STUDY OF ANGIOGENIC POTENTIAL OF MORINGA OLEIFERA LAM. ON CHICKEN CHORIOALLANTOIC MEMBRANE (CAM)

Physiology

Vijaykumar Gupta*

Assistant Professor & PhD Scholar, Dept. of Physiology, D Y Patil University, School of Medicine Navi Mumbai, India. *Corresponding Author

Vivek Nalgirkar

Professor & Head, Dept. of Physiology, D Y Patil University, School of Medicine Navi Mumbai, India.

Geetanjali Gajanan Ranade

Researcher Goldfield Fragrances Pvt. Limited Prabhadevi, Mumbai, India

Yash Kripalani

Tutor and research fellow, dept. of Physiology, D Y Patil University, School of Medicine, Navi Mumbai, India.

ABSTRACT

Angiogenesis plays an important role in many physiological and pathological conditions. Currently, the drugs possessing pro-angiogenic property as compared to anti-angiogenic property are limited. The primary objective of study was to find the angiogenic potential of hot methanolic extract of leaves of Moringa Oleifera Lam. The hot methanolic extract of moringa leaves was prepared using soxhlet extraction. Phytochemical analysis was done by thin layer chromatography, ultra violet and infrared spectrophotometer. The egg candling instrument was prepared indigenously to observe the angiogenesis in Chick Chorioallantoic Membrane (CAM) model of hen's egg. The eggs obtained from Aarey Poultry, Goregaon (Mumbai) were observed for their fertility and Embryonic Development Day (EDD). The growth of embryo was observed every day till 7th day of EDD. The test sample was loaded on 8th day and the angiogenic effect was observed and photographed every day till 11th day. The Plermin™ ointment ($\beta\beta$ PDGF) (Platelet Derived Growth Factor) was used as positive control. Quantification of blood vessels was done with growth index [(No of BLVS11th day – no of blood vessels 8th day)/No of BLVS11th day], Abgel© sponge assay and compared with PDGF.

KEYWORDS

Arteries, Chorioallantoic Membrane, Angiogenesis, Moringa Oleifera, Chromatography, Healing

INTRODUCTION

Angiogenesis is the development of new blood vessels from the preexisting blood vessels. It is a highly regulated process from fetal life till the death of an organism. Angiogenesis plays a significant role in various physiological functions, normal growth and various pathologies. Many diseases can occur due to uncontrolled angiogenesis like vascular tumors, solid carcinomas, macular degeneration etc. Similarly, several complications like delayed wound healing, ischemia, ulcers, decreased somatic growth etc. can occur due to insufficient angiogenesis. Therefore, many diseases can invariably be treated or controlled by modulating the process of angiogenesis⁽¹⁾.

The list of drugs used to modulate angiogenesis is small and that of compounds from plant origin to modulate angiogenesis is still smaller. Therefore, therapeutic intervention to improve angiogenesis can be of significant value in clinical practice. There is wide use of Moringa as a wound healing agent in folklore medicine⁽²⁾.

The Pro-angiogenic property of Moringa oleifera commonly known as drumstick tree was tested. The extracts of dried leaves of Moringa are tested for their efficacy on angiogenesis. To experimentally study angiogenic potential, the Chick Embryo Chorioallantoic Membrane (CAM) model was chosen. The model was chosen because of its simplicity, rapidity, and low cost. The CAM is also used widely in pre-clinical in vivo studies⁽³⁾. The CAM is an extra embryonic membrane used to observe the angiogenic effect of drug. The membrane starts developing on 3rd day of embryonic development day (EDD) and the density of vessels can be changed and photographed for analysis. If the drug is pro-angiogenic then the density of blood vessels in CAM increases and if the drug is anti-angiogenic then the density of blood vessels decreases around the drug testing area on the membrane. The plant extract is loaded on CAM on 8th day and results are visualized on 11th day of (EDD). The efficiency of leaf extract is compared against Plermin™ an ointment containing ($\beta\beta$ PDGF) a known compound for angiogenesis⁽²⁾⁽⁴⁾.

