IN-VITRO AND *IN-VIVO* ANTI-ANGIOGENIC EFFECT OF SUPERCRITICALLY EXTRACTED CURCUMIN (CP-95%)

A Dissertation Submitted to St. Teresas College (Autonomous), Ernakulam in Partial Fulfilment of The Requirement
For The Award Of

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY

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CERTIFICATE

This is to certify that the dissertation entitled **'*In-vitro* and *in-vivo* Anti-Angiogenic Effect Of Supercritically Extracted Curcumin (CP-95%)'** submitted to St. Teresa's College (Autonomous), Ernakulam, in partial fulfilment of the requirement of award of degree of Master of Science in Zoology is an authentic work carried out by **Ms. FIDHA LATHEEF** (SM20ZOO002) in the academic year 2020 – 2022 under the guidance and supervision of **Dr. P Sreejith** (External Guide) Assistant Professor, Department of Zoology, University of Kerala, Karyavattom Campus, Thiruvananthapuram and **Mrs. Indu Vasudevan** (Internal Guide), Assistant Professor, Department of Zoology, St. Teresa's College, Ernakulam.

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This is to certify that the project work entitled, **"*In-vitro* and *In-vivo* Anti-Angiogenic Effect Of Supercritically Extracted Curcumin (CP-95%)"** is an authentic record of the project carried out by Ms. **Fidha Latheef** of St.Teresa's College, Ernakulam, Kerala, under the guidance of **Dr. P. Sreejith,** Assistant Professor, **Department of Zoology, University of Kerala, Karyavattom Campus, Thiruvananthapuram** during the period from 1st April 2022 to 30th April 2022 in partial fulfillment of the requirement of the award of Degree of Master of Science in Zoology. I further certify that she has completed all the work and duties to my complete satisfaction.

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DECLARATION

I hereby declare that the dissertation entitled **'*In-vivo* and *in-vitro* Anti-Angiogenic Effect Of Supercritically Extracted Curcumin (CP-95%)'** submitted to St. Teresa's College (Autonomous), Ernakulam in partial fulfilment of the requirements, for the award of the Degree of Master of Science in Zoology is a record of original research work done by me under the supervision and guidance of **Dr P Sreejith** (External Guide) Assistant Professor, Department of Zoology, University of Kerala, Karyavattom Campus, Thiruvananthapuram during the period from 1st April 2022 to 30th April 2022 and **Mrs. Indu Vasudevan** (Internal Guide), Assistant Professor, Department of Zoology, St. Teresa's College, Ernakulam, to the best of my knowledge and belief, this project contains no material previously published or written by another person, except where due reference is made.

FIDHA LATHEEF

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LIST OF ABBREVIATIONS

1.	%	Percentage
2.	℃	Degree Celsius
3.	µg	Microgram
4.	ml	Milli Litre
4.	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
5.	q-RT PCR	Real-time Quantitative Reverse Transcription PCR
6.	MDA-MB-231	M.D. Anderson-Metastatic Breast 231
7.	CAM	Chorio-allantoic Membrane
8.	VEGF	Vascular Endothelial Growth Factor
9.	BAX	BCL2 Associated X
10.	COX	Cyclo-oxygenase
11.	LOX	Lysyl Oxidase

12.	NF-Kβ	Nuclear Factor kappa β
13.	HEP2	Human Epidermoid Carcinoma 2
14.	HER2	Human Epidermal Growth Factor Receptor 2
15.	CO_2	Carbon dioxide
16.	BCL-XL	B-cell lymphoma-extra large
17.	bFGF	Basic fibroblast growth factor
18.	cAMP	Cyclic Adenosine Monophosphate
19.	PDE2	Phosphodiesterase 2
20.	PDE4	Phosphodiesterase 4
21.	TNBC	Triple negative breast cancer
22.	DMEM	Dulbecco's Modified Eagle Medium
23.	PBS	Phosphate buffered saline
24.	BME	Basal Medium Eagle
25.	DMSO	Dimethyl sulfoxide
26.	ELISA	Enzyme-linked immunosorbent

		assay
27.	rpm	Revolutions per minute
28.	DNA	Deoxyribonucleic acid
29.	cDNA	Complementary Deoxyribonucleic acid
30.	dNTP	Deoxynucleotide triphosphate
31.	mRNA	Messenger RNA
32.	pH	Potential hydrogen
33.	mm	Millimetre
34.	hr	Hour
35.	µL	Microlitre
36.	g	Gram
37.	ng	Nanogram
38.	RT	Room temperature
39.	Vol	Volume
40.	CUR	Curcumin
41.	qPCR	Quantitative polymerase chain reaction

42.	ddH_2O	Double distilled water
43.	RNA	Ribonucleic acid
44.	PCR	Polymerase chain reaction
45.	NACL	Sodium chloride
46.	NCTC	National Collection of Type Cultures
47.	Na_2HPO_4	Sodium hydrogen phosphate
48.	KH_2PO_4	Potassium dihydrogen phosphate
49.	KCL	Potassium chloride
50.	IU	International unit
51.	CP	Curcumin powder

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ABSTRACT

Angiogenesis, the formation of new blood vessels from host vasculature, is critical for tumor growth and metastases. Curcumin, a novel small-molecular-weight compound, has been shown to inhibit carcinogenesis in different organs and the common link between these action is anti-angiogenic effect. The purpose of the present study was to evaluate the effect of supercritically extracted curcumin in anti-angiogenesis through *in-vitro* and *in-vivo* assays. Angiogenesis assay and MTT assay were performed to analyse the effect of 95% curcumin powder, result was confirmed by testing q-RT PCR analysis. Since cancer cell lines provide the most suitable model for the preliminary studies of herbal compounds' anti-cancer potential, the MDA-MB-231 cell line was used for the analysis. Initially, the anti-viability of the compound was analyzed using an MTT assay. The results supported the anti-cancer potential of the compound. When comparing with the control, there was a dose-dependent decrease in cell viability with increasing concentration. The angio-inhibitory effect of 95% *curcumin* was assessed from the CAM Assay. Reduction in the blood vessel and hemoglobin content in the chick embryo emphasized the anti-angiogenic capacity. The results supported the anti-angiogenic potential of the compound. Also, the result showed that at a concentration of 25µg/ml, the percentage of anti-angiogenesis is higher when comparing to other two concentrations.The result was confirmed by testing q-RT-PCR analysis of two different gene expression. Analysis of breast cancer cell line MDA-MB-231 by checking VEGF (which serve as a potent angiogenic factor favouring tumour growth and metastasis) mRNA gene expression and BAX (which act as pro-apoptotic regulators) mRNA gene expression in concentration of 20 and 40 µg/ml of curcumin comparing with untreated control. According to the RT- PCR result, the proangiogenic protein VEGF gene expression shows down regulation in 40 µg/ml curcumin, Similarly the pro- apoptotic regulator BAX gene expression shows upregulation in 40 µg/ml curcumin which shows anti-cancerous effect of curcumin in 40 µg/ml. Curcumin’s effect on the overall process of angiogenesis compounds its enormous potential as an antiangiogenic drug.

INTRODUCTION

Cancer remains the second leading cause of morbidity and mortality globally. Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. There are as many types of cancer cells as there are types of cancer. Of the hundred-plus types of cancer, most are named for the type of cancer cells in which the disease began, Carcinomas are cancers that arise in epithelial cells that line body cavities. Sarcomas are cancers that arise in mesenchymal cells in bones, muscles, blood vessels, and other tissues. Leukimias, lymphomas, and myeloma are blood-related cancers that are arise from the bone marrow (leukemias and multiple myelomas) or the lymphoid tissues (lymphomas). They are "fed" by nutrients in the bloodstream and lymph fluid such that they don't need to form tumors. (Lynne. 2022).

