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Short Communication

Complementary/Alternative Medicine Section

Assessment of the Effect of Moringa Oleifera Leaves Extract on Angiogenesis using the Novel Yolk Sac Model: An In-ovo Experimental Study

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ABSTRACT

Introduction: In recent times, the focus of cancer research has shifted to naturally occurring compounds derived from plants that may have the potential for anticancer activity. Moringa oleifera is a softwood tree whose fruits, roots, and leaves have been advocated for medicinal and industrial uses. Angiogenesis plays an important role in many physiological and pathological conditions and is considered a hallmark of tumour development and progression. An efficient anticancer drug is expected to have a significant inhibitory effect on angiogenesis. However, there are a limited number of studies reported to explore the anti-angiogenic activities of Moringa Oleifera Leaf extract (MOL).

Aim: To assess the effect of MOL on angiogenesis using the Yolk Sac Model (YSM).

Materials and Methods: A preliminary in-ovo study was conducted in the in the specialised Regenerative laboratory of Dr. D.Y. Patil Dental College and Hospital, Pimpri, Pune, Maharashtra, India, between September 2022 and February 2023 after obtaining necessary scientific and ethical permissions. The calculated sample size for YSM analysis was seven in each study and control group (total of 14); however, the authors used a total of 28 YSMs (seven in each of the three study concentration groups and seven in the control group). In this method, freshly fertilised White Leghorn chicken (Gallus domesticus) eggs, procured from the hatchery, were incubated and treated with different concentrations (10 µg/mL, 100 µg/mL, and 500 µg/mL) of the treatment substance (MOL) along with a control group (Avastin). The anti-angiogenic effect of MOL extract was determined by calculating vessel density, total vessel network length, total branching points, total segments, mean segment length, and width in the three groups compared to Avastin after 48 hours of treatment using WimCam software for analysing the images. Descriptive statistical analysis and one-way Analysis of Variance (ANOVA) were then applied to compare the parameters in the four groups.

Results: Statistical analysis by one-way ANOVA showed a significant ($p<0.05$) percentage reduction in the number of blood vessels in each treatment group after 48 hours of treatment. Among the different concentrations of MOL used, 500 µg/mL showed greater anti-angiogenic capacity.

Conclusion: The results indicate that MOL could suppress abnormal angiogenesis in a dose-dependent manner and may be considered a potential anti-angiogenic agent in various diseases, including cancer.

Keywords: Anti-angiogenic, Anticancer, In-ovo model

INTRODUCTION

Oral cancer is a disease with profound medical, economic, and social impact, especially in the Indian subcontinent, where the burden of oral cancer is extremely high, being amongst the three most common cancers [1]. The age-adjusted rate in the country is 20 per 10,000 population, and it is estimated to account for 30% of all types of cancers [2].

One of the major reasons for the high incidence of debilitation in cases of oral cancer is delayed diagnosis and the extreme side effects of therapies. The treatment of oral cancer poses a major financial challenge for patients, and most of the patients leave the treatment midway, further increasing the mortality rate. Hence, the delivery of affordable and equitable cancer care is one of the greatest challenges for India's public healthcare system [2,3].

In recent years, there has been a paradigm shift in the management of oral cancer with a focus on natural derivatives. Many natural derivatives have been reported to show potential effects against various forms of cancers in general, and oral cancer in particular [3]. Plants have been used since ancient times in traditional medicine and Ayurveda to treat various diseases and provide essential nutrients. Many plant derivatives, like curcumin and quercetin, have been reported to work effectively as anticancer, antioxidant, and antimicrobial agents [4]. Such naturally occurring substances, especially those with low toxicity and high potency, may also have important implications for chemotherapy and chemoprevention.

One such plant with immense healing potential and medicinal properties is Moringa oleifera, known as the "Wonder tree" in Ayurveda due to its array of medicinal properties [5]. Various studies have reported its potent hypoglycaemic, antibiotic, hypertensive, anti-inflammatory, and antitrypanosomal activities [6,7].

