Original Article

Chick Chorioallantois Membrane (CAM) Assay as an *In Vivo* Model to Study the Anti-angiogenesis and Anti-inflammatory activity of Nano-HSP

Ritika Chauhan, Gangasani Narasimha Reddy, Muniyasamy Muneeswari, Deivasigamani Arivukodi, Nagarajan Niveditha, Boopathy Usharani, Chandrasekar Shobana*

Department of Biochemistry, School of Life Sciences, Vels Institute of Sciences, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai
DOI: 10.47750/pnr.2022.13.S08.142

Abstract

Background: Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and was first described as an essential growth factor for vascular endothelial cells. VEGF is up-regulated in many tumours and its contribution to tumour angiogenesis is well defined. COX2 is an enzyme that speeds up the formation of substances that cause inflammation and pain. It may also cause tumour cells to grow. Some tumours have high levels of COX-2 and blocking its activity may reduce tumour growth. Also called cyclooxygenase-2 and prostaglandin-endoperoxide synthase 2. CD105 is a cell membrane glycoprotein mainly expressed on endothelial cells and overexpressed on tumour-associated vascular endothelium, which functions as an accessory component of the transforming growth factor -β receptor complex and is involved in vascular development and remodelling. VEGF, COX2 and CD105 biomarkers could represent a selective antiangiogenic and anticancer activity. Aim: To evaluate the impact of Hesperidin onto VEGF, COX2 and CD105 tumour cell marker on the rapidly growing capillary plexus of the chick embryo chorioallantois membrane (CAM). Materials and Methods: The in-ovo CAM assay was performed for the Hesperidin and Nano-HSP against humanized VEGF, COX2 and CD105 tumour cell marker. Results: Haemorrhagic damage was induced in the capillaries, which led to the early death of the embryos. Congestion leads to reduced blood flow out from tissues, which may be localized or systemic as seen in capillaries. Upon morphological staining, with haematoxylin and eosin stain, there was evidence of mild oedema, vascular disruption and extravasation of red blood cells in the chorion. Signs of vacuolization of the covering epithelium were also observed. Conclusion: Hesperidin blocking endogenous VEGF, COX2 and CD105 by chick embryo CAM assay might represent a valuable approach to impairing or inhibiting angiogenesis in steroidogenic-derived embryonic tissues. Therefore Hesperidin shows antiangiogenic and anti-inflammatory activity that is showing anti-VEGF, anti-COX2 and anti-CD105 antibody properties.

Keywords: Nanoformulated-Hesperidin, Nanotechnology, Angiogenesis, Inflammatory

1. INTRODUCTION

The existence of a broad well-associated microvascular blood vessel matrix is significant for the proper delivery of metabolites and the removal of waste products which is essential to carry-out tissue stability in the body. In the truancy of angiogenesis, contradictory effects such as oxygen deprivation and tissue death can occur [1,2]. The microvasculature is also important for assembling physiological responses including hormone responses and inter-organ communication, and injury responses including immune and inflammatory responses [3]. But it leads to big trauma when its presence leads to uncontrollable multiplication, therefore, leading to the formation of cancerous cells through the process of angiogenesis [4,5]. Cancer is the second foremost cause of death worldwide, just after cardiovascular diseases [6]. In fact, there were an estimated 18.1 million new cancer

cases and 9.6 million cancer deaths in 2018 around the world [7]. Cancerous growth depends on angiogenesis; so, targeting it could be a crucial step to arresting cancer growth and invasion into tissues [2]. Growing cancer requires a wide arrangement of vessels to flexibly supplement oxygen and invade distant organs [8].

Angiogenesis is the origination of newly developed blood vessels arising from pre-existing ones during embryo growth, inflammation, organogenesis and wound healing process[9]. It is an organised event, and the growth of new blood vessels is regulated by the delicate balance between several pro-angiogenic and anti-angiogenic growth factors. Pro-angiogenic growth factors which stimulate, platelet-derived growth factors (PDGFs), vascular endothelial growth factors (VEGF), transforming growth factor-α (TGF-α) [10], TGF-β [11], hepatocyte growth factor [12], and tumour necrosis factor- α (TNFα) basic fibroblast growth factor (b-FGF), hypoxia-inducible factor-1α (HIF-1α) [13], matrix metalloproteinase (MMP) action [14], or endothelial cell multiplication-movement [15]. However, inhibitors like thrombospondins (TSP) , angiostatin and endostatin [16] can lead to anti-angiogenic effects. The release of pro-angiogenic growth factors activates proteolytic enzymes to remodel the extracellular matrix (ECM) of blood vessels, leading to the sprouting and reorganisation of new blood vessels [17]. Unregulated angiogenesis has an impact on certain comorbidities like diabetic retinopathy [18], rheumatoid joint pain [19], psoriasis disease development [20], adolescent haemangiomas [21], chronic inflammation[22], atherosclerosis, ocular-related disorder, growth and metastasis of various cancer types[23]. There have been numerous studies concerning angiogenesis inhibitors. Sunitinib exhibited broad and potent antitumor activity by targeting the vascular endothelial growth factor receptor [24], with angiogenesis inhibitors, Marimastat and TNP-470, exhibiting antitumor activity in preclinical studies [25,26]. However, these inhibitors each have different limitations. A broad range of herbal products and bioactive compounds were proven as angiogenic inhibitors with extension to cancer research [27]. An urgent requirement is for a more safe and efficient system for screening angiogenesis and angiogenesis inhibitors for the treatment of various comorbidities. A few earlier studies, either in vivo or in vitro, recorded the anticancer competence of flavonoids.

Hesperidin (HSP) belongs to the flavanone family of bioflavonoids and it has a beneficial effect on blood vessels. HSP has a wide range of pharmacological activities, having influential anti-oxidant, anti-inflammatory, anti-atherosclerotic, cardioprotective, neuroprotective, anti-allergic, anti-viral, anti-microbial, and anti-cancer compounds[28,29]. Its role in protection against malignant transformation and progression has been described in multiple preclinical studies, acting through diverse cellular signalling pathways[30,31]. HSP can affect diverse molecular targets involved in the survival, division, and death mechanisms of tumour cells[32,33]. HSP modulate tumour ANG via VEGF, MMP, b-FGF, endothelial cell proliferation, migration and metastasis owing to their anti-angiogenic potential[34,35]. In 2009, anti-ANG potential of HSP in-vitro in Hep G2 human hepatocellular carcinoma cells where HSP inhibits cytosolic MMP-9 expression via decreasing AP-1, JNK signalling pathway and NF-j B signalling pathway) [36,37]. This was further supported by findings from other studies in 2010 which reported HSP inhibited 12-O- tetradecanoylphorbol-13-acetate (TPA)-induced cytosolic MMP-2 and MMP-9 and cyclooxygenase-2 (COX-2) expression via modulating NF-j B and AP-1 induced tumour cell invasion and metastasis in lung cancer and hepatocellular carcinoma [38,39]. In 2015, the anti-angiogenic effect of HSP in human umbilical vascular endothelial cells (HUVECS) and mouse embryonic stem cell (mES)- derived endothelial-like cells by blocking AKT and mTOR signalling pathways. In MCF-7 and HUVECs cells where HSP inhibited proliferation and capillaries formation in human breast cancer cells by suppressing NFATc3 (nuclear factor of activated T- cells) expression. In female BALB/c nude mice, a xenograft tumour model in vivo HSP inhibited tumour growth, by suppressing NFATc3, VEGF, and VEGFR2 expression via the NFAT signalling pathway[40]. Anti-ANG capability of HSP in pancreatic cancer by targeting MKK3/6 and p38 intracellular signalling pathways[41]., HSP inhibit tumour proliferation capability in A549 non-small cell lung cancer cells via targeting SDF-1a (stromal cell-derived factor 1) by suppressing CXCR-4 (C-X-C chemokine receptor type 4), p-Akt, p-Ij B (phosphorylated-I kappa B), and p-p65 expression (SDF-1/ CXCR-4 signalling cascade)[42]. HSP was also reported to inhibit angiogenesis in human osteosarcoma MG-63 cells via wound healing and matrigel assay and in vivoin male BALB/c xenograft mice model[43]. Due to HSP's high potency and lower side effects, it has a promising role in treating cancer and other disorders [44]. But its clinical usage is confined due to low solubility and less bioavailability. There is the urge to prevail in this matter by using nanotechnology[43]. Much work has been done with HSP-based nanoparticles to improve the bioavailability, absorption, and biodistribution of these flavonoids[44,45,46,47,48]. Further to develop new targeting strategies have developed HSP-coated solid lipid nanoparticles targeted delivery for the treatment of various dis- orders[49,50]. Evaluated Cerebro- protective

