
Turmeric (*Curcuma longa*) Decoctum Had no Effect on Vascular Endothelial Growth Factor (VEGF) and Vascular Endothelial Cadherin (VE-cadherin) Expression in the Chick Embryo

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Abstract Turmeric (*Curcuma longa*) as a medicinal plant has widely consumed by pregnant women. The absence of dose standardization and the regulation of turmeric consumption in pregnancy raise concerns, especially if taken in early pregnancy. Angiogenesis is the process of new blood vessel formation from previously existing blood vessels, and plays an important role in embryogenesis and placentation during pregnancy. Angiogenesis is regulated by angiogenic molecules, such as VEGF and VE-cadherin. Turmeric has antiangiogenic effects in which the crude extract is pharmacologically more potent compared to the pure curcumin form. This study aimed to determine whether a turmeric decoctum affects the expression of VEGF and VE-cadherin in chick embryos. Turmeric was extracted by the decoction and freeze-dried methods to obtain turmeric decoctum powder. This was tested on embryonated chicken eggs, which were divided into four groups; control group (2% DMSO) and treatment groups receiving various doses of the turmeric decoctum (200 ppm, 300 ppm, and 400 ppm). The eggs used were less than 7 days after oviposition and incubated for 16 hours prior to injection of the treatment solution in ovo to the center of the yolk, followed by reincubation for up to 48 hours. Intraembryonic VEGF and VE-cadherin expression were assessed by whole-mount immunohistochemistry and quantified using image analysis techniques. This study showed administration of turmeric decoctum up to 400 ppm had no effect on the expression of VEGF and VE-cadherin in chick embryos.

Introduction

Turmeric (*Curcuma longa*) is a medicinal plant with multiple uses (Prasad & Aggarwal, 2011). Based on surveys, it is known that the users of herbal medicines are mostly women, and some of them are pregnant (Holst et al., 2009). Limited information about dose standardization and regulations regarding the consumption of herbal medicines during

pregnancy have gained significant attention in global health debates (Tilburt & Kaptchuk, 2008; Fakeye et al., 2009). In Indonesia, traditional herbal medicines, known by the term “jamu”, are made from many kinds of medicinal plants, including turmeric (Elfahmi et al., 2014). Turmeric preparations are usually made using the decoction method, in which the turmeric is thinly sliced, placed in boiling water and allowed

to boil for a period of time, after which it is ready for consumption.

Turmeric is a plant of Zingiberaceae family. There is quantitative variation in each component of turmeric compounds depending on the variety, origin, planting site, conditions during growth and the age at harvest. Several studies have shown significant effects of curcumin on vascularization and embryo development (Yue et al., 2015). Other studies have shown that the crude extract of turmeric has a greater antiangiogenic effect compared to the pure curcumin form (Li et al., 2011).

Angiogenesis is the process of blood vessel formation from pre-existing blood vessels (Ribatti and Crivellato, 2012). Angiogenesis is regulated by angiogenic molecules such as vascular endothelial growth factor (VEGF) and vascular endothelial cadherin (VE-cadherin) (Ferguson et al., 2005). The binding of VEGF to its receptor plays a critical role in vascular permeability, the migration and proliferation of endothelial cells, tube formation, and vascular lumen development (Ferrara & Kerbel, 2005). VE-cadherin as an adherent's junction protein, plays a role in maintaining polarity between endothelial cells and stabilizing blood vessels [16]. Abnormalities in angiogenesis could potentially cause a disruption in vascularization during embryonic growth and placentation and could even cause embryonic death (Ferguson et al., 2005).

Angiogenesis in the embryo can be studied using chick embryos (Ferguson et al., 2005). Chick embryos (*Gallus gallus*) are applicable as a model for studies of biological growth, embryology and teratology that precisely target specific developmental stages in the absence of maternal metabolism (Drake et al., 2006). After 48-56 hours of incubation, the chick embryo is comparable to 24 days of human gestation. This study aimed to determine whether a decoctum of turmeric affects VEGF and VE-cadherin expression in chick embryos.

