Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2020 Apr 15; 8(A):297-301.
https://doi.org/10.3889/oamjms.2020.4292
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pharmacology*

Ethanolic Extract of *Nerium indicum* Mill. Decreases Transforming Growth Factor Beta-1 and Vascular Endothelial Growth Factor Expressions in Keloid Fibroblasts

Hernita Taurustya[1], Mae Sri Hartati Wahyuningsih[2,3]*, Indwiani Astuti[2]

[1]Department of Pharmacology and Therapy, Faculty of Medicine and Health Sciences, Universitas Bengkulu, Bengkulu, Indonesia; [2]Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia; [3]Center for Herbal Medicine, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Yogyakarta, Indonesia

Edited by: Sinisa Stojanoski
Citation: Taurustya H, Wahyuningsih MSH, Astuti I. Ethanolic Extract of *Nerium indicum* Mill. Decreases Transforming Growth Factor Beta-1 and Vascular Endothelial Growth Factor Expressions in Keloid Fibroblasts. Open Access Maced J Med Sci. 2020 Apr 15; 8(A):297-301. https://doi.org/10.3889/oamjms.2020.4292
Keywords: Keloid fibroblast; *Nerium indicum* Mill.; Oleandrin; Transforming growth factor beta-1; Vascular endothelial growth factor
***Correspondence:** Mae Sri Hartati Wahyuningsih, Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. Phone: +62 812 1569 484. E-mail: maeshw@ugm.ac.id
Received: 11-Jan-2020
Revised: 19-Feb-2020
Accepted: 21-Feb-2020

Funding: This research was supported financial by UGM DAMAS 2018 grant from Universitas Gadjah Mada, Yogyakarta
Competing Interests: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Keloid is a benign fibroproliferative dermis tumor characterized by an increase in growth factors which induce fibroblast proliferation, excessive migration, and synthesis of collagen. *Nerium indicum* Mill. extract had been studied as a keloid therapy agent. 5α-oleandrin contained in *N. indicum* has antikeloid activity by inhibiting keloid fibroblast proliferation, fibroblast migration, collagen deposition, and transforming growth factor beta-1 (TGF-β1) synthesis.

OBJECTIVE: This study aimed to determine the effect of administration of *N. indicum* extract on TGF-β1 and vascular endothelial growth factor (VEGF) expression in keloid fibroblast.

METHODS: This research was a quasi-experimental research with a post-test only control group design. The research subjects were fibroblast cells passage IV-VII isolated from patients' keloid tissue with explant techniques. Treatment groups received *N. indicum* extract with a serial concentration of 2 μg/ml, 1 μg/ml, and 0.5 μg/ml, and control group received medium only. The supernatant was obtained after 72 h incubation period. Examination of TGF-β1 and VEGF expressions was performed using ELISA procedure.

RESULT: The expression of TGF-β1 in the treatment groups of the extract *N. indicum* (2 μg/ml, 1 μg/ml, and 0.5 μg/ml) was significantly lower than a control group of keloid fibroblasts ($p < 0.05$), according to increased concentration. VEGF expression in the treatment groups of *N. indicum* extract was lower compared to the control group of keloid fibroblasts. A significant decrease in keloid fibroblast VEGF levels occurred at extract concentrations of 2 μg/ml and 1 μg/ml ($p < 0.05$).

CONCLUSION: *N. indicum* extract could decrease TGF-β1 and VEGF expressions compared to control medium in keloid fibroblast cultures.