potential of hesperidin nanoparticles for the effective treatment of cerebral ischemia in rats[51]. To increase the drug delivery potential for topical applications, studied fabricating textile-based HSP-loaded nano capsules[52,53]. These fabric-based nanocapsules were found to be suitable for the sustained release of drugs. There is a failure of reports where HSP-coated nanoparticles were clinically tried for cancer treatment. Therefore, more progressive attempts are required to integrate hesperidin-based nanoparticles for the effective treatment of cancer and other diseases.

The chorioallantois membrane (CAM) assay is extensively popular *in ovo* experimental organisms appropriate for the replacement of other laboratory animals[54]. Diverse application for the CAM assay in the field of nanotoxicology. Several animal models were evolved, extending from zebrafish to mice, to examine the process of angiogenesis and to perceive the effects of pro-angiogenic and anti-angiogenic factors. It scrutinizes the angiogenic capacity of pristine factors and unharmed cells [55,56,57]. The in vivo CAM assay is a very useful and well-established method to determine the angiogenic, anti-angiogenic and anti-inflammatory effects of natural products like natural compounds or plant extracts[58]. The CAM is formed by the amalgamation of the mesodermal layers of two developmental structures; the Allantois and the Chorion of the avian embryo. At ~3.5 days of incubation, the allantois of the chick embryo appears [59]. A very rich vascular network develops in the chorioallantois membrane (CAM) from day 4 to 14 [60]. Multifarious growth factors (fibroblast growth factor-2, transforming growth factor-β and tumour necrosis factor-α) and protein kinase c were demonstrated to produce CAM angiogenesis[61-65]. The concept of a tissue-specific angiogenic pathway was developed following the characterization of a novel human endothelial cell mitogen, the vascular endothelial growth factor (VEGF), COX2 and CD105 which showed previously angiogenesis effects in various cell lines and animal models[66,67]. Targeting the respective receptors of VEGF, COX2 and CD105 induces the proliferation, migration and survival of endothelial cells under physiological and pathological conditions. Moreover, VEGF, COX2 and CD105 overexpression has been correlated with several types of tumour. Due to the low cost and time consumption of CAM assay, the present study aimed to evaluate the effects of the drug Hesperidin and Nano-HSP against VEGF, COX2 and CD105 tumour cell markers in the chick embryo CAM which have not been previously reported

2. MATERIAL AND METHODS

2.1 Samples and reagents required

Hesperidin (≥97.0% pure), and sodium dodecyl sulphate were obtained from Sigma-Aldrich. Ethanol (99% pure) was purchased from SRL laboratories Pvt. Ltd., and N-Hexane (95% pure) and dimethyl sulphoxide (DMSO, 99% pure) were purchased from SRL laboratories Pvt. Ltd.

2.2. Designing of Nano-formulated Hesperidin (NF-HSP)

The hesperidin was nano-formulated using the solvent evaporation method [68]. Briefly, ~4µm hesperidin was dissolved in ethanol to achieve a final concentration of 5 mg/ml. To the above solution, a known quantity of 0.2% sodium dodecyl sulphate solution (SDS) was added and stirred. Under constant stirring, hexane (antisolvent) was added to the mixture where the ratio of solvent and antisolvent was maintained at 1 : 20. Then, the mixture was continuously stirred for 3 h at 150 rpm and held at 30°C overnight. After complete evaporation of the solvent, the powder formed was crushed. It was subjected to various physiochemical characterization studies to determine the morphology and molecular vibrations in NF-HSP.

2.3. Characterization Techniques

The NF-HSP particle size distributions were determined using the dynamic light scattering (DLS) technique (Malvern Zetasizer, USA). The particle size distributions of the nano-formulated drugs were recorded by dissolving 100 µl of prepared nanoformulation into 900µl of solvent. Fourier transforms infrared (FTIR) spectroscopy was used with attenuated total reflection mode to elucidate the molecular vibrations that exist in the pristine and nano-formulated drugs. An adequate quantity of samples was placed on the crystal surface, and the spectra of NF-HSP were recorded in the range 4000 cm^{-1}–500 cm^{-1} (Model BRUKER-ALPHA, Germany) using OPUS software. FTIR spectroscopy characterizes the characteristic functional groups present in the test samples originating from the different molecular bond's unique vibration and rotational energies.

2.4. Solubility test of Nano-formulated Hesperidin

Hesperidin is insoluble in water in its pristine form. After nano-formulation, the drug become water-soluble and was validated through the solubility test using Milli-Q water. A solubility test was performed by suspending 1mg of NF-HSP in 10 ml of Milli-Q water and comparing it with its pristine counterpart [68].

2.5. CAM Assay preparation

The effect of HSP and Nano-HSP on angiogenesis was evaluated using the CAM assay. Devising of the chick embryo's chorioallantois membrane was performed according to the procedure formerly reported by Ribatti *et al* [69].

The fertilized chicken eggs were kept previously in humidified egg incubator for 72 h at 37°C and a relative humidity of 80%. The eggs were placed into a parallel position and rotated several times to prevent attachment of the embryo to the eggshell. After 3 days of incubation, a 1 cm^2 window was carefully created on the broad side of the egg to assess the extent of embryonic blood vessels. The normal development was verified, and embryos with malformations or dead embryos were excluded. Then, about 2 mL of albumen was aspirated from each egg through the small window to lower the embryo and CAM away from the eggshell. After removal of albumen, covered with Parafilm and the egg specimens were re-incubated for the next four days. From incubation day 7, one group (n=3) was used as a control and was treated with distilled water, whereas the other two groups (n=6, 3 specimens for each two groups) were treated with 10μM HSP and 10μM Nano-HSP were directly placed on the small window created before by membrane dialysis method. For every test compound, 3eggs were utilized. All samples were tested in triplicate at different times.

2.6. Macroscopic view of CAM assay

Treated and control CAM specimens were macroscopically monitored at 24 hours i.e. on day 8 of incubation, using a Zeiss Stemi DV4 SPOT stereomicroscope equipped with a Sony Cybershot camera and blood vessels were photographed. The antiangiogenic effects of HSP and Nano-HSP on the CAMs were quantified, mean vessel area as a percentage of the total area, mean vessel length and mean a number of branch points which were marked using Wimasis software[70].

2.7. Haematoxylin and Eosin Staining view of CAM assay

Due to the low survival rate of the treated specimens and the rapid appearance of vascular changes, membrane specimens were collected on day 9 of incubation, fixed in formalin for 24 h and were then paraffin-embedded. Five-micrometre serial sections were made from each specimen. The haematoxylin-eosin stain was used for the morphological study of the collected chick embryo CAMs.