Some studies have also reported the potential anticancer activities of Moringa oleifera, particularly its apoptotic properties [8,9]. Before it can be established as a potent anticancer agent, its effects on all the different hallmarks of cancer must be analysed. Angiogenesis is an important hallmark of neoplastic activities, promoting tumour growth, recurrence, and metastasis. A substantial body of evidence suggested that targeting angiogenesis could provide a clinical benefits in the treatment of solid tumours [9,10]. There is sporadic data regarding the use of Moringa oleifera as an anti-angiogenic agent, and consequently, as an anticancer agent. To the best of the authors knowledge, the present study is the first study in the Indian subcontinent that used the novel and recently established YSM to study the effect of Moringa oleifera leaf extract on angiogenesis. If Moringa leaf extracts could be established as a potent anti-angiogenesis agent, it would be a breakthrough in the exploration of its further anticancer effects.

MATERIALS AND METHODS

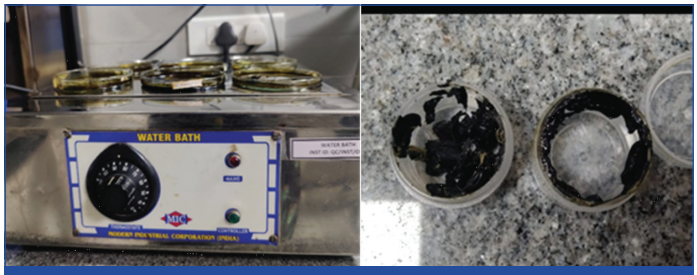
An experimental in-ovo study was conducted in the specialised Regenerative laboratory of Dr. D.Y. Patil Dental College and Hospital, Pimpri, Pune, Maharashtra, India, between September 2022 and February 2023. This timeline included the preparation of the sample drug, preliminary testing for its cytotoxicity, and the study of the anti-angiogenic activity under suitable conditions. The study was carried out after acquiring clearance from the Institutional Scientific Committee and Ethical Committee (Letter no: DPU/IEC/PhD/612(6)/2019).

Sample size calculation: Sample size calculation was done using Open Epi, Version 3 for the comparison of two means. The calculated sample size was seven in each group: the study (MOL) and the control (Avastin) group. To have a greater number of models to study and a margin to eliminate the models that were disqualified due to any technical problem, seven eggs were included in each concentration group and control as well.

Study Procedure

Moringa leaves (150 g) were acquired from the local vegetable market, dried, and powdered at the institutional Ayurvedic laboratory. The leaves were then suspended in 1 mL of cold water at four degrees Celsius followed by vigorous vortexing for one minute. Centrifugation was used to remove the water-insoluble parts, and supernatants were collected. The resultant MOL extracts were lyophilised and stored at -20ºC. For the experiments, lyophilised extracts were diluted with ethanol to concentrations of 10 µg/mL, 100 µg/mL, and 500 µg/mL [Table/Fig-1a,b]. These concentrations were selected as per the recommendations of previous researchers and a concomitant study conducted on the cytotoxicity of the test drug as part of this dissertation [7,8]. The safety of Moringa leaves extract, being a natural derivative, is well-documented; however, authentication was achieved from the Ayurvedic and molecular laboratory as per the protocol.

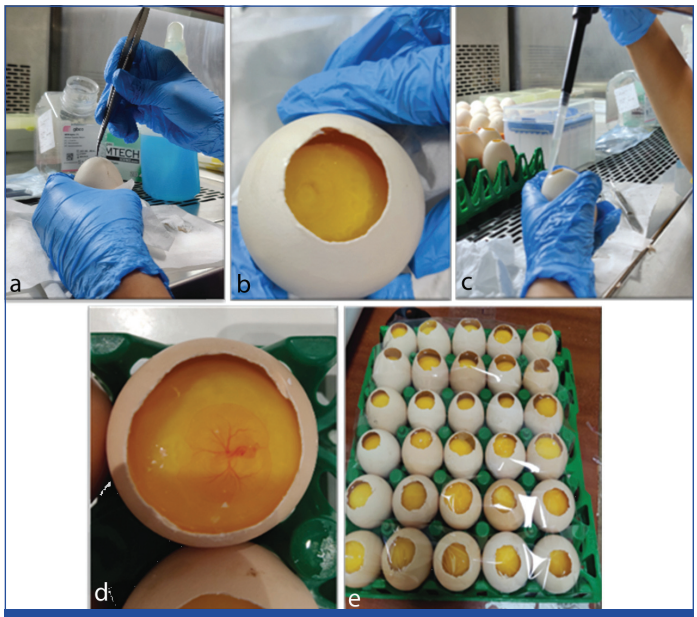
[Table/Fig-1a]: Fresh and dried Moringa Oleifera leaves.