Introduction

Keloid is a dermal fibroproliferation tumor characterized by excessive accumulation of extracellular matrix (ECM) components such as collagen, fibronectin, elastin, proteoglycans, and growth factors [1]. The occurrence of keloids is due to an imbalance between synthesis and degradation of ECM during the wound healing process [2]. In the pathogenesis of keloids, fibroblasts are the dominant cell and keloid fibroblasts have a greater proliferative ability than normal fibroblasts. Increased proliferation of keloid fibroblasts is influenced by increased growth factors such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), fibroblast growth factors, platelet-derived growth factor (PDGF), and cytokines' tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) [3]. Increased TGF-β1 will accelerate mitogenesis of fibroblasts, stimulating the formation of collagen, elastin, and fibronectin. Furthermore, VEGF induces angiogenesis directly through endothelial cell mitogenesis and lead increases vascular permeability and promotes deposition of extravascular fibrin matrix [4]. Keloid management is currently lacking satisfactory results. For example, keloid excision surgical therapy had a recurrence of 45–100% while non-surgical therapy had various side effects such as skin atrophy, telangiectasias, and pigmentation disorders [5]. Various studies were done using plants/herbs that have the potential as keloid therapy agents and milder side effects. *Nerium indicum* Mill. containing active ingredient Oleandrin has been studied potentially as a keloid therapy agent. Oleandrin cytotoxicity (IC_{50} 15.6 nM) was greater than doxorubicin (IC_{50} 26.9 nM) and cisplatin (IC_{50} 79.8 nM) on M19 cell line (melanoma) [6], [7]. 5α-oleandrin has antikeloid activity by inhibiting keloid fibroblast proliferation, fibroblast migration, collagen deposition, and TGF-β1 synthesis [8]. Ethanolic extract

N. indicum inhibits proliferation of keloid fibroblasts with IC_{50} 0.458 µg/ml, also can inhibit collagen deposit with IC_{50} 0.055 µg/ml at 72 h incubation period. In incubation 48 h after administration of ethanol extract of *N. indicum* 1 µg/ml, there was a significant inhibition of migration compared to the control medium [9]. Therefore, based on the above exposures, this study aimed to determine the molecular mechanism of *N. indicum* extract activity on the expressions of TGF-β1 and VEGF which play an important role in pathogenesis of keloid tumors.

Materials and Methods

Keloid fibroblast cultured cell

This research received approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada based on the Certificate of Ethical Eligibility number: KE/FK/0485/EC/2018. This research was a quasi-experimental research with post-test only control group design.

Keloid fibroblasts used were a subculture passage IV-VII, obtained from the Laboratory of Health Technology, Dermatology Venereology Division, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada. Materials used were *N. indicum* leaves collected from Sleman-Yogyakarta Special District of Indonesia on May 2018, identified at the laboratory plant systematics, and voucher specimen no: UGM/FA/4533/M/03/02 deposited in the Laboratory of Plant Systematics, Faculty of Pharmacy, Universitas Gadjah Mada. Additional materials included amphotericin B-Fungizone (Gibco™), Dulbecco's Modified Eagle's Medium Low Glucose (Gibco), dimethyl sulfoxide (DMSO) (Merck), MTT (Sigma), penicillin-streptomycin (Gibco BRL), phosphate buffer saline (Gibco), Povidone-iodine 10% (Gibco), and Trypsin EDTA 0.25% (Gibco).

Extraction of N. indicum

About 1 kg of dried powder from *N. indicum* leaves was macerated by ethanol (70%) (2 L). The mixture was stirred periodically for 24 h. The filtrate was separated by filtration (Buchner funnel), and maceration was repeated 3 times. The filtrates obtained were combined and evaporated *in vacuo* to dryness.

Tested concentration preparation

Ethanol extract of *N. indicum* as much as 5 mg was dissolved with 100 µL of DMSO to obtain a stock solution concentration of 50,000 µg/ml. The stock solution concentration was made into three serial levels: 0.5 µg/ml, 1 µg/ml, and 2 µg/ml.

The 24 well plate culture preparation

Cell suspension was counted based on a number of groups in the study in triplicate. Fibroblast cell culture was harvested, washed, and made into suspension. Cells (3×10^4 cells/well) were seeded into 24 well plates and further incubated with *N. indicum* extract (0.5; 1; and 2 µg/ml) and control medium for 72 h.

Measurement of TGF-β1 expression

The protocol of TGF-β1 measurement was performed according to the procedure issued by Koma Biotech Inc. as the manufacturer of the human TGF-β1 with Catalog No: K0332110.

Measurement of VEGF expressions

The protocol of VEGF measurement was performed according to the procedure issued by Koma Biotech Inc. as the manufacturer of the human VEGF with Catalog No: K0331132.