2.8. Immuno-histochemical view of CAM assay

Additional sections underwent for immunohistochemical staining. Cut 3mm sections on charged slides and incubate at 60-70°C for 1 hour. Deparaffinize by 2 changes of xylene for 5 minutes each. Hydrate through descending grades of alcohol as follows: a) Absolute alcohol – two changes five minutes each; b) 90% alcohol – 5 minutes; c) 70% alcohol – 5 minute; d) Running water – 5 minutes, and Antigen retrieval for 15-20 minutes in MERS. The pH of the retrieval buffer may be either 6, 8 or 9.5 according to the marker. Wash in running water for 5 minutes. Wash in PBS/TBS for 2 minutes. Do endogenous peroxidase blocking by adding H2O2 on the section, and keep for 5 minutes. Wash in the wash buffer for 2 minutes, twice. Add primary VEGF or COX2 or CD105 antibodies and keep for 30 minutes in a moist chamber. Then wash in wash buffer 2 times, 2 minutes each. Add Polyexcel Target binder and keep for 15 minutes. Wash in two changes of buffer, 2 minutes each. Add Polyexcel HRP and incubate for 15 minutes. Wash with buffer, 2 minutes 2 changes. Add working DAB Chromogen and keep it for 2-5 minutes, then wash in distilled water. Counterstain with haematoxylin for 30 seconds, and wash with water. Dehydrate, clear and mount as usual.

3. RESULTS

1. Designing of Nano-formulated Hesperidin

The hesperidin compound was nano-formulated through the solvent evaporation method and subjected to DLS and FTIR characterization studies.

2. Solubility test of Nano-formulated Hesperidin and HSP

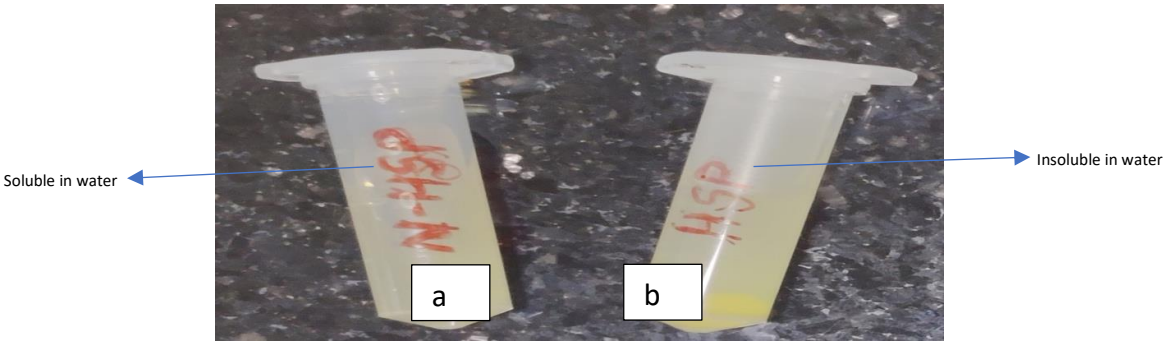
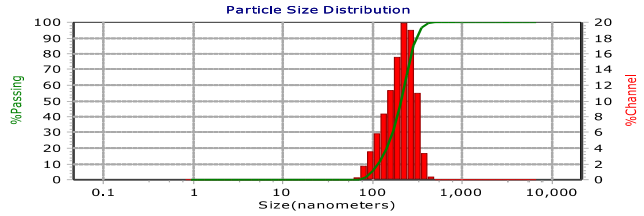


Figure 1. Solubility test of a) Nano-formulated Hesperidin and b) HSP with distilled water

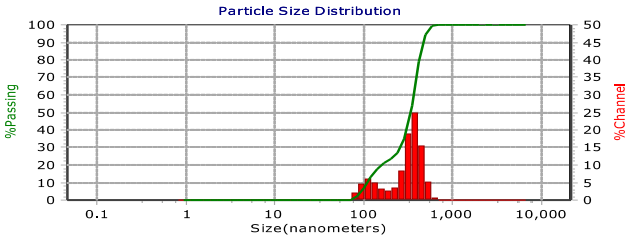
The solubility of NF-HSP prepared through the solvent evaporation method was evaluated by dispersing it in Milli-Q water. When nano-formulated HSP drug was added to the water, it was easily distributed, ensuring a hydrophilic nature (Figure 1(a)). In contrast, when pristine hesperidin was added to water, the drug was found insoluble. It was sedimented in of the water, validating the hydrophobic nature of the drug (Figure 1(b)). Therefore, it was substantiated that the nanoformulation converts the wetting behaviour of the drug from hydrophobic to hydrophilic in nature.

3. Characterization studies of Nano-formulated Hesperidin

To confirm the nano arrangement of prepared NF-HSP compared to the micron-sized pristine drugs, DLS measurements were recorded. The nano-formulated drugs were ultra- sonicated for 1h before recording DLS measurement. The average particle size distributions of normal hesperidin and NF-HSP was found as 687 nm and 486 nm, respectively, having a polydispersity index (PDI) of 0.2239(hesperidin) and 0.1256 (NF-HSP) (Figures 2(a) and 4(b)).



(a)



(b)

Figure 2: DLS measurements of a)HSP and b)Nano-HSP

The FTIR spectra of the pristine HSP and synthesized NF-HSP were shown in Figures 3 and 4. The spectrum of pristine hesperidin exhibited bands at 3539cm^{-1} and 3413 cm^{-1}, confirming the presence of the O-H stretch. The aromatic groups and C-O, C-H, C=C, and C=O were observed at 437 cm^{-1},586 cm^{-1},611 cm^{-1},741 cm^{-1},876 cm^{-1},911 cm^{-1},1065 cm^{-1},1181 cm^{-1},1274 cm^{-1},1358 cm^{-1},1443 cm^{-1},1517 cm^{-1},1644 cm^{-1},1986 cm^{-1} cm^{-1},2321 cm^{-1} and 2919 cm^{-1} respectively. The FTIR spectrum of NF-HSP also shows similar peaks at 438 cm^{-1},586 cm^{-1},611 cm^{-1},741 cm^{-1},876 cm^{-1},911 cm^{-1},1065 cm^{-1},1182 cm^{-1},1274 cm^{-1},1358 cm^{-1},1443 cm^{-1},1645 cm^{-}

^{1},1984 cm^{-1},2323 cm^{-1} and 2918cm^{-1} ascribed to the aromatic groups and C-O, C-H, C=C, and C=O functional groups, respectively. Even though similar bands were observed in the spectrum of both pure hesperidin and NF-HSP, the transmittance intensity originated from NF-HSP was lower than the pristine form. This phenomenon was due to the presence of amorphous constituents in the prepared NF-HSP. The presence of amorphous constituents improves the solubility of the drug in water than pristine hesperidin. Moreover, the peak obtained at 2850 cm^{-1} ascribed to C-H stretching vibration originating from the long-chain C of SDS substantiates that the SDS molecules were absorbed with hesperidin while nanoformulation.