Materials and methods

Animals

Fertile chicken eggs were purchased from the avian breeder group "Lestari Sejahtera" in Mojokerto, East Java, Indonesia. The eggs were stored in coolers set at approximately 15°C to 20°C for fewer than 7 days after oviposition and incubated at 37.5 °C to 39.5 °C up to stage 3+ or 16 h of incubation (Drake et al., 2006; Fassenko, 2007).

Herbal Preparation

Fresh turmeric rhizomes were purchased from UPT Materia Medica Batu, Health Department of East Java, Indonesia. An aqueous extract was prepared using the decoction method. A total of 500 g of turmeric rhizomes were thinly sliced, then placed in 2,500 mL of boiling distilled water and left to simmer for 15 minutes (Silva et al., 2012). The solution was allowed to cool, filtered through sterile muslin cloth and stored at -20 °C. A freeze-dried extract was obtained from the solution to produce a turmeric decoctum powder, which was stored at 4 °C (Tacouri et al., 2013).

Phytochemical Detection

Phytochemical detection was performed by a qualitative phytochemical test, thin layer chromatography (TLC) (Waksmundzka-Hajnos et al., 2008). TLC was conducted to test for both curcuminoid and terpenoid compounds in the turmeric decoctum using a standard protocol from the Pharmaceutical Laboratory, Pharmacy Study Program, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia.

Treatment Solutions

A stock solution (500 ppm) was made by dissolving 500 mg of turmeric decoctum powder into 2 % DMSO. The stock solution was used to prepare treatment solutions for at various doses, i.e. 200 ppm (P1), 300 ppm (P2) and 400 ppm (P3). The control group received 2 % DMSO without the turmeric decoctum powder.

Administration of Treatment Solutions on Chick Embryo

Treatment solutions (200 μ L) were injected into the center of the egg yolks after 16 h of incubation. Eggs were placed horizontally with respect to the long axis, and a small hole was made in the blunt end of each egg with the use of a probe. A 1-mL disposable syringe (23 gauge) was inserted horizontally and treatment solutions were delivered into the center of each egg yolk. After injection, the hole was sealed with vinyl tape and the egg was turned 180° and reincubated until 48 h of incubation (Drake *et.al.*, 2006).

Collecting Data

Each egg was cracked into a petri dish. The square frame of thick filter paper (Whatman 3MM) with external dimensions of about 1.4 x 1.4 cm and with an internal window of about 1 cm² was laid down onto the surface of the egg yolk such that the embryo was in the center of the window, and the filter paper was allowed to become wet. The vitelline membrane outside of the frame was cut with spring scissors and lifted gently using forceps, then washed in a dish of 0.9% NaCl solution to remove adherent yolk. The embryo was fixed in 4% neutral paraformaldehyde solution and stored at 4°C. The whole mount immunohistochemistry technique was used to detect VEGF expression and VE-cadherin expression in intraembryonic endothelial cells of the chick embryo using anti-VEGF antibody and anti-VE-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were observed under a stereomicroscope with a magnification of 20 x and images were captured by a Panasonic Lumix DMC-GH2 digital camera. The expression of VEGF and VE-cadherin was defined as mean density and was quantified using ImageJ software (Rahayu *et al.*, 2006).

Ethical Approval

All materials and methods for the experiments included in this study were

approved by the Ethical Committee of the Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia (262/EC/KEPK-S2/06/2016).

Statistical Analysis

Data are presented as mean \pm SD. Statistical analysis was performed using the IBM SPSS (version 24) statistical package. The relationships between turmeric decoctum and VEGF expression or VE-cadherin expression were assessed using Pearson correlations. The comparisons between VEGF expression or VE-cadherin expression in various treatment groups were analyzed using one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered to indicate statistical significance for the correlation and comparison.

Results and discussion

Phytochemical Detection

TLC identified curcuminoids in the turmeric decoctum, while terpenoids were not detected or were present in very small amounts.