METHODOLOGY

Preparation of Plant Extract.

The fresh leaves of Moringa oleifera were obtained from single tree from Palghar district of India. The leaves were shade dried for 20 days till the crackling sound were heard when they are crushed with hands. The Hot Methanolic extract was prepared from leaves and preserved in amber colored bottle. The property of extract was determined

according to protocols available in Ayurvedic Pharmacopeia⁽⁵⁾.

Chick Chorioallantoic Membrane (CAM) Study

The fresh fertilized white leghorn eggs *Gallus gallus* were obtained from Central poultry development organization, Aarey milk colony, Goregaon Mumbai. The embryonic development day (EDD) of the eggs is determined by trans-illumination using egg. It is confirmed that all the eggs have same EDD. The eggs were cleaned with 70% alcohol and kept in humidified Incubator and the temperature was maintained at 37th Centigrade. The eggs were marked and turned every 1 hour to facilitate normal growth of embryo till 7th day of EDD⁽⁶⁾. On 2nd day the pointed end of the egg is swabbed with neat Betadine and a small hole made in the shell using a dental drill bit 1mm and subsequently 2ml of albumen is aspirated from egg this provides the larger working area in the egg for subsequent procedures. The eggs were just observed unopened till 5th day and on 6th day the eggs were opened from broad end. The opened end of the eggs is covered with adhesive tape to avoid the loss of moisture from eggs. The test sample was applied on CAM on 8th EDD⁽⁷⁾. The eggs were divided in 3 groups. One group was used for test sample extract. Second group is used as a positive control group. And third group is used as a blank control.

Disc Method for Sample Application

The test sample is applied by loading 20 microliters of methanolic extract on 2mm disc of Whatman filter paper no. 1 and air dried for 1 hour before loading sample on CAM. Similarly, Plermin™ by Dr. Reddy's, ($\beta\beta$ PDGF) (Platelet Derived Growth Factor) is used as a positive control. And only methanol is loaded on blank control. One egg is also kept in incubator as an incubator control.

Figure I. CAM on 8th Day

Figure II. CAM on 11th Day

Sponge Method for sample application

In this method the test and standard samples were prepared of suitable concentration using 0.9% normal saline as a diluent. The sterilized small blocks of size 2mm³ of AbGel™ was cut from its large block. The small blocks were dipped into the samples and were soaked for 4 hrs. The soaked blocks were then placed on CAM. The angiogenic activity of the drug will allow the vessels to infiltrate the sponge and these sponge blocks are sectioned from CAM. The blocks are stored in 1% formaldehyde solution and then analyzed by histopathology using H & E staining technique (7). The neovascularization can be observed and counted by stained endothelial cells (figure 1).

Figure III. Slide showing sponge-tissue interphase at 100x, H & E stain
Large number blood vessels can be seen in sponge

Statistics

Using the one-way analysis of variance (ANOVA) the P value obtained is 0.0021. It is considered significant for this study. The mean value for each column vary significantly greater than expected.

Table I. Comparison between Moringa and Plermin

Comparison	Mean Difference	q	P value
MOG vs Plermin	-21.297	2.741 ns	P> 0.05

Table II. Mean difference between Moringa and Plermin

Difference	Mean Difference	95% Confidence interval	
		From	To
MOG - Plermin	-21.297	-49.097	6.504

The table above indicates the use of Tukey-Kramer multiple comparison test. Here Moringa Oleifera (MOG) is compared to Plermin and conclusions are derived accordingly. The second column indicates the mean difference, whereas, the third and fourth column indicate q and P values respectively. Here the q value is less than 3.578 hence the P value is greater than 0.05 according to the test.

Assumption test were done using the Bartlett's method where ANOVA assumed that the samples were collected from populations with identical standard deviations. This test could be only performed when each column has more than five values.

ANOVA also assumed that the data sampled from the populations follow Gaussian distributions. This assumption was done with Kolmogorov and Smirnov method. The following table contains the information for this method.