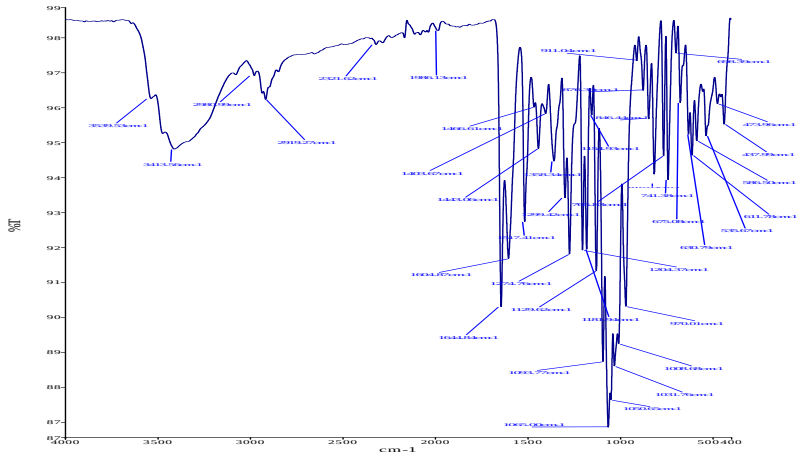


Figure 3. FTIR-HSP

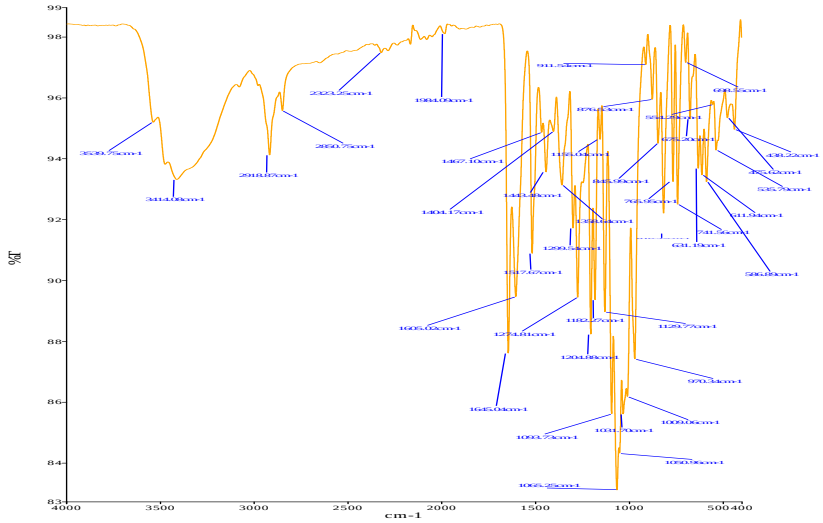
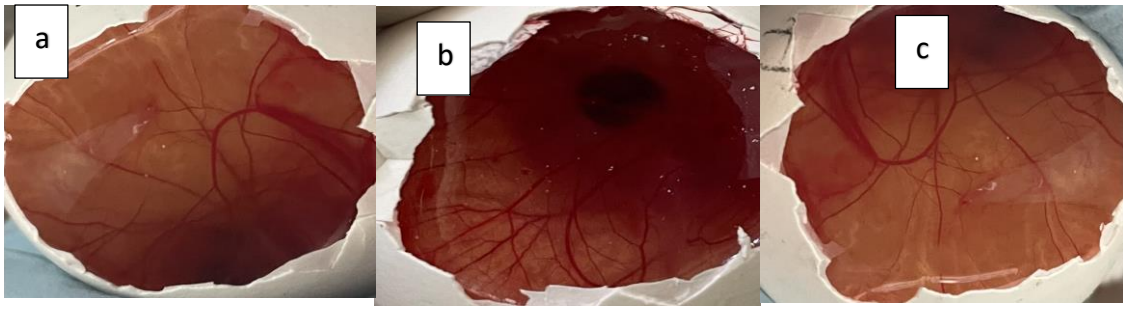
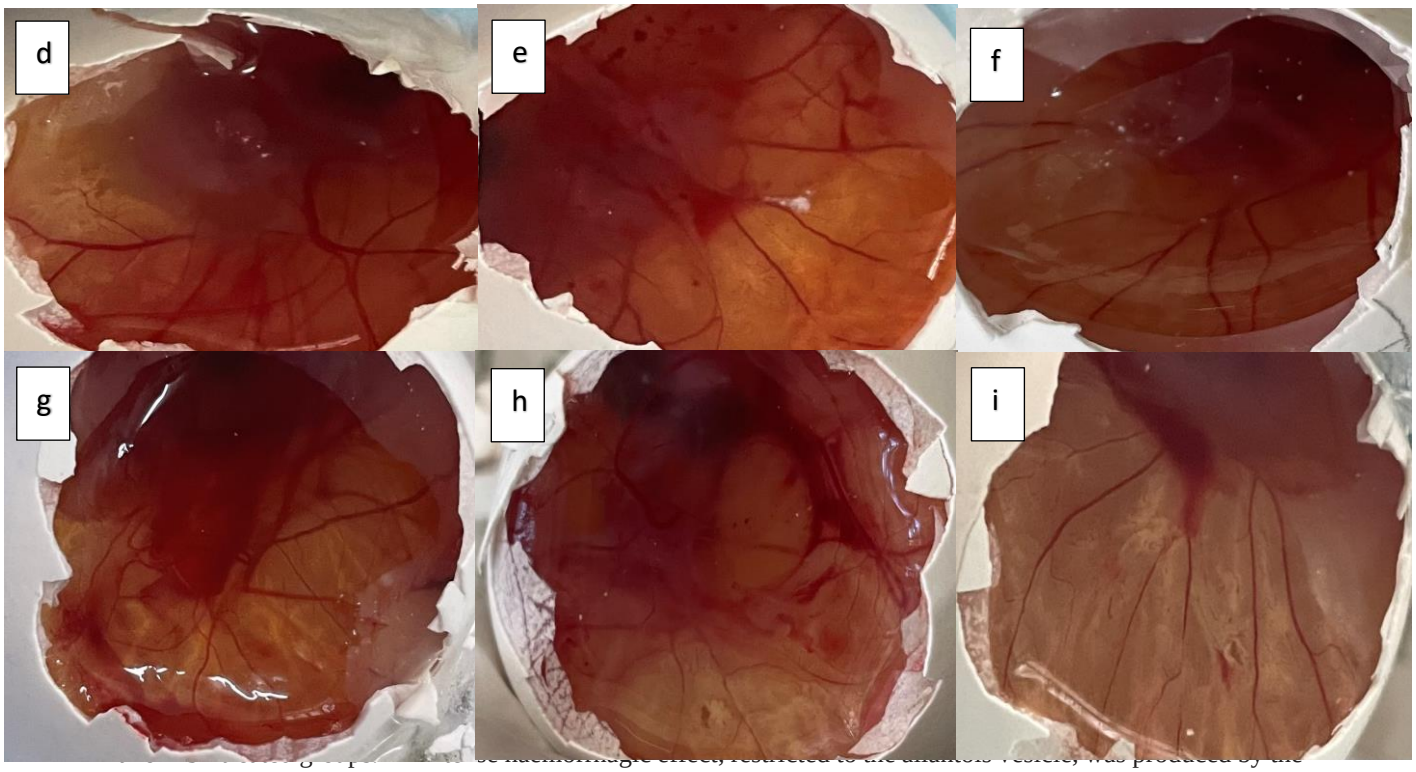


Figure 4. FTIR- Nano HSP

4. Macroscopic view of CAM on incubation day 8, one day after application of HSP and Nano HSP





normal HSP (Figure 5d,5e,5f), while the highest seen by Nano-HSP produced vascular changes extensive to the whole CAM surface, of chick embryos (Figure 5g,5h,5i)

5. Hematoxylin-eosin staining of CAM on incubation day 9, 2 days after application of HSP and Nano HSP against endocrine gland related VEGF, COX2 and CD105 tumour markers.

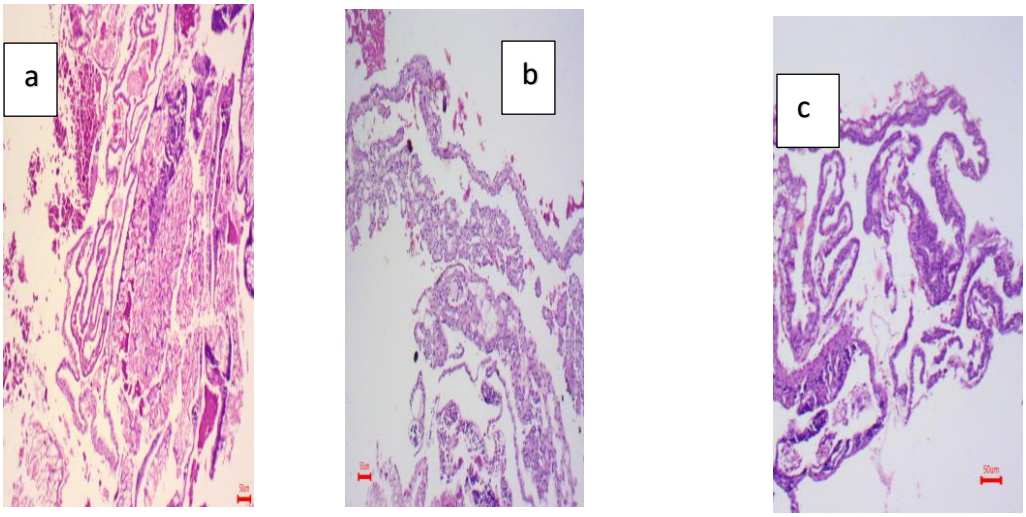


Figure 5. a: Control; b: HSP, c: Nano-HSP; ×10 magnification.