Effect of The Turmeric Decoctum On VEGF and VE-Cadherin Expression

The results of the whole mount immunohistochemistry assay of VEGF expression are shown Figure 1 and those for VE-cadherin expression are depicted in Figure 2. Statistically, turmeric decoctum had no effect on VEGF or VE-cadherin expression in chick embryos (Table 1). Descriptively, our results show that all doses of the turmeric decoctum caused a decrease in VEGF or VE-cadherin expression. At the turmeric decoctum dose of 200 ppm, VEGF expression decreased, but VEGF expression tended to increase compared to the control group at the doses of 300 ppm and 400 ppm, as depicted in Figure 3. VE-cadherin expression increased with a turmeric decoctum dose of 200 ppm and decreased with turmeric decoctum doses of 300 and 400 ppm, as shown in Figure 3.

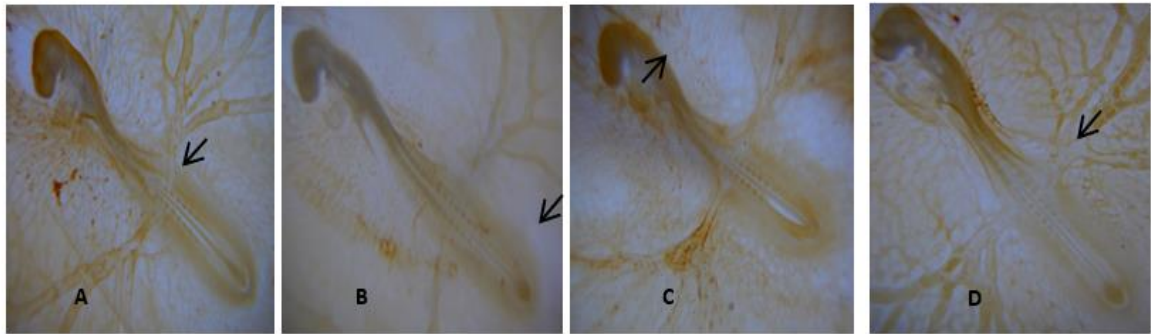


Figure 1. Whole mount immunohistochemistry using a specific antibody recognizing VEGF. VEGF expression in chick embryos (curved arrows) exposed to (A) 2% DMSO, (B) the turmeric decoctum at 200 ppm, (C) the turmeric decoctum at 300 ppm and (D) the turmeric decoctum at 400 ppm (whole mount IHC: A-D, 20x).

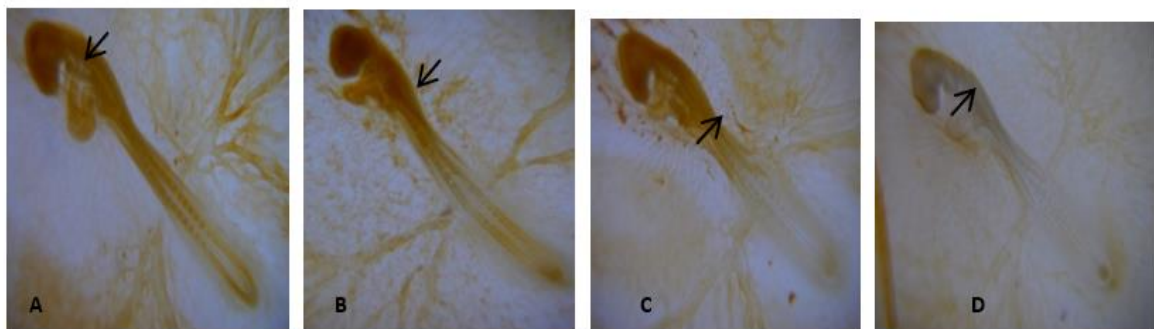


Figure 2. Whole mount immunohistochemistry using a specific antibody against VE-cadherin. VE-cadherin expression in chick embryos (curved arrows) exposed to (A) 2% DMSO, (B) the turmeric decoctum at 200 ppm, (C) the turmeric decoctum at 300 ppm and (D) the turmeric decoctum at 400 ppm (whole mount IHC: A-D, 20x).

Table 1. Number of VEGF and VE-cadherin expression of view of chick embryo specimens from the studied groups.