Cancer represents a group involving about 100 diseases. Two main characterizations are, firstly, there is no control for the growth of cancer cells, and secondly, the cancer cells can metastasize and migrate from the original site to different parts of the body. They are named based on the tumor's location or where it first started growing in the body. Lung, breast, colon, and prostate cancer are the most common forms of cancer. Chemotherapy and radiation therapy are conventional therapies in cancer treatment. Tamoxifen, a non-steroidal anti-estrogen drug, is used to treat estrogen receptor (ER)-positive breast cancer patients and as chemoprevention in high-risk women but is not effective against ER-negative breast tumors (Gupta et al. 2006). Non-selectiveness of medicines is one of the significant drawbacks of the chemotherapeutic drug, a high percentage of healthy cells will be lost with cancer cells. The side effects of either solid or haematological cancer include nausea, vomiting, diarrhoea, constipation, hypercalcemia, pain, loss of appetite, anaemia, fatigue, cachexia, leucopenia, neutropenia, and thrombocytopenia. Among the terrestrial plants and marine environments, the search for novel anticancer drugs is being conducted, (Greenwell and Rahman. 2015). The search for plant-derived anticancer agents started in the 1950s. It started with discovering and developing the vinca alkaloids, vinblastine, vincristine, and the cytotoxic podophyllotoxins' isolation. The adverse side effects can be reduced by natural therapies using plant-derived products in cancer treatment. In chemoprevention, safety of the participants is the first priority and should be considered of the utmost importance since essentially healthy people will receive the chemo preventive treatment for a long period of time. Moreover, the toxicity of the agents could impact patient accrual in larger scale studies in real clinical practice. To this end, unlike

synthetic compounds, the safety of natural compounds present in fruits, vegetables, and spices are well established through their long-term consumption in human history. Therefore, taking natural compounds for cancer prevention can be a well justified and effective strategy for people with increased risk for cancer development – such as those with premalignant lesions of intraepithelial neoplasia (Amina AR. et al. 2009).

In comparison with synthetic drugs, natural compounds are naturally available, cheaper, and easy to administer orally, and have low or minimal side effects. They are found to be rich in various biologically active chemotypes. National Cancer Institute has approximately screened 35,000 plant species for their potential anticancer activities has found that about 3,000 plant species have shown reproducible anticancer activity (Desai et al., 2008). Phytocompounds from these plant sources can prevent cancer initiation, promotion, and progress by exerting antioxidant effects mediated by integrating NF-κB, Nrf2, and AP-1 signaling pathways. Medicinal plants such as *Allium sativum, Annona muricata, Berberies aristata, Catharanthus roseus, Linum usitatissimum, Podophyllum hexeandrum, Rubia cordifolia, Withania somnifera*, etc., show potential role in the inhibition of cancer cell proliferation (Roy et al., 2017). *Andrographis paniculata is* described as a "diterpene lactone," containing diterpenes, flavonoids, stigmasterols, a potent chemoprotective agent, and effective against various infectious diseases and a powerful oncogenic agents, (Puri et al. 1993). *Curcuma longa is popular with its ability to* down-regulate the expression of genes, Activator Protein 1 (AP-1), NF-kappa B, cyclooxygenase 2 (COX2), lysyl oxidase (LOX), Epidermal growth receptor 1 (EGR-1), nitric oxide synthase (NOS), matrix metallopeptidase 9 (MMP-9), and tumor necrosis factor (TNF), (Agarwal, (2003); Surh, (2001). It is reported that *Phyllanthus amarus* showed a significant decrease in n-nitrosodiethylamine induced tumor incidence with the decline in tumor marker enzymes and injury markers (Jeena. 1999).

Curcumin's effect on the overall process of angiogenesis compounds its enormous potential as an antiangiogenic drug (Bhandarkar, et al. 2007). *Curcuma longa* L. is popularly known as turmeric in English, haridra in Sanskrit and haldi in Hindi. The rhizome of the plant is traditionally used in cooking. The active ingredient of this plant is curcumin (diferuloylmethane, chemical structure shown below), a polyphenol derived from the rhizome of the plant. Turmeric is used for both cancer prevention and treatment. Main features of this perennial herb are they have a short stem, with large oblong leaves and bears branched and brownish-yellow coloured ovate, pyriform or oblong rhizomes. *Curcuma longa* L. belongs to

the family Zingiberaceae, seen throughout tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China.

The most representative polyphenol component extracted from the rhizomes of *Curcuma longa* (known as turmeric) is curcumin. It was isolated for the first time in 1815 by two scientists, Vogel and Pelletier, from Harvard College Laboratory. Since then, the scientific interest towards curcumin has increased and, more and more, its health benefits have been discovered. Curcumin belongs to a chemical class of polyphenols; it is known as diferuloylmethane and its IUPAC name is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, with a chemical formula of $C_{21}H_{20}O_6$ and a molecular weight of 368.38. The chemistry of curcumin is at the basis of its several biological activities. Cancer is one of the primary causes of death in industrialized countries. In recent years, the early diagnosis and increase in therapeutic options has reduced the death rate. However, the growth of drug-resistant cancers necessitates the search for innovative and more effective drugs (Barone D et al. 2019). It is worth noting that cancer cells are characterized by deregulated signalling pathways involving proliferation, apoptosis, and angiogenesis.

In this scenario, curcumin represents a promising candidate as an effective anticancer drug to be used alone or in combination with other drugs. It affects different signalling pathways and molecular targets involved in the development of several cancers (G. Antonio et al. 2019).

In present study, 95% Curcumin powder were identified for the study, to find out their comparative efficiency and various properties of this compound were analysed.

Turmeric was described as *C. longa* by Linnaeus, and its taxonomic position is as follows:

Kingdom: Plantae

Phylum: Spermatophyta

Class: Liliopsida

Subclass: Commelinids

Order: Zingiberales

Family: Zingiberaceae

Genus: *Curcuma*

Species: *longa*

Figure1: curcumin

(//encrypted-tbn0.gstatic.com/images?q=tbn:ANd9GcSI3S-Pql8E1MuVx7kVo4bDKnQEV5ZvwOyeA&usqp=CAU)

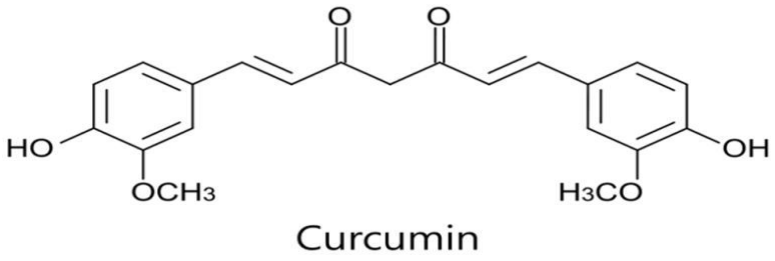
The anticancer potential of curcumin is associated with its ability to inhibit proliferation in a wide variety of tumor cell types. The anti-proliferative properties of curcumin may be related to its ability to down-regulate the expression of a number of genes, including NF-kappa B, Activator Protein 1 (AP-1), Epidermal growth receptor 1 (EGR-1), cyclooxygenase 2 (COX2), lysyl oxidase (LOX), nitric oxide synthase (NOS), matrix metallopeptidase 9 (MMP-9), and tumour necrosis factor (TNF). Moreover, turmeric reduces the expression of various chemokines, cell surface adhesion molecules, cyclins and growth factor receptors, including epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (HER2). In addition to its effects on gene expression, turmeric inhibits the activity of c-Jun N-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases. Turmeric has also been shown to inhibit tumor cell invasion and metastasis *in vitro* by reducing MMP-2 activity and by inhibiting HEp2 (epidermoid carcinoma cell line) cell invasion. A number of studies have shown that curcumin induces apoptosis, inhibits proliferation and interferes with cell cycle progression. Curcumin is suggested to exert its anti-proliferative and apoptotic effects by inhibition of protein tyrosine kinase activity, inhibition of protein kinase C activity, suppression of c-myc mRNA levels and up-regulation of B-cell lymphoma 2 (Bcl-2) mRNA expression. Curcumin has been shown to cause apoptosis *in vitro* by bringing about a rapid decrease in mitochondrial membrane potential, release of cytochrome c, activation of caspases 3 and 9, and downregulation of anti-apoptotic proteins Bcl-XL and Inhibitor of Apoptosis Protein (IAP). In LNCaP prostate cancer cells, curcumin was shown to increase apoptosis by enhancing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), promoting cleavage of pro-caspases 3, 8 and 9, and inducing cytochrome c release. Recent studies also suggest that heat shock proteins may play a role in the induction of apoptosis by curcumin.