Statistical analysis

All data are expressed as means ± standard deviation (SD). Differences between control and experimental groups were analyzed by one-way ANOVA followed by Bonferonni's post-hoc test, with $p < 0.05$ considered as statistically significant. All calculations were performed using SPSS statistical software (IBM Corp., Chicago).

Results

TGF-β1 expression

The expressions of TGF-β1 statistically significance of *N. indicum* extract with various concentrations 0.5 µg/ml (9.27 ± 2.9 pg/ml), 1 µg/ml (9.26 ± 2.32 pg/ml), and 2 µg/ml (6.23 ± 2.02 pg/ml) were lower than control group (21.35 ± 5.03 pg/ml). The effect of the ethanolic extract of *N. indicum* on the expression of TGF-β1 in keloid fibroblasts is shown in Figure 1.

Figure 1 shows that TGF-β1 expression was significantly lower in the ethanolic extract *N. indicum* groups compared to those in keloid fibroblast without treatment, according to increased concentration ($p < 0.05$).

VEGF expression

The expression of VEGF supernatant in treatment groups of *N. indicum* extract with various concentration

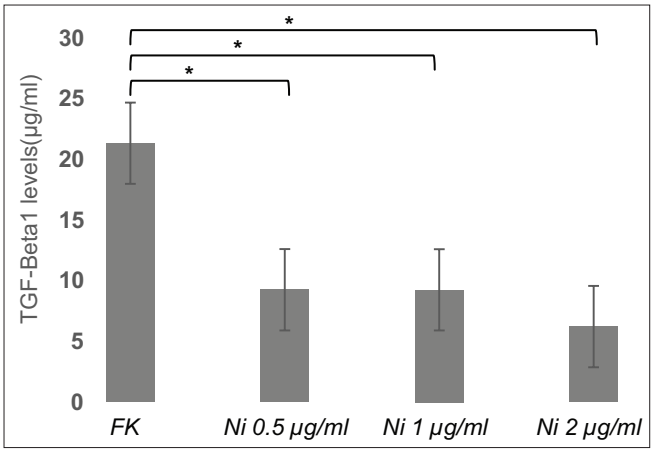

*Figure 1: Effect of Nerium indicum extract on keloid fibroblasts transforming growth factor beta-1 expression (mean ± standard deviation). Description: FK=Fibroblasts keloid, N. indicum extract concentrations 0.5 µg/ml; 1 µg/ml; 2 µg/ml. * p < 0.05 versus control FK*

0.5 µg/ml (38.31 ± 8.46 pg/ml), 1 µg/ml (28.57 ± 4.53 pg/ml), and 2 µg/ml (20.14 ± 1.5 pg/ml) were lower than control group (50.33 ±12.59 pg/ml).The effect of ethanolic extract of *N. indicum* on the expression of VEGF in keloid fibroblasts is shown in Figure 2.

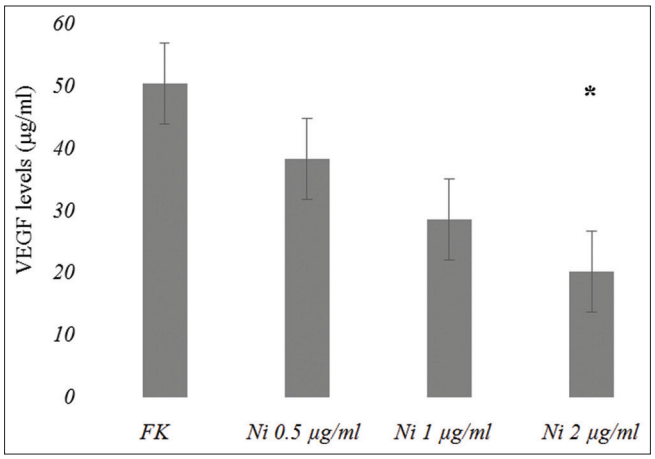

*Figure 2: Effect of Nerium indicum extract on keloid fibroblasts vascular endothelial growth factor expression (means ± standard deviation). Description: FK= Fibroblasts Keloid, N. indicum extract concentrations 0.5 µg/ml; 1 µg/ml; 2 µg/ml. *p < 0.05 versus control FK*

VEGF-A expression of keloid fibroblasts at the concentration of *N. indicum* 2 µg/ml and 1 µg/ml extract showed a significantly lower VEGF-A level compared to the medium control ($p < 0.05$).