Variable	Control 2% DMSO	Turmeric decoctum 200 ppm	Turmeric decoctum 300 ppm	Turmeric decoctum 400 ppm	P-value	
					Pearson Correlations	ANOVA
VEGF expression	161.6±8.7	151.3±9.6	153.8±5.3	155.9±10.9	0.403	0.254
VE-cadherin expression	161.3±9.5	172.7±21.8	164.5±13.1	150.5 ±13.8	0.177	0.120

Data is represented as mean ±SD. DMSO: Dimethyl sulfoxide; ppm: part per million. Significant $p < 0.05$ for Pearson Correlation and ANOVA.

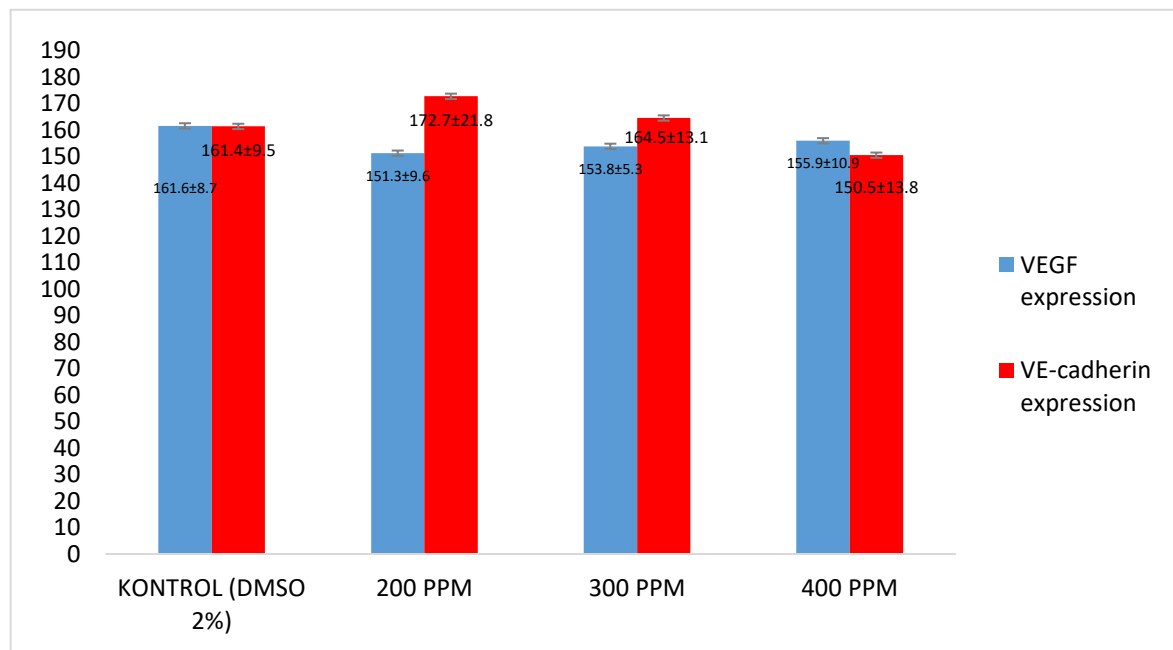


Figure 3. Trend of VEGF and VE-cadherin expression from the studied groups. Graph represents the average number of VEGF and VE-cadherin expression in each group. At the treatment of turmeric decoctum dose of 200 ppm, VEGF expression decrease, but VEGF expression tends to rise toward the control group at the dose of 300 ppm and 400 ppm. VE-cadherin expression increases in turmeric decoctum dose of 200 ppm group and decrease in 300 and 400 ppm.

Turmeric has antiangiogenic activity. The active curcuminoid compound, curcumin, is known to downregulate HIF-1 α and interrupt VEGF gene transcription, resulting in a decrease in VEGF expression (Bae et al., 2006). The results of this study show that VEGF expression was lower in all the turmeric decoctum treatment groups than in the control group. A decrease in VEGF expression is thought to restrain the migration and proliferation of endothelial cells, interrupt the formation of tubes and the vascular lumen, and disrupt vascular permeability, resulting in vascularization defects that interfere with embryo organogenesis (Ferrara & Kerbel, 2005). However, the decreased expression of VEGF in the treatment groups was not statistically significant, so these results need to be strengthened with data on macroscopic extraembryonic vascularization (not done).