Curcumin and its derivatives demonstrated significant inhibition of VEGF and bFGF-mediated corneal neovascularization and directly inhibited angiogenesis *in vivo* and *in vitro*. In addition to its antitumor effects *in vitro*, curcumin has been shown to prevent colon and gastric cancers in rodents. The mechanism underlying the protective effect of curcumin is suggested to be related to its ability to inhibit the growth of several tumor-associated and angiogenesis-associated genes. Additionally, curcumin has been shown to inhibit the growth of nearly nineteen different strains of *H. pylori* (Avani G.D et al. 2008).

A number of new analogs and formulations have already been developed with higher systemic bioavailability and potency. More standardized clinical trials for bioavailability and randomized control trials for efficacy should validate the potential of these newer agents and

formulations. First, specific trials can improve the application of curcumin through changing the route of administration, achieve targeted delivery straight to the lesion sites by increasing tumor-specific affinity, and develop different analogues that can bypass or minimize the first-pass metabolism occurring in the gastrointestinal mucosa and liver. Second, to minimize its metabolism before reaching the targeted site, different preparations of curcumin may improve its delivery to the target and therefore increase its bioavailability. Third, formulating curcumin using nanoparticles and microparticles, which are among the most innovative modalities that can maximize delivery to a target tissue and increase sensitivity and specificity, may enhance its therapeutic index. In the future, targeting specific patient populations with certain biomarkers, so-called tailored chemoprevention, is necessary. Defining critical biomarkers will help to better design a personalized plan for tailored chemoprevention. Progress in personal genome-based risk assessment and profiling of individual patients may also help to identify the patient population best suited to curcumin chemoprevention in the future, (Wungki. et al. 2013).

Figure 2: curcumin



(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3693758/figure/F1/)

The present study**,** analysed the effect of **Supercritically Extracted (95%) Curcumin Powder** in anti-angiogenesis through *in-vivo* and *in-vitro* assays . **Supercritical fluid extraction** is the process in which by using supercritical fluids as the extracting solvent, one component is separated from another. The most commonly used supercritical fluid is CO_2 and also modified co-solvent such as ethanol and methanol are used. The optimal condition for this process is the critical temperature of 31℃ and a critical pressure of 74 bar. Already many studies are carried out to find the medicinal and healing efficiency of turmeric and antioxidant, anti-angiogenesis property of curcumin. However, here in this study, in-vitro and in-vivo anti-angiogenic effect of this compound in cancer cell lines are determined. The MTT assay is mainly done to find the effective concentration at which they show their maximum ability; these concentrations may be considered in the medicinal formulations to make them more

useful. Through these assays, the lethal concentration was also found out, the effectiveness of these compounds in cancer cell lines helps to identify the anti-cancer property.

To study the effect of supercritical extracted curcumin in anti-angiogenesis, *in-vivo* and *in-vitro analysis* were used. Different properties of this compound such as cell viability and angiogenic properties, were analysed. Regular turmeric that we consume contains only 2.5% of Curcumin. To test the properties of 95% extracted curcumin powder was given a different dimension to this work. The cell proliferation of test compound in MDA-MB-231 cell line was assed using MTT assay. The angiogenic property of the compound was examined using CAM Assay. To validate the result used q RT-PCR analysis.

AIM AND OBJECTIVES

AIM:

Aim of the study is to know the curcumin's (one of the most abundant polyphenol isolated from *Curcuma longa)* effect against angiogenesis. The cell proliferation of test compound in MDA-MB-231 cell line was assed using MTT assay. The angiogenic property of the compound was examined using CAM Assay and validate the result using q RT-PCR analysis.

OBJECTIVE:

1. To study the *in-vitro* and *in-vivo* anti-angiogenic effect of supercritically extracted curcumin.
2. To validate the result using q RT-PCR analysis.

RELEVANCE OF THE STUDY:

This study was mainly carried out to assess the biological efficiency of Supercritically extracted 95% Curcumin Powder performing with various assays. This is a novel work, since there is no work presently done in analysis of angiogenic potential using supercrtically extracted Curcumin. Different properties of this compound such as cell viability and angiogenic properties, were analysed. Regular turmeric that we consume contains only 2.5% of Curcumin. To test the properties of 95% extracted curcumin powder was given a different dimension to this work.

REVIEW OF LITERATURE

Curcumin, a polyphenol extracted from *Curcuma longa* in 1815, has gained attention from scientists worldwide for its biological activities (e.g., antioxidant, anti-inflammatory, antimicrobial, antiviral), among which its anticancer potential has been the most described and still remains under investigation. The study conducted by Antonio.G et al. (2019) describes that: curcumin exhibits anticancer ability by targeting different cell signalling pathways including growth factors, cytokines, transcription factors, and genes modulating cellular proliferation and apoptosis. However, curcumin is not immune from side effects, such as nausea, diarrhoea, headache, and yellow stool. Moreover, it showed poor bioavailability due to the fact of low absorption, rapid metabolism, and systemic elimination that limit its efficacy in diseases treatment. Further studies and clinical trials in humans are needed to validate curcumin as an effective anticancer agent.

Among many such natural compounds, ***curcumin*** has drawn special attention for its chemoprevention potential because of its safety, multi-targeted anticancer effects, and easy accessibility. Bhandarkar et al. (2007) evaluated that: Curcumin, a novel small-molecular-weight compound, has been shown to inhibit carcinogenesis in different organs and the common link between these actions is its antiangiogenic effect. Angiogenesis, the development of new blood vessels from the existing vasculature, is essential in normal developmental processes and is a hallmark of over 50 different disease states including cancer, rheumatoid arthritis and psoriasis (Carmeliet, Jain. 2000). Curcumin is a direct inhibitor of angiogenesis and also downregulates various proangiogenic proteins like vascular endothelial growth factor and basic fibroblast growth factor. Curcumin's antiangiogenic effect is also in part due to its inhibitory effect on signal transduction pathways, including those involving protein kinase C and the transcription factors NF-kappaB and AP-1. Curcumin has an inhibitory effect on two groups of proteinases involved in angiogenesis that are the members of the matrix metalloproteinase family and the urokinase plasminogen activator family. Cell adhesion molecules are upregulated in active angiogenesis and curcumin can blocking this effect, adding further dimensions to curcumin's antiangiogenic effect. Curcumin shows a dose-dependent inhibition on tumor necrosis factor, a versatile cytokine, which has its effect on angiogenesis through the signal transduction pathways, expression of proangiogenic factors, and cell adhesion molecules.

Modulation of pathological angiogenesis by curcumin (diferuloylmethane), the active principle of turmeric, seems to be an important possibility meriting mechanistic investigations. The report written by A.E Gururaj et al. (2002), explained: the effect of curcumin on the growth of Ehrlich ascites tumour cells and endothelial cells in vitro. Further, regulation of tumor angiogenesis by modulation of angiogenic ligands and their receptor gene expression in tumor and endothelial cells, respectively, by curcumin was investigated. Curcumin proved to be a potent angio-inhibitory compound, as demonstrated by inhibition of angiogenesis in two in vivo angiogenesis assay systems, viz. peritoneal angiogenesis and chorio-allantoic membrane assay. According to their result on Northern blot analysis clearly indicated a time-dependent (0–24 h) inhibition by curcumin of VEGF, angiopoietin 1 and 2 gene expression in EAT cells, VEGF and angiopoietin 1 gene expression in NIH3T3 cells, and KDR gene expression in HUVECs. Further, decreased VEGF levels in conditioned media from cells treated with various doses of curcumin (1 μM–1 mM) for various time periods (0–24 h) confirm its Angio inhibitory action at the level of gene expression. Because of its non-toxic nature, curcumin could be further developed to treat chronic diseases that are associated with extensive neovascularization.