Evaluation of microscopic specimens confirmed the macroscopic observations. On haematoxylin-eosin-stained CAM samples treated with HSP and Nano-HSP, we observed mild oedema, vascular disruption and extravasation of red blood cells in the chorion. Signs of vacuolization of the covering epithelium, a high rate of extravasation of red blood cells in the mesenchymal tissue of the CAM, dilated medium and large vessels and were also observed (Figures 6b and 6c). Significant differences in vessel density between treated and control groups (Figure 6a), were registered.

6. Immuno-histochemical view of CAM assay- Immunohistochemical (IHC) staining of CAM on incubation day 9, two days after application of antiangiogenic drug HSP and Nano HSP against VEGF, COX2 and CD105 tumors markers.

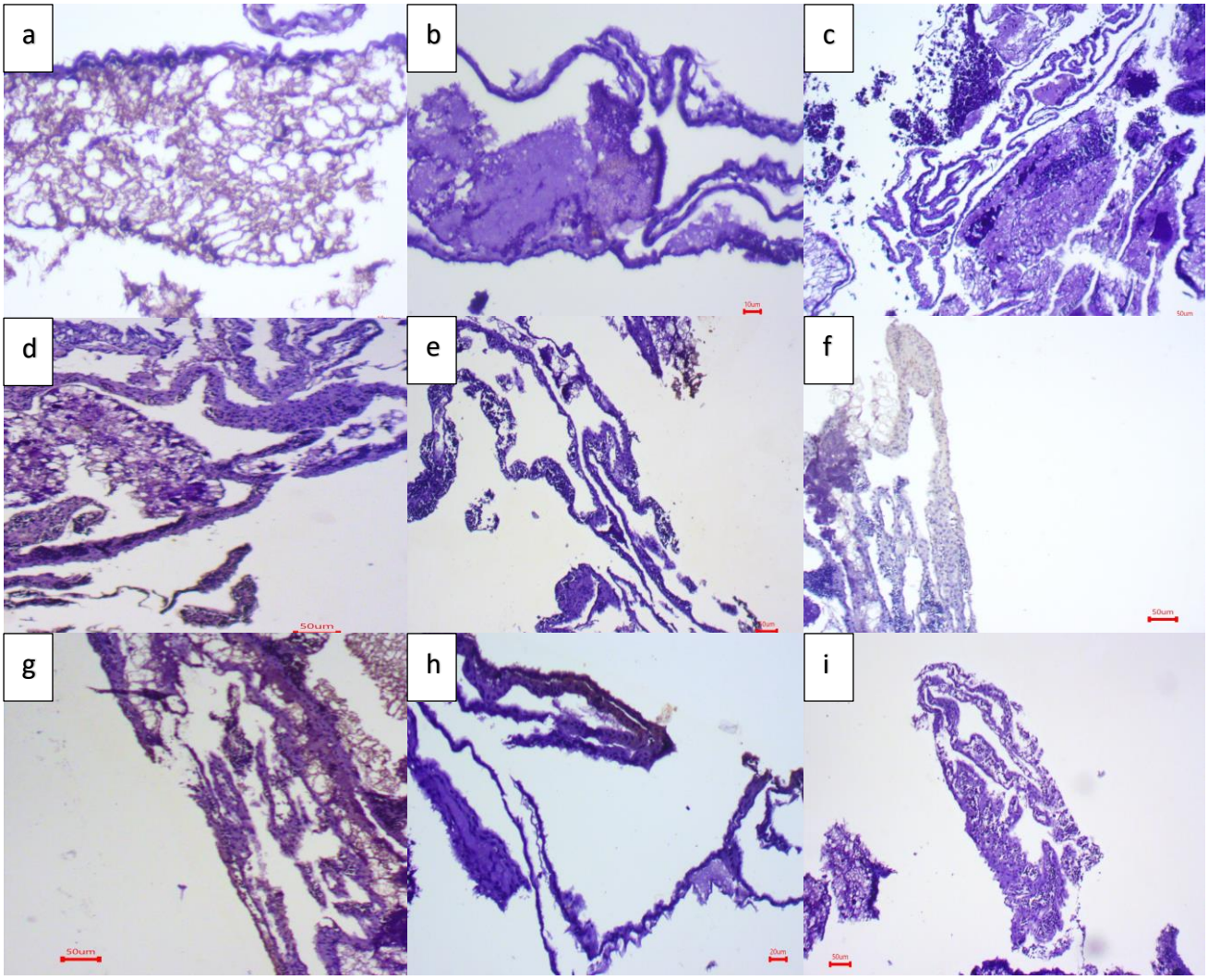


Figure 7. Immunohistochemical (IHC) staining of chorioallantois membranes (CAMs) on incubation day 9, two days after application of HSP and Nano HSP against VEGF, COX2 and CD105.. a: Control VEGF; b: Control COX2, c: Control CD105; d: HSP-VEGF; e: HSP-COX2; f: HSP-CD105; g: Nano-HSP-VEGF; h: Nano HSP-COX2; i: Nano HSP- CD105; magnification ×10.

Immunohistochemical reaction revealed a discontinuous pattern seen more in the endothelium vessels from CAM, treated with Nano-HSP in comparison with normal HSP. The effect of the HSP and Nano-HSP on VEGF, COX2 and CD105 was not restricted to the vascular endothelium. Chorionic epithelial cells were modified by the action of the HSP and Nano-HSP against VEGF, COX2 and CD105 tumour cell marker, and more by Nano-HSP. Epithelial cells had many vacuoles in the cytoplasm and the adjacent stroma presented was denser using the Nano formulated HSP (Figure 7g, 7h and 7i), compared with the normal mesenchyme from the control group (Figure 7a, 7b and 7c) and normal HSP group(Figure 7d, 7e and 7f). Staining for the proliferation was found to be slightly positive in HSP-treated epithelial cells of the chorion and less proliferation was seen in the Nano-HSP treated epithelial cells from the main vessels and capillaries exposed to the VEGF, COX2 and CD105 tumour cell marker (Figure 7d, 7e and 7f) (Figure 7g, 7h and 7i). This switching gives the impression of being linked to the oppression

of condensed stromal blood vessels which was liable to become assembled vascular bundle-like structure without cavity or impairment of breakage of endothelial cell connections (Figure 7g, 7h and 7i). The VEGF, COX2 and CD105 expression was observed scattered in both normal HSP and more scattered with Nano-HSP.

DISCUSSION

VEGF, COX2 and CD105 biomarkers are intricated in several biological processes, including angiogenesis[71]. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and was first described as an essential growth factor for vascular endothelial cells. VEGF is up-regulated in many tumours and its contribution to tumour angiogenesis is well defined[72,73]. COX2 is an enzyme that speeds up the formation of substances that cause inflammation and pain. It may also cause tumour cells to grow. Some tumours have high levels of COX-2 and blocking its activity may reduce tumour growth. Also called cyclooxygenase-2 and prostaglandin-endoperoxide synthase 2[74]. CD105 is a cell membrane glycoprotein mainly expressed on endothelial cells and overexpressed on tumour-associated vascular endothelium, which functions as an accessory component of the transforming growth factor -β receptor complex and is involved in vascular development and remodelling[75,76]. VEGF, COX2 and CD105 biomarkers could represent a selective anticancer and also anti-inflammatory strategy. The effects of VEGF, COX2 and CD105 were extensively studied on human tissue specimens. In birds, the CAM is similar to placental structures in humans and has the same function. Despite this, no data about the effects of Nano-formulated HSP on VEGF, COX2 and CD105 on chick embryo CAM have been previously reported and thus, to our knowledge, no data on the role of VEGF, COX2 and CD105 exist in an avian form. For this reason, and, given the fact that there is a 30% homology between the human and avian genomes[77,78]. we chose the chick embryo CAM as an experimental model for testing the effects of bioactive compound HSP and Nano-HSP against human angiogenic growth factors VEGF, COX2 and CD105 *in vivo*.

