo-dalton)	Control (C_D)	*A. aspera* (A_D)	Standard (S_D)
	-	-	>97.4
	97.4	97.4	-
	81.7	81.7	81.7
	78.0	78.0	78.0
	66.0	-	-
	-	61.0	-
	57.5	-	-
	-	55.0	-
	51.8	-	-
	47.0	-	-
	43.6	-	-
	41.7	-	-
	-	31.5	-
	19.5	19.5	19.5
	19.0	-	-
	17.2	-	-
	-	15.2	15.2
	<14.3	<14.3	<14.3

Immunocompromised wound model:

Remarkable variation in the protein profile was observed in all the three groups of the immunocompromised wound model (Table 3, Figure 1 C). *A. aspera* (A_I) expressed maximum numbers of proteins, whereas standard group exihibited only 3 numbers of proteins. Fourteen new proteins of high, medium and low molecular weight were expressed only in the granulation tissue of A_I treated group, which were supressed in C_I and S_I treated groups.

Table 3. ***Comparative study of protein profile in the granulation tissues of control, A. aspera, and standard group in immunocompromised wound model.***

	Control (C_I)	*A. aspera* (A_I)	Standard (S_I)
Protein profile (Molecular weight in kilo-dalton)	>97.4	>97.4	>97.4
	97.4	97.4	97.4
	-	95.0	-
	-	91.6	-
	-	81.7	-
	-	-	78.0
	-	73.4	-
	-	69.4	-
	67.5	-	-
	62.8	-	-
	55.0	-	-
	51.4	51.4	-
	47.0	-	-
	45.0	45.0	-
	42.6	42.6	-
	41.8	41.8	-
	-	38.0	-
	-	33.4	-
	-	31.5	-
	-	27.0	-
	-	23.8	-
	22.0	-	
	-	21.7	-
	-	19.5	-
	17.2	-	-
	15.2	-	-
	-	14.3<	-
	-	<14.3	-

DISCUSSION

Normal healing process can be impeded at any step along its path by a variety of factors. Impaired wound healing may be a consequence of pathologic states associated with diabetes, immune disorders, ischemia, venous stasis and injuries such as burn, frostbite, and gun-shot wounds[17].

A. aspera is one of the widely used plants in inflammatory condition in traditional medicine system of North East India. Our previous studies have shown anti-inflammatory and anti-bacterial activity of the methanol extract of the leaves of this plant. Studies on wound healing activity of *A. aspera* in different wound *viz.* burn, diabetic as well as immunocompromised

models showed reduction in wound area along with elevation of various enzymatic and non- enzymatic anti-oxidants like catalase, super oxide desmutase, reduced glutathione, hydroxy proline, ascorbic acid and protein when compared with the vehicle treated control group. Histopathological study of the granulation tissues in *A. aspera* treated animals also showed similar wound healing activity characterized by fibroblast proliferation, angiogenesis and formation of basement membrane showing collagen fibers in various wounds. On the basis of the above study, attempts were made to understand the mechanism involved at molecular level by expression and / or suppression of various proteins, which might assist in wound healing activity of the plant extract.

In the burn model, a unique similarity was observed in protein profile of *A. aspera* and Himax treated group. It is assumed that the mechanism by which *A. aspera* and Himax enhanced the healing process was probably similar. As mentioned above, protein with molecular weight 69.4 kD were expressed in both the treated groups indicating that this particular protein might be involved in the pro-healing activity. Similar obsertvations were reported in the *Hippophae rhamnoides* treated group[22], where granulation tissue also showed differential expression of some proteins as compared with the control group in burn wound model.

In diabetic wound model, there was distinct disparity in the protein profile of the both *A. aspera* and Himax treated groups suggesting both the plant extract as well as the standard drug have their own unique mechanism in healing wound tissues. Few proteins, namely 81.7, 78.0, 19.5 and <14.3 kD were expressed in all the three groups, that might play an important role in normal wound healing. Three characteristic proteins (61.0, 55.0 and 31.5kD) were expressed in *A aspera* treated group suggesting that these proteins play an important role in wound healing.