Sulochona et al. (2007) showed that: a dose-dependent inhibition on tumor necrosis factor, a versatile cytokine, which has its effect on angiogenesis through the signal transduction pathways, expression of proangiogenic factors, and cell adhesion molecules. Curcumin's effect on the overall process of angiogenesis compounds its enormous potential as an antiangiogenic drug.

The review done by Avani et al. (2014) focused on: the various plant-derived chemical compounds that have, in recent years, shown promise as anticancer agents and will outline their potential mechanism of action. Many studies have focused on the chemoprotective properties of plants such as anticarcinogenic properties of *Abrus precatorius* on Yoshida sarcoma in rats, fibrosarcoma in mice and ascites tumor cells, (Reddy et al. 1969). Similarly, Dhar et al. (1968) have examined: the anticancer properties of *Albizzia lebbeck* on sarcoma in mice and *Alstonia scholaries* on benzo[a]pyrene-induced forestomach carcinoma in humans. Other plants that have shown anticarcinogenic properties include *Anacardium occidentale* in hepatoma, *Asparagus racemosa* in human epidermoid carcinoma, *Boswellia serrata* in human epidermal carcinoma of the nasopharynx, *Erthyrina suberosa* in sarcoma, *Euphorbia hirta* in Freund virus leukemia, *Gynandropis pentaphylla* in hepatoma, *Nigella sativa* in Lewis lung

carcinoma, *Peaderia foetida* in human epidermoid carcinoma of the nasopharynx, *Picrorrhiza kurroa* in hepatic cancers, and *Withania somnifera* in various tumors .

Niklinska et al. *(*1998) showed that: Curcumin and its derivatives demonstrated significant inhibition of VEGF and bFGF-mediated corneal neovascularization and directly inhibited angiogenesis *in vivo* and *in vitro*. In addition to its antitumor effects *in vitro*, curcumin has been shown to prevent colon and gastric cancers in rodents have experimented by (Ikezaki et al., 2001). The mechanism underlying the protective effect of curcumin is suggested to be related to its ability to inhibit the growth of several tumor-associated and angiogenesis-associated genes (Kerbel et al. 2002). Additionally, Mahady et al., (2002) said that: curcumin has been shown to inhibit the growth of nearly nineteen different strains of *H. pylori*.

According to Carolyn et al. (2009), she had reviewed that: the main technical challenge in any study of angiogenesis is the selection of the most appropriate assay. The ideal angiogenesis assay would be robust, rapid, reproducible with reliable readouts, automated computational analysis, multi-parameter assessment, including positive and negative controls and should relate directly to results seen in the clinic. Despite the increasing numbers of both *in vitro* and *in vivo* assays, a 'gold-standard' angiogenesis assay has yet to be developed; therefore, a combination of assays is required to identify the full range of effects of a test protein or to identify the molecular and/or cellular events in angiogenesis.

Angiogenesis assays allow for the evaluation of pro- or anti-angiogenic activity of endogenous or exogenous factors (stimulus or inhibitors) through investigation of their pro-or anti-proliferative, migratory, and tube formation effects on endothelial cells. Zachary et al. (2019) reviewed: current state of angiogenesis assays as well as their mechanisms, advantages, and limitations. There are mainly three types of assays, *in-vitro*, *ex-vivo* and *in-vivo* assays, The chick chorioallantoic membrane (CAM) assay is one of the oldest and most widely used assays for studying angiogenesis *in vivo*. It was developed by Folkman et al, (2004) and exploits the fact that chorioallantoic membranes are present in the fertilized eggs of all avian species and contain a copious number of blood vessels (Goodwin et al, 2007), Staton et al, (2004). These characteristics are ideal for in vivo assays because chicken eggs are inexpensive, the method is easy to reproduce technically and suits large-scale screening, and visualization of new vascularization is simple under a microscope. As an in-vitro assay, MTT assays make it facile to evaluate cell viability based on the absorbance of the resulting formazan solution. However, there is a disadvantage for this technique, the metabolic processes of ECs can be affected by

the same angiogenic agents that are being evaluated. In other words, it cannot be concluded with absolute certainty that a change in proliferation of the ECs occurred because of angiogenesis-specific effects of the drug or metabolic effects of the agents unrelated to blood vessel formation. Also, because this method depends on the metabolism of living cells, it gives no information on the cytotoxicity of the drug itself because there is essentially no way of measuring the extent of apoptosis due to exposure to a potentially toxic chemical as opposed to apoptosis due to metabolic processes. Therefore, it may be worthwhile to confirm the results of an MTT assay using another angiogenesis assay.

The review of Naoyo. N et al. (2006) has explained: about angiogenic inhibitors, While normal angiogenesis is critical for development and tissue growth, pathological angiogenesis is important for the growth and spread of cancers by supplying nutrients and oxygen as well as providing a conduit for distant metastasis, Pathological angiogenesis is a hallmark of cancer and various ischaemic and inflammatory diseases. Concentrated efforts in this area of research are leading to the discovery of a growing number of pro- and anti-angiogenic molecules, some of which are already in clinical trials (Peter. C et al. 2000). Unlike normal blood vessels: 1) tumor vessels have no or a detached pericyte and basement membrane (Inai et al., 2004; Tong et al. 2004; Winkler et al., 2004) the diameter of the vessel is smaller, (Yuan et al. 1996; Tong et al. 2004; Winkler et al. 2004); 3) the vascular density is heterogeneous (Izumi et al. 2002; Tong et al. 2004; Winkler et al. 2004); 4) permeability to large molecules is high, (Yuan et al. 1996 Tong et al. 2004; Winkler et al. 2004; Willet et al. 2004) and 5) the pressure of microvascular and interstitial fluid is almost the same, (Lee et al. 2000; Tong et al. 2004; Willet et al. 2004)., These abnormalities contribute to heterogeneity in tumor blood flow. Angiogenesis is regulated by both activator and inhibitor molecules. The switch to the angiogenic phenotype involves a change in the local equilibrium between positive and negative regulators of angiogenesis. This signalling activates certain genes in the host tissue that make proteins which encourage the growth of blood vessels, (Majima et al. 2000, Semenza 2002). Cancer cells require access to blood vessels for growth and metastasis. The discovery of angiogenic inhibitors provides hope for reducing the mortality and morbidity from carcinomas.

The study conducted by M Rajabi and S.A Mousa (2017) focused on: the role of anti-angiogenesis strategies in cancer treatment, Angiogenesis plays a significant role in tumor progression. Effective inhibition of tumor angiogenesis might arrest or halt tumor progression but would not eradicate the tumor as a stand-alone therapy, especially with a single mechanism

anti-angiogenic agent. Hence, the combination of an anti-angiogenesis agent and chemotherapy might be essential for effective tumor treatment.

Vascular endothelial growth factor (VEGF) plays a major role in angiogenesis by stimulating endothelial cells. Increase in cyclic AMP (cAMP) level inhibits VEGF-induced endothelial cell proliferation and migration. Cyclic nucleotide phosphodiesterases (PDEs), which specifically hydrolyse cyclic nucleotides, are critical in the regulation of this signal transduction. The study conducted by A Abusnina et al. (2015) previosuly reported that: PDE2 and PDE4 up-regulations in human umbilical vein endothelial cells (HUVECs) are implicated in VEGF-induced angiogenesis and that inhibition of PDE2 and PDE4 activities prevents the development of the *in vitro* angiogenesis by increasing cAMP level, as well as the *in vivo* chicken embryo angiogenesis.They have also shown that polyphenols are able to inhibit PDEs. Their present study investigated whether PDE2 and PDE4 inhibitors and curcumin could have similar *in vivo* anti-tumour properties and whether the anti-angiogenic effects of curcumin are mediated by PDEs. They have suggested that curcumin exerts its *in- vitro* anti-angiogenic and *in- vivo* antitumour properties through combined PDE2 and PDE4 inhibition.