Hesperidin is insoluble in water and soluble in organic solvents like DMSO and Sodium CMC[79]. It has been studied that impoverished aqueous soluble drugs displayed good solubility through nanoformulation. Previously reported improved solubility of curcumin in water through nanoformulation[80]. The activity was improved due to a reduction in particle size resulting in target drug delivery as bioavailability was improved. In the present study, the size of hesperidin was reduced significantly through the solvent evaporation method. Obtained results show a one-fold reduction. In the present research work, nano-formulated drugs displayed better solubility in water than in their pristine forms.

In the CAM assay, compounds demonstrate an angiogenic function in the form of increased vessel density around the graft, with the vessels radially meeting towards the centre [81]. An inhibitory function of the tested compound is observed when the quantity and density of vessels decrease and eventually disappear around the graft[82]. The instrument and device required for the CAM assay are easily obtainable. Experimental time was reduced with this method, and the protocol and results were simple and better. By this method, initially testing compounds with angiogenic and angiogenic inhibitory activity is feasible and effective.

In this study, we demonstrated that the treatment with angiogenic growth factors VEGF, COX2 and CD105 produced different effects on the main vessels and capillaries of the chick embryo CAM. After treatment with angiogenic growth factors VEGF, COX2 and CD105, in the treated group main vessels became dilated but preserved their structure, whereas the chick embryo CAM capillaries showed endothelial breakage with discontinuities through the intima and massive haemorrhage into the chorion. There was a lack of proliferative endothelial cell activity, which is usually found in the normal chick embryo CAM vessels at this developmental stage, upon treatment with HSP and Nano HSP, whereas more a lack of proliferation activity with Nano HSP. Based on our observation concerning changes found in the chorioallantois stroma and epithelium, we can hypothesize that an avian endogenous VEGF, COX2 and CD105 exist in the avian embryo and acts not only on endothelial cells but also on other epithelial cell types and connective tissue components. Chick embryo CAM is an avian steroidogenic structure [83] and this study showed that it is highly sensitive to blockade with VEGF, COX2 and CD105 angiogenic growth factors in a dose-dependent manner.

Several macroscopic changes were observed compared to control i.e., evidence of vascular disruption, spot-like hemorrhagic areas were observed between modified capillaries, and both main vessels and capillaries had irregular shapes with pronounced dilation and severe stasis. Morphology of chick embryo CAM upon H and E staining we observed hyperemic areas of vascular plexus, extravasation of red blood cells in the chorion and signs of vacuolization of the covering epithelium were observed. With HSP treatment intense hemorrhagic effect

is restricted to allantois vesicle. With Nano HSP treatment produced vascular changes extensive to the whole CAM surface as in the embryos. Upon Immuno-histochemical staining, we observe that with compare to normal HSP, Nano HSP treated CAM, has a reduced number of blood vessels and inflammation due to reduced expression of VEGF, COX2 and CD105. This suggests the presence of an endogenous avian type of VEGF, COX2 and CD105 involved in the development of both, CAM and the embryonic vascular network. Moreover, changes caused by nano-hesperidin more onto VEGF, COX2 and CD105 of avian tissues suggest a high homology between human and avian VEGF, COX2 and CD105. We have explored that nano-hesperidin affects the inhibition of angiogenesis growth factors and inflammatory mediators VEGF, COX2 and CD105 in chick embryo CAM more efficiently than normal Hesperidin.

CONCLUSION

In vivo Chick embryo CAM is a good tool for studying angiogenic bioactive compound HSP and Nano-HSP activity against VEGF, COX2 and CD105 biomarkers and their inhibitory effects. Therefore, Nano HSP shows good anti-inflammatory and anti-angiogenesis effects due to target drug delivery. Hesperidin blocking endogenous VEGF, COX2 and CD105 biomarkers by chick embryo CAM assay might represent a valuable approach to impairing or inhibiting angiogenesis in steroidogenic-derived embryonic tissues. Therefore Nano-Hesperidin shows effective antiangiogenic and anti-inflammatory activity that is showing anti-VEGF, anti-COX2 and anti-CD105 antibody properties. Further studies are required to characterise avian VEGF, COX2 and CD105 its receptors and the mechanisms of action in this experimental model. Characterization of an avian endogenous VEGF, COX2 and CD105 and its corresponding inhibitors would improve the use of chick embryo chorioallantoic membrane as a model for targeting the VEGF, COX2 and CD105 pathways in normal and tumour angiogenesis.

ACKNOWLEDGEMENT

The authors would like to thank Prof. Dr. S. Gowtham Kumar and the management of Chettinad Academy of Research and Education (Deemed to be University) for providing facilities to perform this study. This research was supported by the UGC-CSIR Life Sciences, Government of India, New Delhi.

REFERENCE

1. Giaccia, A.J.; Simon, M.C.; Johnson, R. The biology of hypoxia: The role of oxygen sensing in development, normal function, and disease. *Genes Dev*. **2004**, *18*, 2183–2194. [CrossRef]
2. Grace, P. Ischaemia-reperfusion injury. *Br. J. Surg.* **1994**, *81*, 637–647. [CrossRef]
3. AS, M.N.; Deshpande, R.; Kale, V.P.; Bhonde, R.R.; Datar, S.P. Establishment of an in ovo chick embryo yolk sac membrane (YSM) assay for pilot screening of potential angiogenic and anti-angiogenic agents. *Cell Biol. Int.* **2018**, *42*, 1474–1483. [CrossRef] [PubMed]
4. Ribatti, D.; Crivellato, E. "Sprouting angiogenesis", a reappraisal. *Dev. Biol.* **2012**, *372*, 157–165. [CrossRef] [PubMed]
5. Ausprunk, D.; Knighton, D.; Folkman, J. Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Role of host and preexisting graft blood vessels. *Am. J. Pathol.* **1975**, *79*, 597. [PubMed]
6. Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. J Thorac Dis 2017;9:448–51
7. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mor- tality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424
8. Hanahan D et al (1998) A flanking attack on cancer. Nat Med. 4(1):13–14. https://doi.org/10.1038/nm0198-013
9. Auerbach, R.; Lewis, R.; Shinners, B.; Kubai, L.; Akhtar, N. Angiogenesis assays: A critical overview. *Clin. Chem.* **2003**, *49*, 32–40. [CrossRef] [PubMed]
10. Montesano, R.; Vassalli, J.-D.; Baird, A.; Guillemin, R.; Orci, L. Basic fibroblast growth factor induces angiogenesis in vitro. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 7297–7301. [CrossRef] [PubMed]
11. Baum, O.; Suter, F.; Gerber, B.; Tschanz, S.A.; Buergy, R.; Blank, F.; Hlushchuk, R.; Djonov, V. VEGF-A Promotes Intussusceptive Angiogenesis in the Developing Chicken Chorioallantoic Membrane. *Microcirculation* **2010**, *17*, 447–457. [CrossRef] [PubMed]
12. Leker, R.R.; Toth, Z.E.; Shahar, T.; Cassiani-Ingoni, R.; Szalayova, I.; Key, S.; Bratincsák, A.; Mezey, E. Transforming growth factor α induces angiogenesis and neurogenesis following stroke. *Neuroscience* **2009**, *163*, 233–243. [CrossRef]
13. Yang, E.Y.; Moses, H.L. Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *J. Cell Biol.* **1990**, *111*, 731–741. [CrossRef]
14. Bussolino, F.; Di Renzo, M.F.; Ziche, M.; Bocchietto, E.; Olivero, M.; Naldini, L.; Gaudino, G.; Tamagnone, L.; Coffer, A.; Comoglio, P. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.* **1992**, *119*, 629–641. [CrossRef] [PubMed]
15. O'Reilly, M.S.; Boehm, T.; Shing, Y.; Fukai, N.; Vasios, G.; Lane, W.S.; Flynn, E.; Birkhead, J.R.; Olsen, B.R.; Folkman, J. Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell* **1997**, *88*, 277–285. [CrossRef]
16. Watanabe, K.; Hasegawa, Y.; Yamashita, H.; Shimizu, K.; Ding, Y.; Abe, M.; Ohta, H.; Imagawa, K.; Hojo, K.; Maki, H. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J. Clin. Investig.* **2004**, *114*, 898–907. [CrossRef]
17. Deryugina, E.I.; Quigley, J.P. Chick embryo chorioallantoic membrane models to quantify angiogenesis induced by inflammatory and tumor cells or purified effector molecules. *Methods Enzymol.* **2008**, *444*, 21–41.