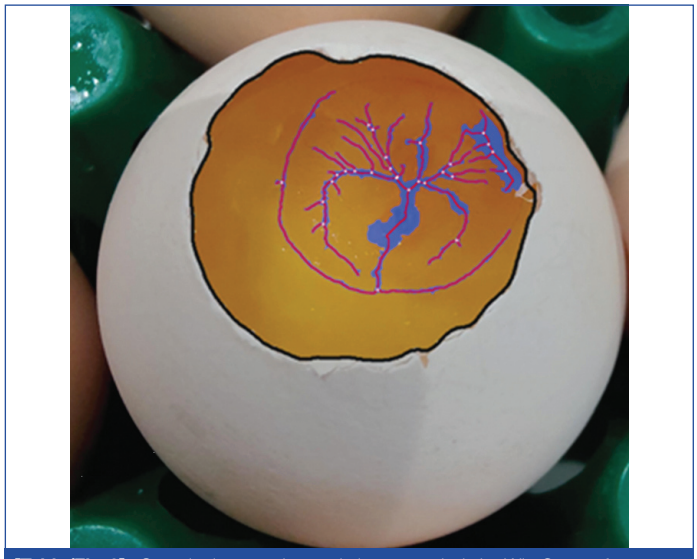
[Table/Fig-1b]: Preparation of lyophilised Moringa Oleifera Leaf (MOL) extract.

Yolk sac membrane model: A total of 28 YSMs were included and studied as described by Nihad ASM et al., [10]. Freshly fertilised hen's eggs were incubated at 37.5 degrees Celsius with 70-80% humidity for 48-72 hours. A 3×3 cm aperture was drilled on each egg, and a small amount of albumen was removed to expose the yolk sac. The eggs were treated with different concentrations of ethanolic extracts of MOL as indicated above. Avastin was used as the positive control. The eggs were then lightly sealed with cell tape and further incubated for 48 hours [Table/Fig-2a-d]. Afterwards, the aperture was widened, and photographs of the angiogenic network were taken for each treatment group [Table/Fig-2e].

The anti-angiogenic effect of MOL extract was determined by calculating the vessel density (%), total vessel network length (in pixels), total branching points, total segments, mean segment length, and width (in pixels) in the three groups compared to Avastin, post-48 hours of treatment using WimCam software for analysing the images [Table/Fig-3]. WimCam has been reported to provide objective and accurate measurements of the vascular structure of the YSM as well as CAM assays, such as the number of vessels and the vessel density of the membrane [11].



[Table/Fig-2]: Cyclic representation of preparation and assessment of YSM: a) Creation of aperture; b) Widening of aperture after 48 hours of inoculation; c) Treatment with test drug; d) Further incubation for 48 hours; e) Widening of aperture and imaging assessment of vasculature.



[Table/Fig-3]: Quantitative morphometric image analysis by WimCam software.

STATISTICAL ANALYSIS

Statistical analysis was conducted using descriptive statistics for different parameters related to vessel density and network characteristics.

RESULTS

The anti-angiogenic activity of different concentrations of MOL is represented in [Table/Fig-4]. The post-48 hours vessel network length in pixels for the yolk sac corresponding to 10 ug/mL and 100 ug/mL of MOL extract was extremely comparable to the control drug. The vessel network length was minimum in the 500 ug/mL of MOL (1508.6 px). The mean segment length for control in pixels (px) was 132.2, 107.8 for 10 ug/mL MOL, 150.9 for 500 ug/mL MOL, and 107.7 for 100 ug/mL MOL solution. The lowest vessel density was observed with 500 ug/mL of MOL extract (1.2%), whereas the

Concentrations of Moringa	Vessels density (%)	Total vessels network length (px)	Total branching points	Total nets	Total segments	Mean segment length (px)	Standard deviation segment length (px)	Mean segment width (px)	Standard deviation segment width (px)
Control 48 F	4.9	4893	15	3	37	132.2	129.1	11.5	2.8
Moringa 10 ug/mL 48 F	11.2	5821.4	26	1	54	107.8	119.8	15.2	11.3
Moringa 100 ug 48 F	3.6	4093.6	16	4	38	107.7	96.5	23	21.3
Moringa 500 ug/mL 48 F	1.2	1508.6	5	1	10	150.9	92.4	19.8	6.9

[Table/Fig-4]: Comparative anti-angiogenic activity parameters of control drug versus different concentrations of MOL extract.

concentration closest to the values seen in control (4.9%) was 100 ug/mL of MOL extract (3.6%).