The report submitted by Urszula et al. (2012) said that: a Japanese quince fruit flavanol preparation (JQFFP) caused favorable changes in *Bax/Bcl-2* mRNA ratio, which rendered normal and cancer cells more resistant and more sensitive, respectively, to apoptosis. The growth and invasiveness of MDA-MB-231 human breast cancer cells were strongly suppressed by JQFFP, which was accompanied with a decrease in MMP-9 activity and stimulation of *TIMP-1* expression.

The study concducted by I.V Auwera et al. (2004) used: real-time quantitative reverse transcriptase-PCR to measure mRNA levels of tumor angiogenesis and lymphangiogenesis-related factors in specimens of inflammatory breast cancer and noninflammatory breast cancer patients. Through their results, they demonstrated that inflammatory breast cancer is a highly angiogenic and lymphangiogenic tumor, which may help explain its potential to metastasize through the hematogenous and the lymphatic route. Their findings suggest that both processes are novel targets for future interventions in the treatment of patients with Inflammatory breast cancer.

MATERIALS AND METHODS

This study was mainly carried out to assess the biological efficiency of Supercritically extracted 95% Curcumin Powder performing with various assays. Different properties of this compound such as cell viability and angiogenic properties, were analysed. Regular turmeric that we consume contains only 2.5% of Curcumin. To test the properties of 95% extracted curcumin powder was given a different dimension to this work.

1. **Compounds used for the study**

Compounds are obtained from Akay Flavours & Aromatics Pvt. Ltd., Ernakulam.

- 95% Curcumin Powder (CC R 01/17)

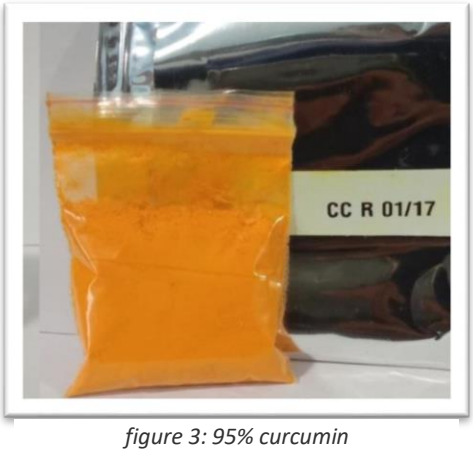


figure 3: 95% curcumin

Cell line used for *in-vitro* analysis:

Human Breast cancer cell line- (MDA MB-231)

The MDA-MB-231 cell line is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma1 and is one of the most commonly used breast cancer cell lines in medical research laboratories. MDA-MB-231 is a highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks oestrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification2,3. Similar to other invasive cancer cell lines, the invasiveness of the

MDA-MB-231 cells is mediated by proteolytic degradation of the extracellular matrix (Cailleau R, et al. 1978).

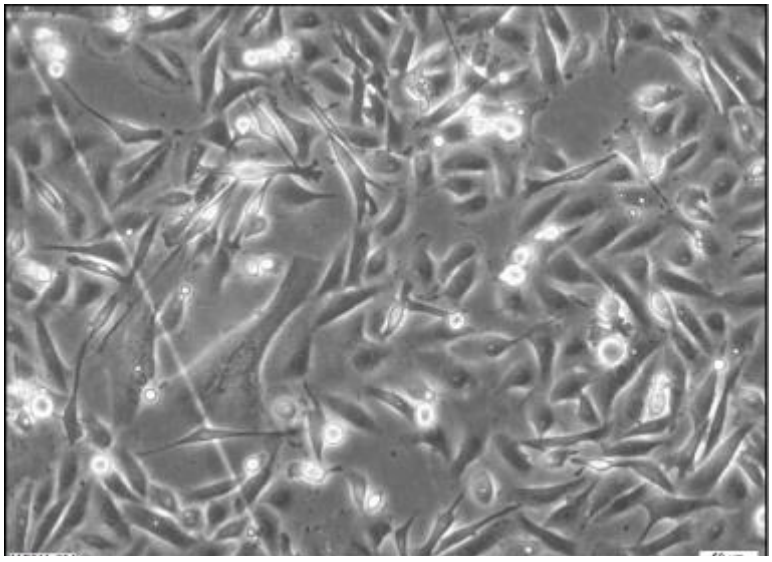


figure 4 breast cancer cell line MDA-MB-231
(https://www.culturecollections.org.uk/media/133182/mda-mb-231-cell-line-profile.pdf)

Culture and Maintenance of Cell Lines

Dulbecco's Modified Eagle's Medium-high glucose:

Basal Medium Eagle (BME) is modified as Dulbecco's Modified Eagle medium (DMEM). It contains four-fold concentrations of the amino acids and vitamins, and the level of glucose has been raised to 4500mg/l in DMEM/High. To culture embryonic mouse cells, the original formulation contained 1000 mg/L of glucose and was used. Since then, to support primary cultures of mouse and chicken cells, it has been modified in several ways as well as a variety of normal and transformed cells. Different combination of L-glutamine and sodium pyruvate were offered in each of these media.

Phosphate-Buffered Saline (10X) pH 7.4, RNase -free (PBS):

Phosphate buffer saline (PBS) is a pH-adjusted phosphate buffer, and saline solutions their osmolarity and ion concentrations of the solution usually match those of the human body (isotonic) and non-toxic to many of the cells. Attains 137 mM NaCl, 2.7 mM KCl, eight mM Na_2HPO_4, and two mM KH_2PO_4 when diluted to a 1X working concentration. It is a salty solution containing potassium chloride, potassium phosphate and sodium phosphate and sodium chloride. PBS is certified RNase-free tested rigorously for contaminating nonspecific endonuclease, exonuclease and RNase activity.

Trypsin:

Trypsin a most frequently used member of serine protease family to detach cell from the adherent substrate. From the proenzyme trypsinogen secreted by the exocrine cell of the pancreas, trypsin is produced, they mainly act on C-terminal side of lysine or arginine. Prewarmed trypsin speed up the detachment and optimum activity are achieved at 37ºC. Incubation with high trypsin concentration for a long time causes cell damage by striping cell surface proteins and kill the cells. Many cell types tolerate trypsin, but various concentration and constituents of trypsin are employed based on the application and type of cell.

Antibiotics:

The culture medium is supplemented with antibiotics to prevent fungal and bacterial infection. Antibiotics function by degrading the cell membrane of the infectious bacterium andfungus, respectively. A working concentration of 50-100 IU/ml penicillin and 50-100µg/ml streptomycin. Penicillin-Streptomycin solution were widely used antibiotics in cell culture.

The Revival of Cell Lines:

A Cryopreserved vial of cells was taken, allowed to thaw in the water bath at 37ºC and transferred the whole content of vial into a sterile 15ml falcon tube and 2ml of media was added slowly within 5 min then it was allowed to centrifuge at 2000 rpm for about 5mins. After the centrifugation, pelleted cells have gently resuspended the cells using 5ml media and it was transferred into a T25 flask. These cells were incubated in 5% CO_2, 37° C incubator for attachment and further growth of the cell. The cells were grown without changing themedium until they were seen to be attached to the flask. After complete attachment of the cell,the media was changed.

Splitting and cell counting using Haemocytometer:

The growth of cells in culture progress from the **lag phase** following the **log phase**, where the exponential proliferation of cells occurs. When all the available substrates were occupied by the adherent cells, then there will be no space left for expansion. For the continued growth and to stimulate further proliferation, the culture has to divide, and new media were supplied to keep them at an optimal density. A decline in pH of the growth medium usually indicates the secretion of lactic acid, which is a by-product of cellular metabolism. The decrease in pH is harmful to cell growth, and lactic acid can be toxic to the cells. The faster exhaustion of the media occurs at high cell concentration than the lower cell concentrations; hence, the

concentration of cells in those medium affects the rate of change of pH. After removing the media, PBS wash was given and then through the side of the flask trypsin was added after that the flask is incubated at room temperature for 2mins to detach the cells. Afterthe detachment of 90% of cells, a prewarmed complete medium was added to this flask to inhibit the action of trypsin. Cells were transferred into 15ml falcon and centrifuge at 2000 rpmfor 7mins. The cell pellet was resuspended using media, and 10ul were taken for cell enumeration and to check the percentage of viability using haemocytometer after staining with trypan blue. The cells were counted in the four-field and average of the count was taken.