18. Folkman J et al (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. 1(1):27–31. https://doi.org/10.1038/nm0195-27 63.
19. Koch AE et al (1998) Review: angiogenesis: implications for rheumatoid arthritis. Arthritis Rheum 41(6):951–962. https://doi.org/10.1002/1529-0131 (199806)41:6<951::AID-ART2>3.0.CO;2-D 64.
20. Ferrara N, Alitalo K et al (1999) Clinical applications of angiogenic growth factors and their inhibitors. Nat Med. 5(12):1359–1364. https://doi.org/10.103 65. 8/70928
21. Ferrara N et al (2004) Vascular endothelial growth factor: basic science and clinical progress, Endocr. Rev. 25(4):581–611
22. Powell J et al (1999) Update on hemangiomas and vascular malformations. 66. Curr Opin Pediatr. 11(5):457–463. https://doi.org/10.1097/00008480-19991 0000-00015
23. Hanahan D et al (1998) A flanking attack on cancer. Nat Med. 4(1):13–14. https://doi.org/10.1038/nm0198-013
24. Mendel DB, Laird AD, Xin X, *et al*: In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacody- namic relationship. Clin Cancer Res 9: 327-337, 2003.
25. Toppmeyer DL, Gounder M, Much J, *et al*: A phase I and phar- macologic study of the combination of marimastat and paclitaxel in patients with advanced malignancy. Med Sci Monit 9: PI99-PI104, 2003.
26. Herbst RS: Targeted therapy using novel agents in the treatment of non-small-cell lung cancer. Clin Lung Cancer 3 (Suppl 1): S30-S38, 2002.
27. Ichikawa H, Nakamura Y, Kashiwada Y, Aggarwal BB. (2007). Anticancer drugs designed by mother nature: Ancient drugs but modern targets. Curr Pharm Design 13:3400–16.
28. BarrecaD,GattusoG,BelloccoE,CalderaroA,TrombettaD,Smeriglio A, Lagana G, Daglia M. Meneghini S, Nabavi SM. Flavanones: citrus phytochemical with health-promoting properties. Biofactors 2017;43:495–506
29. Ahmadi A, Shadboorestan A. Oxidative stress and cancer; the role of hesperidin, a citrus natural bioflavonoid, as a cancer chemoprotective agent. Nutr Cancer 2016;68:29–39
30. Li C, Schluesener H. Health-promoting effects of the citrus flavanone hesperidin. Crit Rev Food Sci Nutr 2017;57:613–31
31. Aishatwi AA, Ramesh E, Periasamy VS, Subash-Babu P. The apoptotic effect of hesperetin on human cervical cancer cells is mediated through cell cycle arrest, death receptor, and mitochondrial pathways. Fundam Clin Pharmacol 2013;27:581–92
32. Roohbakhsh A, Parhiz H, Soltani F, Rezaee R, Iranshahi M. Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases. Life Sci 2015;124:64–74
33. Ahmadi A, Shadboorestan A, Nabavi SF, Setzer WN, Nabavi SM. The role of hesperidin in cell signal transduction pathway for the preven- tion or treatment of cancer. Curr Med Chem 2015;22:3462–71
34. Shanmugam MK, Warrier S, Kumar AP, Sethi G, Arfuso F. Potential role of natural compounds as anti-angiogenic agents in cancer. Curr Vasc Pharmacol 2017;15:503–19
35. Siveen KS, Ahn KS, Ong TH, Shanmugam MK, Li F, Yap WN, Kumar AP, Fong CW, Terganokar V, Hui KM, Sethi G. Y-tocotrienol inhibits angiogenesis-dependent growth of human hepatocellular carcinoma through abrogation of AKT/mTOR pathway in an orthotopic mouse model. Oncotarget 2014;5:1897–911
36. Kamaraj S, Anandakumar P, Jagan S, Ramakrishnan G, Devaki T. Modulatory effect of hesperidin on benzo(a)pyrene induced experi- mental lung carcinogenesis with reference to COX-2, MMP-2 and MMP-9. Eur J Pharmacol 2010;649:320–7
37. Lee KH, Yeh MH, Kao ST, Hung CM, Liu CJ, Huang YY, Yeh CC. The inhibitory effect of hesperidin on tumor cell invasiveness occurs via suppression of activator protein 1 and nuclear factor-kappaB in human hepatocellular carcinoma cells. Toxicol Lett 2010;194:42–9
38. YangY,WolframJ,ShenH,FangX,FerrariM.Hesperetin:aninhibitor of the transforming growth factor-beta (TGF-beta) signaling pathway. Eur J Med Chem 2012;58:390–5
39. Kim GD. Hesperidin inhibits vascular formation by blocking the AKT/mTOR signaling pathways. Prev Nutr Food Sci 2015;20:221–9
40. ZhaoX,LiuJ,FengL,GeS,YangS,ChenC,LiX,PengL,MuY,Wang Y, Gu D, Guo Y, Lin G, Deng B, Cheng Z, Cai D. Anti-angiogenic effects of qingdu granule on breast cancer through inhibiting NFAT signaling pathway. J Ethnopharmacol 2018;222:261–9
41. Lee J, Lee J, Kim M, Kim JH. Fermented extraction of citrus unshiu peel inhibits viability and migration of human pancreatic cancers. J Med Food 2018;21:5–12
42. XiaR,XuG,HuangY,ShengX,XuX,LuH.Hesperidinsuppressesthe migration and invasion of non-small cell lung cancer cells by inhibit- ing the SDF-1/CXCR-4 pathway. Life Sci 2018;201:111–20
43. Du GY, He SW, Zhang L, Sun CX, Mi LD, Sun ZG. Hesperidin exhibits in vitro and in vivo antitumor effects in human osteosarcoma MG-63 cells and xenograft mice models via inhibition of cell migration and invasion, cell cycle arrest and induction of mitochondrial-mediated apoptosis. Oncol Lett 2018;16:6299–306
44. Siddiqui IA, Sanna V. Impact of nanotechnology on the delivery of natural products for cancer prevention and therapy. Mol Nutr Food Res 2016;60:1330–41
45. 101. Wang S, Zhang J, Chen M, Wang Y. Delivering flavonoids into solid tumors using nanotechnologies. Expert Opin Drug Deliv 2013;10:1411–28
46. 102. Majumdar S, Srirangam R. Solubility, stability, physicochemical char- acteristics and in vitro ocular tissue permeability of hesperidin: a nat- ural bioflavonoid. Pharm Res 2009;26:1217–25
47. 103. Duranoglu D, Uzunoglu D, Mansuroglu B, Arasoglu T, Derman S. Synthesis of hesperetin-loaded PLGA nanoparticles by two different experimental design methods and biological evaluation of optimized nanoparticles. Nanotechnology 2018;29:395603
48. 104. Nie S, Xing Y, Kim GJ, Simons JW. Nanotechnology applications in cancer. Annu Rev Biomed Eng 2007;9:257–88
49. 105. Merisko-Liversidge E, Liversidge GG, Cooper ER. Nanosizing: a for- mulation approach for poorly-water-soluble compounds. Eur J Pharm Sci 2003;18:113–20