In immunocompromised wound model too, no similarity was observed in the protein profile of both *A. aspera* and Himax treated group, suggesting that they act by different mechanisms. In this model, a range of high, medium and low molecular weight proteins appeared exclusively in *A. aspera* treated group and might be responsible for healing.

While comparing the three protein profiles, it can be inferred that one characteristic protein of 31.5 kD appeared following treatment with 5% ointment of *A. aspera* extract in diabetic and immunocompromised wound models. For that reason, it may possibly be concluded that *A. aspera* induces expression of a particular protein of molecular weight of 31.5 kD and play an important role in wound healing.

Pascoe et al., 1987 and Chojkier et al., 1988 reported that increase in the level of ascorbic acid enhances the collagen gene expression[23,24]. Our earlier study showed that there was increase in the ascorbic acid level and the histopathological study revealed the formation of collagen fibers. Hence, it might be assumed that the proteins expressed in the healing tissues in *A. aspera* treated animals are associated with gene expression and metabolic cycle which lead to the

development of collagen and other such structural proteins resulting in augmented healing. Histopathological study showed increased angiogenesis in the plant extract treated group. Ferrara et al., 1999 reported that vascular endothelial growth factor (VEGF), an important pro-angiogenic cytokine improves angiogenesis during wound healing by stimulating the migration and proliferation of endothelial cells through the extra cellular matrix[25]. Expression of various proteins like VEGF might as well help in development of structural components related to anatomic continuity and function of skin tissue[25]. The most significant finding of this study was that one characteristic protein of 31.5 kD was invariably expressed in diabetic and immunocompromsied wound models. It signifies that the test drug helps the body to overcome deficit encountered in a diabetic and immunocompromised system and help to conitnue normal wound healing process. The study has been crried out for the first time which will help in unraveling the molecular mechanism involved in healing of tissues after treatment with *Achyranthes aspera* .

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REFERENCES

1. World Health Organisation. Summary of WHO guidelines for the assessment of herbal medicines. Herbal Gram, Volume 28, 1993, pp 13–14.
2. Sofowora, A., Medicinal Plants and Traditional Medicine. In: *Africa*, John Willey & Sons Ltd. New York, 1982, pp. 6.
3. Biswas, T. K., Maity, L. N. and Mukherjee, B., Wound healing potential of *Pterocarpus santalinus* Linn: a pharmacological evaluation. *Int. J. Low Ext. Wounds.*, 2004, **3,** 143–150.
4. Aziz, A., Rahman, M., Mondal, A. K., Muslim, T., Rahman, A. and Quader, A., 3-Acetoxy-6-benzoyloxyapagamide from *Achyranthes aspera*. *Pharma. J* ., 2005, **1**, 1816–1820.
5. Chakraborty, A., Brantner, A., Mukuinaka, T., Nobukuni, Y., Kuchido, M., and Konoshima, T., Cancer chemo preventive activity of *Achyranthes aspera* leaves on Epstein-Barr virus activation and two stage mouse skin carcinogenesis. *Cancer Letters*, 2002, **177**, 1–5.
6. Vetrichelvan, T. and Jegadeesan, M., Effect of alcohol extract of *Achyranthes aspera* Linn. on acute and sub-acute inflammation. *Phytother. Res.*, 2003, **17**, 77–79.
7. Sandhyakumar, K., Boby, R. G. and Indira, M., Impact of feeding ethanolic extracts of *Achyranthes aspera* Linn. on reproductive functions in male rats. *Indian J. Exp. Biol.*, 2002, **40**, 1307–1309.

8. Barua, C. C., Talukdar, A., Begum, S. A., Buragohain, B., Roy, J. D. Borah, R. S., Lahkar, M., Antidepressant like effects of *Achyranthes aspera* Linn in animals models of depression. *Pharmacologyonline,* 2009, **2,** 587-594.

9. Barua, C. C., Begum, S. A., Talukdar, A., Borah, P., Effect of *Achyranthes aspera* Linn on modified forced swimming in rats. *Pharmacologyonline,* 2010**,** 183-191.