The volume of the cell suspension to be taken from the qualified cell suspension

= Average no. of cells × Total vol. × Dilution Factor × 10^4

Dilution factor = 2

Cryopreservation of Cells:

To maintain the stock of cells, cell freezing and cryopreservation are very important. This is done when the cell in the flask becomes confluent. Trypsinize the fully confluent flaskand 10% of the media were added to stop the action of trypsin. Then centrifugation of the vial was carried out at 2000rpm for 7mins. The supernatant was discarded, and 700ml of DMSO and FBS mixed in 1:9 ratio (Freezing Medium) were added to the pellet. Resuspend the cells thoroughly in the freezing medium. The cells were frozen in a controlled rate freezing apparatus, decreasing the temperature approximately 1°C per minute. Cells containing cryo vials are placed in an isopropanol chamber and store them at -80°C overnight. The frozen cells were transferred to liquid nitrogen, and store them in the gas phase above the liquid nitrogen (Freshney, 2015).

Checking Cell proliferation using MTT Assay

The MTT assay is one of the most extensively used and highly reliable colorimetric assays for estimating the viability of cells that are treated with synthetic or natural products which indicate their efficacy to induce cell cytotoxicity. MTT cell viability assay is the first best- known enzyme-based investigation developed for determining mitochondrial dehydrogenase activities in the live cells. The assay relies on the ability of mitochondrial succinate dehydrogenase enzymes present in healthy cells to cleave the tetrazolium ring and transform the yellow water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) substrate into

purple water-insoluble formazan product within the cells. These needle- shaped formazan crystals can be dissolved using isopropanol or dimethyl sulfoxide or an acidified ethanol solution. The number of viable cells is proportional to the colour intensity of the dissolved formazan and can be quantified spectrophotometrically by using an ELISA plate reader.

Cells were trypsinised, enumerated and seeded in a 96 well plate (10000 cells/well). After 24 hours when they attained 90%-100% confluency, the media was removed and 100µl of Supercritical Extracted 95% Curcumin Powder (CP-95%) of concentrations 20µg, 40µg obtained through serial dilutions were added to the well in triplicate manner. Test compounds treated wells are allowed to incubate for 24 hours at 37^0 C. Without removing the media, MTT reagent (40mg MTT/8ml PBS) was added and incubated for 3-4 hours. After that by discarding the media 100µl of DMSO were added and incubated for another 15 minutes. Absorbance was read spectrophotometrically at 590nm.

Checking Angiogenic Potential Using CAM assay (*in-vitro* analysis)

The development of new blood vessels from the pre-existing vessels or endothelial cell progenitors is known as angiogenesis. It shows an essential role in tumor growth and metastasis. The primary function of the chorio-allantoic membrane (CAM) of chick embryo is to exchange gases and nutrients, supported by a dense capillary network. The chorio-allantoic membrane due to its extensive vascularization and ease of use, the CAM is an important assay used by scientists to study the angiogenic nature of the compound.

Fertilized chicken eggs were cleaned with 70% ethanol and incubated under conditions of constant humidity at 37°C. On the 4^{th} day of incubation, a small hole was drilled at the egg's blunt end, and test samples were introduced to the chorio-allantoic membrane. For each sample, 3 eggs were taken. In control, eggs were untreated. 15 µl,25 µl and 35 µl of compound in the concentration 100 µl was added to the CAM through the hole in the chicken eggs. The window was sealed through a sterile para-film, and the egg was re-incubated at 37°C. The window was opened on the 14^{th} day to analyze the microvessel density. The CAM was isolated and homogenized in 5ml Drabkin's reagent and centrifuged at 3500 rpm for 5 minutes. The amount of haemoglobin was determined using nanophotometer at 615 nm.

Percentage of Anti- Angiogenesis =Control- Sample/Control*100

RNA ISOLATION (Trizol Method))

- Scrape the cells using Cell Scrapes in PBS.
- Centrifuge and discard the supernatant
- Add 500 µL Trizol.
- Homogenise using a homogenizer.
- Make the volume 1 ml using Trizol and vortex the mixture.
- Incubate at room temperature for 5 min.
- Add 200 µL of Chloroform added and mixed well by shaking.
- Incubate at room temperature for 10-15 min.
- Centrifuge the mixture at 13,000 rpm for 15 min at 4ºC.
- Collect the supernatant into afresh tube.
- Add 500 µL (ice cold) Isopropyl alcohol and mix gently.
- Incubate at room temperature for 10 min.
- Centrifuge at 13,000 rpm for 10 min at 4ºC.
- Discard the supernatant.
- Add 500 µL (ice cold) 70 ethanol to the pellet and centrifuge 13,000 rpm for 5 min at 4ºC.
- Discard the supernatant and air dry the pellet.
- Dissolve the air dry pellet to 100 µL RNase free water.

CDNA SYNTHESIS:

The volume of each component is for a 20µL final reaction:

	Volume	Final concentration
5X cDNA synthesis buffer	4 µL	1X
dNTP Mix	2 µL	500µM
RNA Primer	1 µL	
RT Enhancer	1 µL	
Verso Enzyme Mix	1 µL	
Template (RNA)	1.5 µL	1ng
Water, nuclease-free (#R0581)	To 20 µL	
Total volume	20 µL	

q RTPCR:

1) Prepare qPCR reaction mixture:

a) Please prepare the q PCR reaction solution according to the list:
The following protocol is recommended for a 20 reaction volume:

Components	Volume
2 AB HS SYBR Green qPCR Mix	10 µL
Forward Primer (2µM)	2 µL
Reverse Primer(2µM)	2 µL
DNA Template	2 µL
ddH_2O	4 µL
Final Volume	20 µL

b) After assembling all the components, gently mix the contents of the tube. Air bubbles may be generated during the mixing, to remove the bubbles apply low-speed centrifugation.

2) Perform quantitative PCR

Perform quantitative PCR using optimized cycling conditions. Provided below is a standard two-step program.

Thermo-cycling conditions for two-step qPCR

Step	Temperature	Time	Number of cycles	Fluorescence Detector
1. Intial denaturation	95 ºC	2 min	1	Off
2. Dentauration	95 ºC	10 sec	40	Off
3. Annealing	60 ºC	20-30 sec	40	On

RESULTS

In the present study, in-vitro and in-vivo anti-angiogenic effect of supercritically extracted curcumin (95%) was analyzed. The cell proliferation of test compound in MDA-MB-231 cell line was assed using MTT assay. The angiogenic property of the compound was examined using CAM Assay. To validate the result used q RT-PCR analysis.

MTT Assay – MDA-MB-231 Cell lines

CTR	VCL	STD	2.5(µg/ml)	5 (µg/ml)	10(µg/ml)	20 (µg/ml)	40 (µg/ml)
100	90.48	94.07	90.6	87.97	66.83	44.5	23.73

TABLE 1*: concentrations of curcumin and its percentage of proliferation*

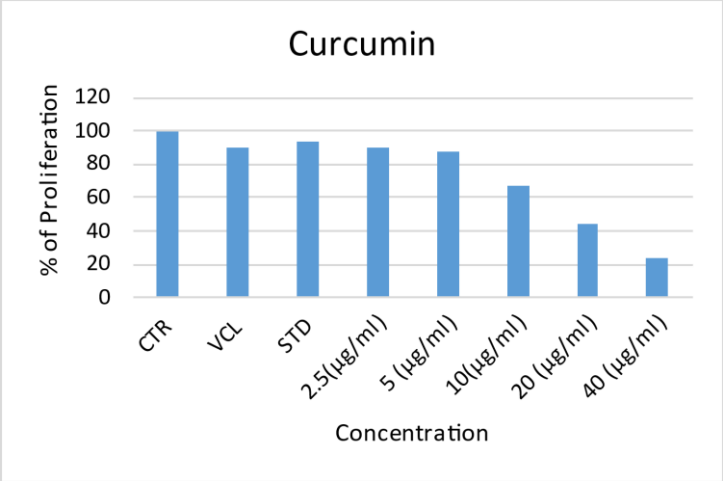


Figure 1: ***Graph showing percentage of cell proliferation when treated with Curcumin powder of different concentration in MDA-MB-231 Cell line.***

MTT Assay- MDA-MB-231 cell lines

Cell proliferation activity can be evaluated using the MTT assay. This assay helps to analyze the concentration in which cells show more proliferation.