Figure 3 shows that, after treatment with the turmeric decoctum at a dose of 200 ppm, VEGF expression decreases, but expression tended to

rise versus the control group at the doses of 300 ppm and 400 ppm. This finding indicates the existence of additional effects of curcumin, the predominant active compound in turmeric. The angiogenic effect of curcumin depends on the dose (Fan et al., 2014). With low-dose exposure (200 ppm), curcumin showed an antiangiogenic effect through the decreased expression of VEGF. However, at higher doses (doses of 300 ppm and 400 ppm), curcumin appeared to have other effects through a different pathway, and may have indirectly affected the expression of VEGF. It is known that the active compound of turmeric (curcumin) can affect various pathways (Aggarwal et al., 2005). In addition to being controlled by its transcription factor, VEGF expression levels are also indirectly influenced by transforming growth factor- β 1 (TGF- β 1), which can induce the transcription and expression of VEGF (Nam et al., 2010). Increased VEGF expression at the doses of 300 ppm and 400 ppm may be associated with a mechanism of action of

curcumin via pathways that induce the expression of TGF- β 1.

This study also showed that VE-cadherin expression in various groups formed a non-monotonic curve, i.e. there was an increase in VE-cadherin expression with a turmeric decoctum dose of 200 ppm and a decrease at the doses of 300 ppm and 400 ppm, as depicted in Figure 5. These results indicate the existence of a hormetic response. A hormetic response in a dose-response interaction may occur as a result of exposure to a substance that has stressor or potentially toxic effects (Reynolds, 2010). Some studies have demonstrated a relationship between angiogenesis and antiangiogenic agents with a hormetic response (Reynolds, 2010). Curcumin is known to induce a hormetic response at the cellular and molecular levels (Ali & Rattan, 2006).

VE-cadherin (cadherin-5, CD144) is the adhesion protein component of adherens junctions between endothelial cells, and plays an important role in regulating adhesion between endothelial cells, endothelial cell polarity and blood vessel stability (Giannotta et al., 2013; Harris & Nelson, 2010). Based on the expression levels of VE-cadherin in this study, a low dose of the turmeric decoctum (200 ppm) may induce stress in endothelial cells, resulting in a defense response to the external stressor by increasing the level of NF- κ B. With an increasing dose of the turmeric decoctum, this defense mechanism may no longer be able to compensate for the external stressor, resulting in decreased expression of VE-cadherin in the of 300 ppm and 400 ppm treatment groups.

The decreased expression of VE-cadherin at the 300 ppm and 400 ppm doses is in line with the results of a study by Aggarwal et al. (2006) showing that the antiangiogenic effects of turmeric, through the active curcuminoid component curcumin, result in decreased regulation and expression of adhesion molecules through the downregulation of NF-

κ B (Aggarwal et al., 2006). Interference in VE-cadherin expression will result in the disruption of adhesion between endothelial cells and endothelial cell polarity and thus destabilize blood vessels. If this condition continues, it will lead to vascular defects and potentially cause embryonic death.

The differences in VEGF and VE-cadherin expression after treatment with the turmeric decoctum were not statistically significant. These results are not in line with other studies that showed a significant antiangiogenic effect of turmeric. Especially when compared to the pure form of curcumin, the antiangiogenic effects of turmeric become pharmacologically more potent when given in a crude form of turmeric extract (Li et al., 2011). The non-significant effect of turmeric decoctum treatment on VEGF and VE-cadherin expression in chick embryos is thought to be influenced by several factors, including the extraction method used and the dose of turmeric decoctum given. The antiangiogenic effects of turmeric are caused by some components of the active compounds contained within it. Approximately 235 compounds, including phenolic and terpenoid compounds, have been identified in various parts of the turmeric plant (Li et al., 2011). There are quantitative variations in each component of turmeric, depending on the variety, origin, planting site, conditions during growth and the age at harvest^[10]. In addition, the extraction method and the solvent type also influence the composition and quality of the active compounds in turmeric that can be extracted (Li et al., 2011).