10. Barua, C. C., Talukdar, A., Begum, S. A., Lahon, L. C., Sarma, D. K., Pathak, D. C. and Borah, P., Antinociceptive activity of methanolic extract of leaves of *Achyranthes aspera* Linn. *(*Amaranthaceae) in animal models of nociception. *Indian J. Exp. Biol.,* 2010, **48,** 817-821.

11. Barua, C. C., Begum, S. A., Talukdar, A., Buragohain, B., Roy, J.D., Pathak, D.C., Sarma, D.K. and Gupta, A., Wound healing activity of *Achyranthes aspera* Linn in immunocompromised rats. The 13th annual conference of ISWM under the aegis of "The Indian Society of wound management" and "Indian Association of surgical oncology",organized at Vardhman Mahabir Medical College, Safdarjang Hospital, New Delhi. 2010.

12. Guiardia, T., Juarez, A. O., Gerreiro, E., Guzman, J. A. and Pelzer, L., Anti-inflammatory activity and effect on gastric acid secretion of dehydroleucorcodine isolated from *Artemsia clocylasiana*. *J. Ethnopharmacol*., 2003, **88**, 195–198.

13. Upadhyay, N. K., Kumar, R., Mandotra, S. K., Meena, R. N. and Siddiqui, M. S. and Sawhney, R. C., Effects of Sea buckthorn (*Hippophae rhamnoides* L.) seed oil on dermal burn wounds. *Food Chem. Toxicol*., 2009, **47**, 1146–1153.

14. Gupta, A. and Raghubir, R., Energy metabolism in the granulation tissue of diabetic rats during cutaneous wound healing. *Mol. Cell Biochem.*, 2005, **270**, 71–77.

15. Gupta, A., Jain, G. K. and Raghubir, R., Time course study for the development of an immunocompromised wound model, using hydrocortisone., *J. Pharmacol. Toxicol.*, 1999, **41**, 183–187.

16. Laemmli, U. K., Cleavage of structural protein during the assembly of the head of the bacteriophage T_4. *Nature*, 1970, **227**, 680–685.

17. Gupta, A., Singh, R.L. and Raghubir, R., Antioxidant status during cutaneous wound healing in immunocompromised rats. *Mol. Cell. Biochem*., 2002, **241**, 1–7.

18. Lusby, P. E., Coombes, A. and Wilkinson, J. M., Honey: a potent agent for wound healing? *J. Wound Ostomy Continence Nurs*., 2002, **29**, 295–300.

19. Simon, A., Traynor, K., Santos, K., Blaser, G., Bode, U. and Molan, P., Medicinal honey for wound care still the 'latest resort'. *Evid. Comple. Alternat. Med.,* 2008, **5**, 475–479.

20. Scortichini, M. and Rossi, M., Preliminary in vitro evaluation of the antimicrobial activity of triterpenes and terpenoids towards *Erwinia amylovor*. *J. Bacteriol*., 1991, **71**, 109–112.

21. Nayak, B. S., Ramdath, D. D., Marshall, J. R., Isitor, G. N., Eversley, M., Xue, S. and Shi, J., Wound healing activity of the skin of the common grape (*Vitis vinifera*) Variant, Cabernet Sauvignon**.** *Phytother. Res.,* 2010, doi: 10.1002/ptr.2999.

22 Upadhyay, N. K., Kumar, R., Siddiqui, M. S. and Gupta A., Mechanism of Wound-healing activity of *Hippophae rhamnoides* L. leaf extract in experimental burns.doi:10.1093/ecam/nep189.

23. Pascoe, G. A., Fariss, M. V., Olafsdottir, K. and Reed, D. J., The role of vitamin E in protection against cell injury. Maintenance of intracellular glutathione precursors and biosynthesis. *Eur. J. Biochem*., 1987, **166**, 241–247.

24. Chojkier, M., Houglum, K., Herruzo, J. S. and Brenner, D. A., Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation. *J. Biol. Chem*., 1988, **263**, 2178–2186.

25. Ferrara, N., Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.,* 1999, **77**, 527–543.