The less percentage of proliferation was shown at the concentration 20µg/ml and 40µg/ml of Curcumin Powder. Higher percentage of proliferation was shown at the concentration 2.5 µg/ml and 5 µg/ml comparing with concentration of control.

ANGIOGENESIS USING CAM ASSAY

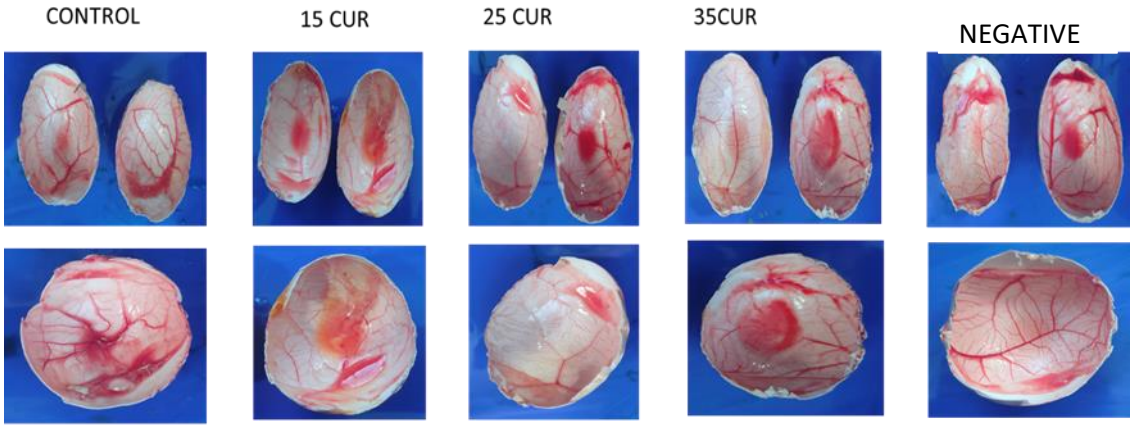
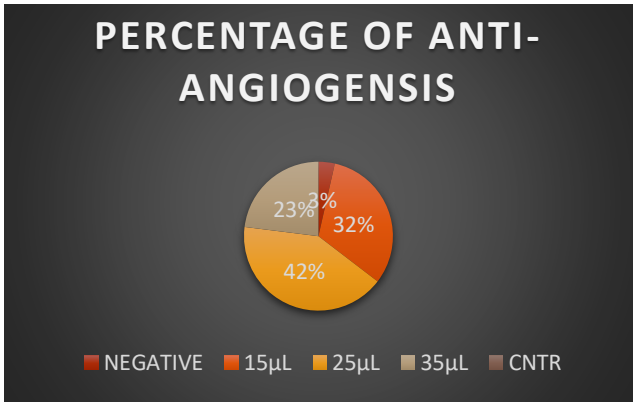


FIGURE 2*: Anti- angiogenesis in 14th day chicken egg*

CONC OF CUR	*PERCENTAGE OF ANTI-ANGIOGENSIS*
NEGATIVE	7.25%
15µL	65.25%
25µL	85.15%
35µL	47.14%
CNTR	

Table 2: *percentage of anti-angiogenesis by different concentrations of test compound*



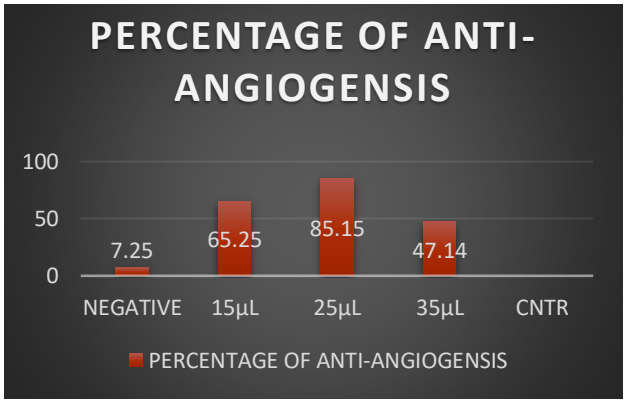


Figure 3: *Pie chart showing percentage of anti-angiogenesis*, **figure 4***: Bar graph showing percentage of anti-angiogenesis*

The anti-angiogenic potential of **supercritically extracted Curcumin** of various concentration was assessed using CAM Assay. 15µg/mL, 25 µg/mL, 35 µg/mL concentration of test compounds and control and negative control which are untreated with the test compound was introduced into the 4th day chick embryo. The 14th day result showed the anti-angiogenic potential of the compound. When comparing with the control, the compound treated groups showed a significant decrease in neovascularization. Also, the results showed a decrease in the amount of hemoglobin in the compound treated groups compared to the control group. Among the three concentrations, 25µg/mL showed a high percentage of anti-angiogenesis. This signifies that a particular concentration of curcumin which shows anti-angiogenic potential, which increase or decrease in concentration reduces the anti- angiogenic potential of curcumin. This shows the dose-dependent character of curcumin.

q- RT PCR for gene expression of VEGF and BAX

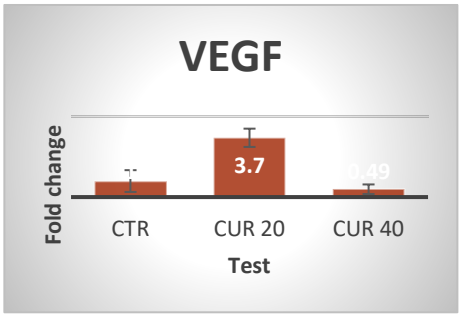
VEGF -Gene expression

CTR	CUR 20	CUR 40
1	3.7	0.49
0.68	0.57	0.3

BAX – Gene expression

CTR	CUR 20	CUR 40
1	1.74	12.71
1.06	1.06	1.37

TABLE 3: ***Shows gene expression of VEGF on q-RT PCR analysis*****, TABLE 4*****: shows gene expression of BAX on q-RT PCR analysis***



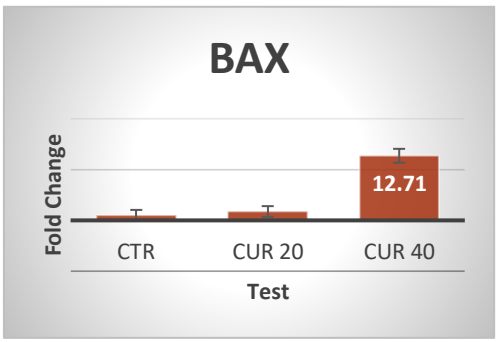


Figure 4: ***Graph showing test result of VEGF gene expression when treated with curcumin of different concentrations,*** **Figure 5*****: Graph showing test result of BAX gene expression when treated with curcumin of different concentrations***

To validate the result q-RT PCR analysis was used. Analysis of breast cancer cell line MDA-MB-231 by checking VEGF (which serve as a potent angiogenic factor favouring tumour growth and metastasis) mRNA gene expression and BAX (which act as pro-apoptotic regulators) mRNA gene expression in concentration of 20 and 40 µg/ml of curcumin comparing with untreated control. According to the RT- PCR result VEGF gene expression shows down regulation in 40 µg/ml curcumin, Similarly the pro- apoptotic regulator BAX gene expression shows upregulation in 40 µg/ml curcumin which shows anti-cancerous effect of curcumin in 40 µg/ml.