The antiangiogenic effects of turmeric are mainly mediated by active curcuminoid compounds. Curcuminoids have been identified as the most prevalent components of turmeric. Various extraction techniques and solvents can affect the amount and composition of curcuminoids extracted. According to Li et al. (2011), Soxhlet extraction with ethanol as the

solvent provides excellent curcuminoid extraction, i.e. up to 27% (w/w), while hydrodistillation can extract only 2.1% (w/w) curcuminoids (Li et al., 2011). Curcuminoids are poorly soluble in water, but soluble in ethanol, methanol, acetone and DMSO (Li et al., 2011). The limit of curcumin solubility in water is 0.29% (w/w) (Liu et al., 2008).

Other active compounds in turmeric which are thought to have antiangiogenic effects are essential oils (terpenoid compounds). When essential oils, especially the sesquiterpenoid group, work together with curcuminoid, this produces synergistic effects in bioactivity (Li et al., 2011). Aromatic turmerone (ar-turmerone) shows antiangiogenic effects through the inhibition of endothelial cell proliferation and motility, suppression of new blood vessel formation, and disruption of vascular tube formation, either in vitro or in vivo. Ar-turmerone is also able to increase curcumin transport into intestinal cells, such that curcumin absorption in the body is significantly increased (Yue et al., 2015).

It is known that the curcuminoid group is difficult to dissolve in water, and the terpenoid class (monoterpenoids and sesquiterpenoids) have similarly limited solubility in water; however, these compounds are soluble in ethanol (Ganora, 2011). The poor solubility of the active compounds of turmeric in water may have influenced the results of this study, particularly the absence of significant effects on VEGF and VE-cadherin expression in chick embryos following treatment with the turmeric decoctum.

This conclusion is supported by the TLC results on the freeze dried turmeric decoctum. The identified active compounds belonged to the curcuminoid group, while the essential oil compounds of the terpenoid group (monoterpenoids and sesquiterpenoids) were not detected or present in very small amounts. In relation to the active compound in turmeric

that exerts the antiangiogenic effect, if it was extracted in a small amount through the decoction method, as suspected in this study, it is likely that the amount of that active compound was too small to have a significant effect on VEGF and VE-cadherin expression in chick embryos.

Based on the results of this study, there was no effect of the turmeric decoctum on VEGF and VE-cadherin expression in chick embryos. However, it has not yet been determined whether turmeric decoctum are safe to take during pregnancy.

Conclusions

This study shows that the administration of turmeric decoctum up to a dose of 400 ppm had no effect on the expression of VEGF and VE-cadherin in chicken embryos.

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References

- Aggarwal, S., Ichikawa, H., Takada, Y., Sandur, S., Shisodia, S., Aggarwal, B. 2006. Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of I κ B α kinase and Akt activation. *Mol Pharmacol*, 69 (1): 195-206.
- Ali, R. and Rattan, S. 2006. Curcumin's biphasic hermetic response on proteasome activity and heat-shock protein synthesis in human keratinocytes. *Ann N. Y. Acad Sci.*, 1067: 394-399.
- Bae, M., Kim, S., Jeong, J., Lee, Y., Kim, H., Kim, S., et al. 2006. Curcumin inhibits hypoxia induced angiogenesis via