Table III. Normality test of Moringa and Plermin

Group	KS	P value	Passed normality Test
MOG	0.2090	>0.10	Yes
Plermin	Too few values to test		

The first column contains the groups of the test samples used in the

study which are moringa Oleifera and Plermin. The second and third column indicates the KS value and P value. The fourth column indicates whether it passed the normality test. The values provided are for only Moringa Oleifera as Plermin had too few values to test.

Table IV. Intermediate Calculations and ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean Square
Treatments (between columns)	2	4755.3	2377.7
Residuals (within columns)	20	5572.8	278.64
Total	22	10328.1	

F = 8.533 (MS treatment/ MS Residual)

Table V. Summary of Data

Groups	Number of points	Standard mean	Standard error of Deviation	Mean	Median
MOG	10	32.870	19.079	6.033	38.750
Plermin		54.167	12.332	7.120	60.000

Table VI. 95% confidence interval

Group	Minimum	Maximum	From	To
MOG	0.000	50.000	19.222	46.518
Plermin	40.000	62.500	23.529	84.804

Table VII. Collection of all Data

Collection of Titles	MOG (A)	Plermin (B)
Mean	32.87	54.166667
Standard Deviation (SD)	19.079	12.332
Sample Size (N)	10	3
Standard Error of Mean (SEM)	6.033	7.120
Lower 95% Confidence limit	19.222	23.529
Upper 95% Confidence limit	46.518	84.804
Minimum	0.000	40.000
Median (50th Percentile)	38.750	60.000
Maximum	50.000	62.500
Normality Test KS	0.2090	Too few values
Normality Test P value	>0.10	
Passed Normality test?	Yes	

Table VIII. Values of Moringa Oleifera abgel dose against growth

Concentration of Moringa Oleifera	Growth
1685	50.0
1685	40.0
3370	37.5
3370	50.0
5055	33.3
5055	42.9
6740	50.0
6740	0.00
8425	25.0
8425	0.00

Graphical representation in the form of Bar graph is provided below

Figure IV.

Figure V.

Linear Regression
 Number of points = 10
 Best – Fit standard 95% confidence level interval

Table IX. Linear Regression in tabular format.

Parameter	Best-Fit Value	Standard Error	95% Confidence interval	
			from	to
Slope	-0.004970	0.002031	-0.009653	-0.0002875
Y intercept	57.995	11.349	31.825	84.165
X intercept	11668			

Correlation Coefficient (r) = -0.6544

$r^2 = 0.4282$

Standard deviation of residuals from line (S_{y.x}) = 15.303

We need to know if the slope is significantly different from 0. The p value here is 0.0401, which is considered significant. This result was obtained from the following ANOVA table

Table X. Source of variations.

Source of variation	Degrees of Freedom	Sum of squares	Mean Square
Linear regression (model)	1	1402.8	1402.8
Deviations from linearity (Residual)	8	1873.4	234.17
Total	9	3276.2	

F = 5.991

Standard Curve representation

Figure VI. In the above graphical representation growth of moringa Oleifera is plotted on Y axis against concentration of Moringa Oleifera on X axis.

RESULTS

The Methanolic extract of Moringa leaves have shown strong angiogenic property when compared with Plermin[®] owing to its inflammatory response in CAM. Its phytochemical constituents are Arbutin, Anthraglycosides, Alkaloids, Flavonoids, Cardiac glycosides and Bitter principles the study suggests that Moringa Leaves possess a very strong angiogenic property and can be used as a natural substance instead of synthetic substances for Angiogenesis. In the photograph of

CAM on 11th day, there is visible increase in growth of Blood vessels in Moringa and Plermin. According to Tukey-Kramer Multiple Comparisons Test the p value is greater than ($P > 0.05$) of moringa compared to Plermin. If the value of q is greater than 3.578 then the P value is less than 0.05. A visible growth of blood vessels is seen in the CAM where moringa is applied as an angiogenic agent. It is comparable to Plermin which is known for its angiogenic potential. This shows that moringa Oleifera can be used as a potent natural substance to produce angiogenesis instead of a synthetically manufactured product.