Discussion

Our study was able to find lower expression in TGF-β1 and VEGF levels in *N. indicum* Mill. extract treatment groups compared to the control medium group, which showed the antikeloid effect of *N. indicum* ethanol extract. A significant decrease in keloid fibroblast TGF-β1 levels occurred at all of the *N. indicum* extract concentrations (2 µg/ml, 1 µg/ml, and 0.5 µg/ml) compared to those in the control group ($p < 0.05$). This is in accordance with previous studies by Dachlan (2019) that showed 5α-oleandrin isolates from ethanol extraction of *N. indicum* leaves significantly decreased TGF-β1 expression compared to control medium ($p < 0.05$) [8]. Increased TGF-β1 activity in fibroblast cells can enlarge the ability to stimulate tissue remodeling and the excessive process of wound healing causes keloid tissue. TGF-β1 plays an important role in pathogenesis of fibrosis because of its ability to initiate and maintain activation of keloid fibroblasts. The downstream pathway of TGF-β1 signaling is activated by Smad 2/3 protein which regulates increased proliferation in fibroblast cells as well as transcription factors that can activate genes which produce cytokines and TGF-β1 [10]. The presence of increased TGF-β1 in keloids can also induce transcription factor activator protein-1 (AP-1) which increases collagen type 1 production and extracellular protein synthesis [11]. In addition, there is also a 6/7 Smad protein which plays a role in Smad 2/3 activation termination (negative feedback). Protein inhibition of Smad 2/3 can suppress genes that produce TGF-β1, type I collagen synthesis, and decrease ECM deposition so that it can inhibit fibrosis [12], [13].

The active compound Oleandrin contained in *N. indicum* extract can inhibit activation of signal transduction such as AP-1 which is mediated through inhibition of JNK and MEK, thereby suppressing the genes that synthesize TGF-β1 growth factor [14]. There are obstacles of TGF-β1 signaling activity from the active compound Oleandrin contained in *N. indicum* extract which can be an indicator that supports keloid therapy. Mechanical transduction is also reported to affect cell function. On the surface of keloid tissue, there is often a higher mechanical emphasis than the surrounding tissue, and this is influenced by the expression of TGF-β1 which can regulate the expression of smooth muscle actin (SMA). TGF-β1 can increase cell rigidity through interactions between TGF-β1 receptors and SMA axis so that it can also be targeted for keloid therapy [15]. Recent research using TGF-β1 oligonucleotide can provide long-term effects in inhibiting fibrosis both *in vitro* and *in vivo.* Administration of anti-TGF-β1 antibodies in experimental animals was also reported to reduce fibrosis [16].

In this study, the VEGF-A expression of keloid fibroblasts was measured at the concentration of *N. indicum* 2 µg/ml and 1 µg/ml extract and showed a significantly lower level compared to the control medium ($p < 0.05$). This finding is in accordance with the research of Wahyuningsih *et al*. in 2018 that found ethanolic extract of *N. indicum* can inhibit the proliferation of keloid fibroblasts for a 48-h incubation period [8]. In another study, the administration of taginin

C isolates from plants significantly reduced VEGF-A expression in keloid fibroblast cultures compared to the control medium group ($p < 0.05$) [17].

Oleandrin compounds from *N. indicum* extract have a steroid chemical structure that can bind to glucocorticoid receptors (GR) on keloid fibroblast cells so that they can inhibit transcription factor signaling which induces genes' expression of VEGF-A. In addition, the lactone chemical structure which is also present in Oleandrin plays a role in signaling pathway receptor tyrosine kinase (VEGFR) through inhibition of extracellular regulated kinase (ERK) proteins so that VEGFR phosphorylation does not occur [18].