DISCUSSION

Curcumin, a polyphenol extracted from *Curcuma longa* in 1815, has gained attention from scientists worldwide for its biological activities (e.g., antioxidant, anti-inflammatory, antimicrobial, antiviral), among which its anticancer potential has been the most described and still remains under investigation. Antonio et al., (2019) reviewed on the cell signalling pathways involved in cancer development and proliferation, and which are targeted by curcumin. Cancer is one of the primary causes of death in industrialized countries (WHO, 2015). In recent years, the early diagnosis and increase in therapeutic options has reduced the death rate. However, the growth of drug-resistant cancers necessitates the search for innovative and more effective drugs (Baron et al. 2018). It is worth noting that cancer cells are characterized by deregulated signaling pathways involving proliferation, apoptosis, and angiogenesis (Al- Eijeh et al. (2010), Udagawa et al. 2010). In this scenario, curcumin represents a promising candidate as an effective anticancer drug to be used alone or in combination with other drugs. It affects different signaling pathways and molecular targets involved in the development of several cancers.

In present study, supercritical extracted curcumin powder (CP- 95%) was used to analyse the effect of compound in anti- angiogenesis through *in-vitro* and *in-vivo* assays. Different properties of this compound such as cell viability and angiogenic properties, were analysed. Regular turmeric that we consume contains only 2.5% of Curcumin. To test the properties of 95% extracted curcumin powder was given a different dimension to this work. The MTT assay is mainly done to find the effective concentration at which they show their maximum ability; these concentrations may be considered in the medicinal formulations to make them more useful. Through these assays, the lethal concentration was also found out, the effectiveness of these compounds in cancer cell lines helps to identify the anti-cancer property.

Wright et al. 2013 demonstrated growth inhibitory effects of the curcuminoids in breast cancer have previously been attributed to their targeting of cell cycle and/or apoptotic cellular pathways our demonstration of equipotent effects of the curcuminoids on cell viability, as measured by MTT assay is consistent with a pro-apoptotic, rather than anti-proliferative effect.

The MTT assay was done in breast cancer cell line to find the cell proliferative and apoptotic property of the compounds and found that there is a significant reduction in percentage of proliferation when concentration of test compound is increased.

Angiogenesis plays a critical role in the pathogenesis of several diseases such as retinopathy, rheumatoid arthritis, diabetes, psoriasis, and cancer. Extensive neovascularization is a key feature of cancer cells. Several studies indicated that natural plant products could meet inexpensive, less toxic, effective anti-angiogenic molecules, and novel (Lu et al. 2016). Angiogenesis is regulated by both activator and inhibitor molecules. The switch to the angiogenic phenotype involves a change in the local equilibrium between positive and negative regulators of angiogenesis. This signaling activates certain genes in the host tissue that make proteins which encourage the growth of blood vessels (Majima et al. (2000); Semenza (2002). Cancer cells require access to blood vessels for growth and metastasis. The discovery of angiogenic inhibitors provides hope for reducing the mortality and morbidity from carcinomas.

The study conducted by M Rajabi and S.A Mousa (2017) focused on the role of anti-angiogenesis strategies in cancer treatment, Angiogenesis plays a significant role in tumor progression. Effective inhibition of tumor angiogenesis might arrest or halt tumor progression but would not eradicate the tumor as a stand-alone therapy, especially with a single mechanism anti-angiogenic agent. Hence, the combination of an anti-angiogenesis agent and chemotherapy might be essential for effective tumor treatment.

The angio-inhibitory effect of 95% *curcumin* was assessed from the CAM Assay (figure 2 and 3). Reduction in the blood vessel and hemoglobin content in the chick embryo emphasized the anti-angiogenic capacity. The results supported the anti-angiogenic potential of the compound. Also, the result showed that at a concentration of 25µg/ml, the percentage of anti-angiogenesis is higher when comparing to other two concentrations.

Sulochana et al. (2007) reviewed the effect of curcumin as an inhibitor of angiogenesis. Curcumin, a novel small-molecular-weight compound, has been shown to inhibit carcinogenesis in different organs and the common link between these actions is its antiangiogenic effect. Curcumin is a direct inhibitor of angiogenesis and also downregulates various proangiogenic proteins like vascular endothelial growth factor and basic fibroblast growth factor. Curcumin's antiangiogenic effect is also in part due to its inhibitory effect on signal transduction pathways, including those involving protein kinase C and the transcription factors NF-kappaB and AP-1.

Curcumin has an inhibitory effect on two groups of proteinases involved in angiogenesis that are the members of the matrix metalloproteinase family and the urokinase plasminogen activator family. Cell adhesion molecules are upregulated in active angiogenesis and curcumin can block'this effect, adding further dimensions to curcumin's antiangiogenic effect. Curcumin shows a dose-dependent inhibition on tumor necrosis factor, a versatile cytokine, which has its effect on angiogenesis through the signal transduction pathways, expression of proangiogenic factors, and cell adhesion molecules. Curcumin's effect on the overall process of angiogenesis compounds its enormous potential as an antiangiogenic drug.

CONCLUSION

Humans, driven by their instinct, taste, and experience, treat illnesses using plants; hence, medicinal plants' history is as long as humans' history. Medicinal plants played a vital role as sources for drug lead compounds. They have been used against various diseases for thousands of years, and 80% of the world's population still depends on herbal medicine. The bioactive molecules present in the plant gives them unique therapeutic property. The disease-inhibiting capabilities make them extremely useful as a natural drug, provide essential bioactive compounds that are less toxic and more efficacious, and bring chemical and biological means of modification and extraction of natural products into a potent drug. Among various malignancies, breast cancer is considered the most prevalent malignancy in females, the therapy for treatment gains much attention worldwide. Chemotherapy is considered the most prevalent treatment for cancer. However, the treatment has multiple side effects, and long-term treatment leads to other diseases. Various studies are being carried out, searching for an alternative for the chemical drugs.

Curcumin (diferuloylmethane), is the major natural polyphenol found in the rhizome of turmeric has been shown different biological effects viz., anti-inflammatory, anti-oxidant, anti-carcinogenic and anti-infectious effects. The present study was to demonstrate the *in-vitro* and *in-vivo* anti-angiogenic effect of supercritical extracted curcumin. Angiogenesis assay and MTT assay were performed to analyse the effect of 95% curcumin powder, result was confirmed by testing q-RT PCR analysis. Since cancer cell lines provide the most suitable model for the preliminary studies of herbal compounds' anti-cancer potential, the MDA-MB-231 cell line was used for the analysis. Initially, the anti-viability of the compound was analyzed using an MTT assay. The results supported the anti-cancer potential of the compound. When comparing with the control, there was a dose-dependent decrease in cell viability with increasing concentration. The angio-inhibitory effect of 95% *curcumin* was assessed from the CAM Assay. Reduction in the blood vessel and hemoglobin content in the chick embryo emphasized the anti-angiogenic capacity. The results supported the anti-angiogenic potential of the compound. Also, the result showed that at a concentration of 25µg/ml, the percentage of anti-angiogenesis is higher when comparing to other two concentrations.The result was confirmed by testing q-RT-PCR analysis of two different gene expression. Analysis of breast cancer cell line MDA-MB-231 by checking VEGF (which serve as a potent angiogenic factor favouring tumour growth and metastasis) mRNA gene expression and BAX (which act as pro-apoptotic regulators) mRNA gene

expression in concentration of 20 and 40 µg/ml of curcumin comparing with untreated control. According to the RT- PCR result VEGF gene expression shows down regulation in 40 µg/ml curcumin, Similarly the pro- apoptotic regulator BAX gene expression shows upregulation in 40 µg/ml curcumin which shows anti-cancerous effect of curcumin in 40 µg/ml.

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