50. 106. Gu SF, Wang LY, Tian YJ, Zhou ZX, Tang JB, Liu XR, Jiang HP, Shen YQ. Enhanced water solubility, antioxidant activity, and oral absorp- tion of hesperetin by D-alpha-tocopheryl polyethylene glycol 1000 succinate and phosphatidylcholine. J Zhejiang Univ Sci B 2019;20:273–81
51. 107. Ansar S, Abudawood M, Alaraj ASA, Hamed SS. Hesperidin allevi- ates zinc oxide nanoparticle induced hepatotoxicity and oxidative stress. BMC Pharmacol Toxicol 2018;19:65
52. 108. Ferrari PC, Correia MK, Somer A, Ribeiro MA, Astrath NGC, Sato F, Novatski A. Hesperidin-loaded solid lipid nanoparticles: develop- ment and physicochemical properties evaluation. J Nanosci Nanotechnol 2019;19:4747–57
53. 109. Praveen Kumar P, Sunil Kumar KT, Kavya Nainita M, Sai Tarun A, Raghu Ramdu BG, Deepika K, Pramoda A, Yasmeen C. Cerebroprotective potential of hesperidin nanoparticles against bilat- eral common carotid artery occlusion reperfusion injury in rats and in silico approaches. Neurotox Res 2019; doi: 10.1007/s12640-019-00098-8. [Epub ahead of print]
54. Hasan, J.; Shnyder, S.D.; Bibby, M.; Double, J.A.; Bicknel, R.; Jayson, G.C. Quantitative angiogenesis assays in vivo—A review. *Angiogenesis* **2004**, *7*, 1–16. [CrossRef] [PubMed]
55. Howell, M.G.; Grandis, J.R. Molecular mediators of metastasis in head and neck squamous cell carcinoma. *Head Neck* **2005**, *27*, 710–717. [CrossRef] [PubMed]
56. Nicoli, S.; Presta, M. The zebrafish/tumor xenograft angiogenesis assay. *Nat. Protoc.* **2007**, *2*, 2918–2923. [CrossRef] [PubMed]
57. Norrby, K. In vivo models of angiogenesis. *J. Cell. Mol. Med.* **2006**, *10*, 588–612. [CrossRef] [PubMed]
58. Passaniti, A.; Taylor, R.M.; Pili, R.; Guo, Y.; Long, P.V.; Haney, J.A.; Pauly, R.R.; Grant, D.S.; Martin, G.R. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab. Investig.* **1992**, *67*, 519–528. [PubMed]
59. Ribatti D, Nico B, Vacca A, Roncali L, Burri PH and Djonov V: Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat Rec 264: 317-324, 2001.
60. Ausprunk DH, Knighton DR and Folkman J: Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol 38: 237-248, 1974.
61. Ribatti D, Vacca A, Roncali L and Dammacco F: The chick embryo chorioallantoic membrane as a model for in vivo research on angiogenesis. Int J Dev Biol 40: 1189-1197, 1996.
62. Wilting J, Christ B and Bokeloh M: A modified chorioallantoic membrane (CAM) assay for qualitative and quantitative study of growth factors. Studies on the effects of carriers, PBS, angio- genin, and bFGF. Anat Embryol (Berl) 183: 259-271, 1991.
63. Olivo M, Bhardwaj R, Schulze-Osthoff K, Sorg C, Jacob HJ and Flamme I: A comparative study on the effects of tumor necrosis factor-alpha (TNF-alpha), human angiogenic factor (h-AF) and basic fibroblast growth factor (bFGF) on the chorioallantoic membrane of the chick embryo. Anat Rec 234: 105-115, 1992.
64. Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V and Nuseir N: Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. Nature 329: 630-632, 1987.
65. Leene W, Duyzings MJ and van Steeg C: Lymphoid stem cell identification in the developing thymus and bursa of Fabricius of the chick. Z Zellforsch Mikrosk Anat 136: 521-533, 1973.
66. Jakob W, Jentzsch KD, Mauersberger B and Heder G: The chick embryo choriallantoic membrane as a bioassay for angiogenesis factors: reactions induced by carrier materials. Exp Pathol (Jena) 15: 241-249, 1978.
67. Knighton DR, Fiegel VD and Phillips GD: The assay of angio- genesis. Prog Clin Biol Res 365: 291-299, 1991.
68. Y. Santhosh Kumar, Langeswaran Kulanthaivel, G. S. Hikku, R. Saravanan, Thangavelu Lakshmi, and Gowtham Kumar Subbaraj: Improved Antibacterial Activity of Water-Soluble Nanoformulated Kaempferol and Combretastatin Polyphenolic Compounds**.** Hindawi International Journal of Polymer Science Volume 2021, Article ID 5682182.
69. Ribatti D, Vacca A, Roncali L and Dammacco F: The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis. Int J Dev Biol *40*: 1189-1197, 1996.
70. Hulya Tuba Kıyan, Betu ̈l Demirci, Kemal Hu ̈snu ̈Can Bas ̧er, and Fatih Demirci: The in vivo evaluation of anti-angiogenic effects of Hypericum essential oils using the chorioallantoic membrane assay.http://informahealthcare.com/phb. ISSN 1388-0209 print/ISSN 1744-5116 online .
71. LeCouter J and Ferrara N: EG-VEGF and Bv8. A novel family of tissue-selective mediators of angiogenesis, endothelial phenotype, and function. Trends Cardiovasc Med *13*: 276-282, 2003.
72. Goi T: Angiogenesis and tumor proliferation/metastasis of human colorectal cancer cell line SW620 transfected with endocrine gland-derived vascular endothelial growth factor, as a new angiogenic factor. Cancer Res *64*: 1906-1910, 2004.
73. Monnier J and Samson M: Prokineticins in angiogenesis and cancer. Cancer Lett *296*: 144-149, 2010.
74. A.A. Badawy, M.A. El-Magd, S.A. AlSadrah, Therapeutic effect of camel milk and its exosomes on MCF7 cells in vitro and in vivo, Integr. Cancer Ther. 7 (4) (2018) 1235–1246.
75. Duff SE, Li C, Garland JM and Kumar S: CD105 is important for angiogenesis: evidence and potential. applications. FASEB J *17(9)*: 984-992, 2003. PMID: 12773481. DOI: 10.1096/fj.02- 0634rev
76. Fonsatti E and Maio M: Highlights on endoglin (CD105): from basic findings towards clinical applications in human cancer. J Transl Med 2*(1)*: 18, 2004. PMID: 15193152. DOI: 10.1186/1479- 5876-2-18
77. Raica M, Coculescu M, Cimpean AM and Ribatti D: Endocrine gland derived-VEGF is down-regulated in human pituitary adenoma. Anticancer Res *30*: 3981-3986, 2010.
78. Brouillet S, Hoffmann P, Benharouga M, Salomon A, Schaal JP, Feige JJ and Alfaidy N: Molecular characterization of EG- VEGF-mediated angiogenesis: differential effects on microvascular and macrovascularendothelial cells. Mol Biol Cell *21*: 2832-2843, 2010.
79. Ahmadi A, Shadboorestan A. Oxidative stress and cancer; the role of hesperidin, a citrus natural bioflavonoid, as a cancer chemoprotective agent. Nutr Cancer 2016;68:29–39
80. R. K. B. Bhawana, R. K. Basniwal, H. S. Buttar, V. K. Jain, and N. Jain, "Curcumin nanoparticles: preparation, characteriza- tion, and antimicrobial study," Journal of Agricultural and Food Chemistry, vol. 59, no. 5, pp.2056–2061, 2011.
81. Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L and Presta M: Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. Dev Biol 170: 39-49, 1995.

82. Iurlaro M, Vacca A, Minischetti M, *et al*: Antiangiogenesis by cyclosporine. Exp Hematol 26: 1215-1222, 1998.
83. Albergotti LC, Hamlin HJ, McCoy MW and Guillette LJ: Endocrine activity of extraembryonic membranes extends beyond placental amniotes. PloS ONE *4*: e5452, 2009.