VEGF-A is an active protein homodimer that plays a role in angiogenesis. Angiogenesis occurs together with fibroplasia and is interdependent. Collagen and ECM when formed must always get oxygen and nutrients so that metabolic processes can occur [19]. Some of the most important mechanisms for regulating VEGF-A are hypoxia. The hypoxic condition will induce a hypoxia-inducible factor-1alpha (HIF-1α) to bind with VEGF receptors, triggering the tyrosine kinase pathway to angiogenesis [20]. Some cytokines and other growth factors can also increase VEGF-A expression. TNF-α is an inflammatory cytokine that has a strong biological activity to increase VEGF-A. Other cytokines such as IL-1 and IL-6 can also stimulate VEGF-A. Other growth factors such as TGF-β, EGF, and PDGF-BB can also induce VEGF-A [21].

In keloid tumors, there is an increased VEGF expression that binds to the VEGFR receptor, activating signaling pathways for angiogenesis and neovascularization of tyrosine kinase. Activation of VEGFR by VEGF-A causes autophosphorylation of tyrosine residues at the VEGFR receptors, thereby activating mitogen-activated protein-kinase (MAP-K) which causes cell proliferation and migration as well as activation of AKT/ERK proteins producing eNOS so that vascular permeability increases [22]. Fibrosis in keloid tissue is caused by increased VEGF-A phosphorylation with its receptors. Increased VEGF-A can occur exogenously and endogenously. Exogenous VEGF-A will increase the phosphorylation of VEGFR-A receptors, thereby increasing proliferation of keloid fibroblasts, while endogenous VEGF-A will increase the activation of transcription factors from VEGF-A and IGF gene expressions in keloid tissue fibroblasts [18], [19].

Conclusion

Based on the results of this research, it can be concluded that the ethanolic extract of *N. indicum* Mill. can decrease expressions of TGF-β1 and VEGF in keloid fibroblast cultures. Further research is necessary to examine the effect of *N. indicum* extract on Smad 2/3 and Smad 6/7 expressions, as well as the expression of AP-1 transcription factors in keloid fibroblasts to support the molecular mechanism of *N. indicum* extract as a keloid therapy agent.

References

1. Seo BF, Lee JY, Jung SN. Models of abnormal scarring. Biomed Res Int. 2013;2013:423147.
   PMid:24078916
2. Velnar T, Bailey T, Smrkolj V. The wound healing process: An overview of the cellular and molecular mechanisms. J Int Med Res. 2009;37(5):1528-42. https://doi.org/10.1177/147323000903700531
   PMid:19930861
3. Shih B, Garside E, McGrouther DA, Bayat A. Molecular dissection of abnormal wound healing processes resulting in keloid disease. Wound Repair Regen. 2010;18(2):139-53. https://doi.org/10.1111/j.1524-475x.2009.00553.x
   PMid:20002895
4. Chau CH, Clavijo CA, Deng HT, Zhang Q, Kim KJ, Qiu Y, *et al*. Etk/bmx mediates expression of stress-induced adaptive genes VEGF, Pai-1, and iNOS via multiple signaling cascades in different cell systems. Am J Physiol Cell Physiol. 2005;289(2):C444-54. https://doi.org/10.1152/ajpcell.00410.2004
   PMid:15788485
5. Slemp AE, Kirschner RE. Keloids and scars: A review of keloids and scars, their pathogenesis, risk factors, and management. Curr Opin Pediatr. 2006;18(4):396-402. https://doi.org/10.1097/01.mop.0000236389.41462.ef
   PMid:16914994
6. Wahyuningsih MS, Mubarika S, Bolhuis R, Nooter K, Gandjar IG, Wahyuono S. Cytotoxicity of oleandrin isolated from the leaves of *Nerium indicum* mill. On several human cancer cell lines. Indones J Pharm. 2004;15(2):96-103.
7. Mae SH, Sofia M, Bolhuis RL, Nooter K, Oostrum RG, Subagus W, *et al*. Selectivity of compounds isolated from the leaves of *Nerium indicum* mill. On various human cancer cell lines. Med J Malaysia. 2008;63 Suppl A:24-5.
   PMid:19024965
8. Dachlan I, Wirohadidjojo YW, Wahyuningsih MS, Aryandono T, Soebono H, Afandy D. The effect of 5α-oleandrin on keloid fibroblast activities. BMC Proc. 2019;13 Suppl 11:14. https://doi.org/10.1186/s12919-019-0177-6
   PMid:31890007
9. Wahyuningsih MS, Yuliani FS, Rahmawati DY, Pratiwi AN. Antifibrotic effect of ethanolic extract of *Nerium indicum* mill. Standardized 5α-oleandrin on keloid fibroblasts cells. Trad Med J. 2018;23(1):70-8. https://doi.org/10.22146/mot.35116
10. Tang M, Bian W, Cheng L, Zhang L, Jin R, Wang W, *et al*. Ginsenoside Rg3 inhibits keloid fibroblast proliferation, angiogenesis and collagen synthesis *in vitro* via the tgfβ/smad and erk signaling pathways. Int J Mol Med. 2018;41(3):1487-99. https://doi.org/10.3892/ijmm.2018.3362
    PMid:29328420
11. Gonçalves-de-Albuquerque CF, Silva AR, da Silva CI, Castro-Faria-Neto HC, Burth P. Na/k pump and beyond: Na/k-atpase as a modulator of apoptosis and autophagy. Molecules. 2017;22(4):578. https://doi.org/10.3390/molecules22040578
    PMid:28430151