- downregulation of HIF-1. *Oncology reports.*, 15: 1557–1562.
- Drake V., Koprowski S., Lough J., Smith S. 2006. Gastrulating chick embryo as a model for evaluating teratogenicity : A comparison of three approaches. *Birth Defects Research (Part A): Clinical and Molecular Teratology*, 76: 66–71.
- Elfahmi, Woerdenbag H., Kayser O. 2014. Jamu: Indonesian traditional herbal medicine towards rational phytopharmacological use. *Journal of Herbal Medicine*, 4 (2): 51-73.
- Fakeye T., Adisa R., Musa I. 2009. Attitude and use of herbal medicines among pregnant Women in Nigeria. *BMC Complementary and Alternative Medicine*, 9: 53
- Fan, S., Xu, Y., Li, Xin., Tie, L., Pan, Y., Li, X. 2014. Opposite angiogenic outcome of curcumin against ischemia and Lewis lung cancer models: in silico, in vitro, and in vivo studies. *Biochimica et Biophysica Acta*, 1842: 1742-1754.
- Fasenko G. 2007. *Egg storage and the embryo*. Poultry Science, 86: 1020-1024.
- Ferguson J., Kelley R. W., Patterson C. 2005. Mechanisms of endothelial differentiation in embryonic vasculogenesis. *Arterioscler Thromb Vasc Biol.*, 25: 2246-2254.
- Ferrara N., Kerbel R. 2005. Angiogenesis as a therapeutic target. *Nature*, 438: 967-74.
- Ganora, L. *Solubility of herbal constituents*. 2011. NJ: Merck & Co., Inc, Whitehouse Station.
- Giannotta M., Trani M., Dejana E. 2013. Ve-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Dev Cel, Cell Press*, 26: 441-454.
- Harris, E., Nelson, W. 2010. VE-cadherin: at the front, center, and sides of endothelial cell organization and function. *Current Opinion in Cell Biology*, 22: 651-658.
- Holst L., Wright D., Haavik S., Nordeng H. 2009. The use and the user of herbal remedies during pregnancy. *J Altern Complement Med.*, 15 (7): 787-92.
- Jayaprakasha G. K., Rao L. J., Sakariah K. K. 2005. Chemistry and biological activities of *C. longa*. *Trends in Food Science and Technology*, 16: 533-548.
- Li S., Yuan W., Deng G., Wang P., Yang P., Aggarwal B. 2011. Chemical composition and product quality control of turmeric (*Curcuma longa* L.). *Pharmaceutical Crops*, 2: 28-54.
- Liu, D., Schwimer, J., Liu, Z., Woltering, E. 2008. Antiangiogenic effect of curcumin in pure versus in extract forms. *Pharmaceutical Biology*, 46 (10-11): 677-682.
- Nam, E., Park, S., Kim, P. 2010. TGF- β 1 induce mouse dendritic cells to express VEGF and its receptor (Flt-1) under hypoxic conditions. *Experimental and Molecular Medicine*, 42 (9): 606-613.
- Prasad S. Aggarwal B. 2011. Herbal medicine: biomolecular and clinical aspects. 2nd edition. In: Benzie. Wachte-Galor. Boca Raton. eds. Chapter 13: *turmeric, the golden spice from traditional medicine to modern medicine*. CRC Press Taylor & Francis Group.

- Rahayu, I., Djati, M., Indra, M. 2011. Hambatan EGCG terhadap ekspresi vascular endothelial growth factor (VEGF) dan ekspresi vascular-endothelial cadherin (VE-cadherin) dalam vaskulo-angiogenesis embrio ayam. *J Exp Live Sci*, 1 (2): 56-63.
- Reynolds, A. 2010. Potential relevance of bell-shaped and u-shaped dose-responses for the therapeutic targeting of angiogenesis in cancer. *Dose-Respon*s, 8: 253-284.
- Ribatti, D. and Crivellato, E. 2012. "Sprouting angiogenesis", a reappraisal. *Development Biology*, 372: 157-165.
- Silva R., Couto A., Bresolin T. 2012. Plant bioactives and drugs discovery: principles, practice, and perspectives. 4th Edition. In: Valdir Cechinel-Filho. *Chapter 10: Medical plants and pharmaceutical technology*. New Jersey, John Wiley & Sons, Inc.
- Tacouri D., Baboolall D., Puchooa D. 2013. In vitro bioactivity and phytochemical screening of selected spices used in Mauritian foods. *Asian Pacific Journal of Tropical Disease*, 3 (4): 253-261.
- Tilburt and Kaptchuk. 2008. *Herbal Medicine Research and Global Health: an Ethical Analysis*. *Bulletin of the World Health Organization*, 86: 594-599.
- Waksmundzka-Hajnos M., Sherma J., Kowalska T. 2008. *Thin layer chromatography in phytochemistry*. CRC Press Taylor & Francis Group.
- Yue G., Kwok H., Lee J., Jiang L., Chan K., Cheng L. et al. 2015. Novel anti-angiogenic effects of aromatic-turmerone, essential oil isolated from spice turmeric. *Journal of Functional Foods*, 15: 243-253.