DISCUSSION

Impaired wounds healing may occur in any individual but are more frequent in the elderly and chronically ill people. With an ageing population and a dramatically increasing prevalence of chronic diseases like cancer, diabetes, wound care will certainly become an even more noteworthy issue for health systems. Delayed wound healing is signified with alteration in physical properties of collagen, flattening of the dermo-epidermal junctions, nutritional depletion and cellular immunity leading to abnormal changes in pro-inflammatory and anti-inflammatory cytokines. A number of evidence has been collected to show immense potential of medicinal plants used in various traditional systems.

Moringa Oleifera is known to improve wound healing and aid in stunting bacterial growth. It is widely used in traditional medicine. The extract of this plant is used to study its angiogenic potential in the chorioallantoic membrane of the chick embryo. It is compared against plermin which is well known for inducing angiogenesis as a control. The utilisation of CAM for testing angiogenesis and anti-angiogenesis has become popular during the last 3 decades comprising of over 150 publications in 2003-04. The CAM is an alternative to animal model and provides a natural environment to grow blood vessels. The principle is to explant cells, tissue etc. on CAM directly to test substances implicated in angiogenesis in order to evaluate alterations in its natural of induced angiogenic capacity. Angiogenesis in CAM comprises of two different mechanisms, endothelial sprouting and intussusceptive microvascular growth (IMG). Endothelial sprouting is based on cell migration, proliferation and tube formation. IMG divides the vessels lumens by formation and insertion of tissue structure and pillars or post. The CAM assay has multiple advantages such as low cost, relative ease of preparation and carrying out various studies, simplicity, absence of mature immune system in avian embryos, more reliability, and readily available and feasible as an agent to conduct this study.

The required material was obtained and prepared accordingly, the extract was loaded on the CAM on the 8th day and observations were made throughout.

As per the observation, there was visible increase in blood vessels on the 11th day in both the control and the sample loaded with moringa extract which has been photographed, on the contrary, the CAM sample not loaded with any drug did not show improvement in angiogenesis. The study suggests that Moringa Leaves possess a very strong angiogenic property and can be used as a natural substance instead of synthetic drugs for Angiogenesis.

Author Contributions

Vijaykumar Gupta is the corresponding author and has led the research along with his team of four researchers mentioned above. Dr. Vivek Nalgirkar has been a guide and mentor to Mr. Vijaykumar Gupta and his team in conducting this research. Geetanjali Gajanan Ranade has provided a tremendous help in providing materials required to conduct this research. Dr. Yash kripalani has also provided support in editing and writing up the research manuscript along with observations made throughout the project.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Declaration of conflict of interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

- Judah Folkman, 1972. Antiangiogenesis: New concept for therapy of solid tumors.

- Annals of Surgery. Mar 175(3),409–416.
2. Shanshan Wanga, Zhengui Zheng et al. Angiogenesis and anti-angiogenesis activity of Chinese medicinal herbal extracts. *Jrnl. of life sciences* 74(2004)2467–2478.
 3. Angelica Vargas, Magali Zeisser-Labouèbe, The chick embryo and its chorioallantoic membrane (CAM) for the in vivo evaluation of drug delivery systems, *Advanced Drug Delivery Reviews* 59 (2007) 1162–1176
 4. Maija Bry, Riikka Kivelä et al. Vascular endothelial growth factor-b in Physiology and disease. *Physiol Rev* 94:779–794,2014.
 5. Government of India ministry of health and family welfare, department of ayush The Ayurvedic Pharmacopoeia Of India; Appendix-1, 139-146.
 6. Department of Anatomy and Embryology, University College London, Gower Street, W.C., U.K. A critical period for the turning of hens' eggs *J. Embryol. exp. Morph.* Vol. 5, Part 3, pp.293-299, September 1957.
 7. J. Clifford Murray, *Methods in molecular medicine, Angiogenesis Protocols.*, Humana Press Inc., Totowa, NJ, Vol. 46, 107-130.