12. Wang XQ, Lu SL, Mao ZG, Liu YK. Study on the biological function of vascular endothelial cells in the hypertrophic scar. Zhonghua Shao Shang Za Zhi. 2007;23(3):219-21.
PMid:18019066

13. Bran GM, Goessler UR, Schardt C, Hormann K, Riedel F, Sadick H. Effect of the abrogation of tgf-β1 by antisense oligonucleotides on the expression of tgf-β-isoforms and their receptors I and II in isolated fibroblasts from keloid scars. Int J Mol Med. 2010;25(6):915-21. https://doi.org/10.3892/ijmm_00000422
PMid:20428796

14. Manna SK, Sah NK, Newman RA, Cisneros A, Aggarwal BB. Oleandrin suppresses activation of nuclear transcription factor-κb, activator protein-1, and c-jun nh2-terminal kinase. Cancer Res. 2000;60(14):3838-47.
PMid:10919658

15. Lee CH, Hong CH, Chen YT, Chen YC, Shen MR. Tgf-beta1 increases cell rigidity by enhancing expression of smooth muscle actin: Keloid-derived fibroblasts as a model for cellular mechanics. J Dermatol Sci. 2012;67(3):173-80. https://doi.org/10.1016/j.jdermsci.2012.06.004
PMid:22771320

16. Viera MH, Vivas AC, Berman B. Update on keloid management: Clinical and basic science advances. Adv Wound Care. 2012;1(5):200-6. https://doi.org/10.1089/wound.2011.0313
PMid:24527306

17. Wahyuningsih MS, Nugrahaningsih DA, Budiyanto A. Ethanolic extract of *Tithonia diversifolia* (hemsley) a. Gray inhibits migration activity and degrease the transforming growth factor-beta1, VEGF expression on keloid fibroblasts. Asian J Pharm Clin Res. 2019;12(1):342-5. https://doi.org/10.22159/ajpcr.2019.v12i1.29850

18. Wu WS, Wang FS, Yang KD, Huang CC, Kuo YR. Dexamethasone induction of keloid regression through effective suppression of vegf expression and keloid fibroblast proliferation. J Invest Dermatol. 2006;126(6):1264-71. https://doi.org/10.1038/sj.jid.5700274
PMid:16575391

19. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. Vegf receptor signalling? In control of vascular function. Nat Rev Mol Cell Biol. 2006;7(5):359. https://doi.org/10.1038/nrm1911
PMid:16633338

20. Karamysheva AF. Mechanisms of angiogenesis. Biochemistry (Mosc). 2008;73(7):751.
PMid:18707583

21. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol. 2005;23(5):1011-27. https://doi.org/10.1200/jco.2005.06.081
PMid:15585754

22. Fearnley G, Smith G, Harrison M, Wheatcroft S, Tomlinson D, Ponnambalam SJ. Vascular endothelial growth factor-a regulation of blood vessel sprouting in health and disease. OA Biochemistry 2013;1(1):1. https://doi.org/10.13172/2052-9651-1-1-471