The comparison of the parameters between groups is shown in [Table/Fig-5]. Statistical analysis by one-way ANOVA showed that all measured parameters show highly significant differences across the four groups, as indicated by p-values that are less than 0.001. These findings indicate that the Moringa treatments, at varying concentrations, have a significant impact on vascular properties when compared to the control group.

One-way ANOVA (Fisher's)				
	F	df1	df2	p
Vessel density	2273.97	3	24	<0.001
Total vessels network length	396884.02	3	24	<0.001
Total branching points	151.53	3	24	<0.001
Total nets	9.63	3	24	<0.001
Total segments	1552.53	3	24	<0.001
Mean segment length	6371.97	3	24	<0.001
Mean segment width	1142.40	3	24	<0.001

[Table/Fig-5]: Comparison of the parameters between groups.

DISCUSSION

In this preliminary study, an attempt was made to explore the antiangiogenic effect of different concentrations of MOL compared to Avastin, a well known antiangiogenic agent that achieved promising results. Angiogenesis refers to the growth of new blood vessels from previously existing vasculature. It is a prominent physiological process during embryogenesis and subsequently in various physiological and pathological processes. It is a well-orchestrated mechanism with a fine balance between angiogenesis factors and inhibitors. Excessive and uncontrolled vascular growth is a major hallmark of cancer, and targeting it is a vital requirement of any anticancer therapy [9].

Moringa oleifera is known to have multiple medicinal properties, and its anticancer properties have been a topic of research recently. The YSM has been established as a novel method to study the process of angiogenesis process. The in-ovo techniques are considered superior to in-vitro methods as they can closely replicate biological processes. Amongst the in-ovo methods, the Yolk Sac Membrane model is increasingly popular due to its simplicity, efficiency, and feasibility. The present study utilised the YSM model as described and documented by As MN et al., [10]. YSM has been recognised as an effective experimental model for studying angiogenesis [11]. However, there are certain technical hindrances associated with analysing this model, the most important of which is the lack of a quantifiable method to assess vascular growth. Artificial intelligence tools like Image J software and WimCam have been reported as effective ways to analyse this model in the past [10]. In the present study, the latter software was used for the morphometric analysis of the anti-angiogenic effect, in terms of parameters like vascular density and branching. The study found that Moringa oleifera had a similar effect to the well-established anticancer therapeutic drug, Avastin.

The results suggest that there was a significant reduction in angiogenesis with all treatment concentrations of the ethanolic extract of MOL. Also, there was an increase in anti-angiogenic activity with the rising concentration of the extract. The treatment group with 500 µl/mL extract showed the lowest vascular density after 48 hours. This finding aligns with previous studies by authors like Pachava VR et al., and Dharani S et al., which suggested a dose-dependent anti-angiogenic effect of Moringa extracts [12,13]. According to the study by Pachava VR et al., a dose of 100 µg showed greater inhibition of vascularisation compared to concentrations of 1 and 10 µg/mL [12]. In the present study, a higher concentration of 500 µg/mL was used, and its anti-angiogenic potential was found to be superior to the other two concentrations, clearly indicating a dose-dependent activity. Furthermore, only one of the YSM models treated with 500 µg/mL lost vitality, highlighting the significantly low toxicity of the treatment agents.

Limitation(s)

A meaningful analysis and quantifiable assessment are always the biggest challenges of in-ovo analysis. The technical expertise and training of investigators require resourceful laboratories, which may be challenging in different research settings. Additionally, the present study is a preliminary part of an ongoing study to analyse the overall anticancer activity of MOL extract. Therefore, certain molecular aspects of the same have not been discussed here and will be included in further reports once they are studied completely.

CONCLUSION(S)

At the studied concentration in the study, MOL extracts act as potent anti-angiogenic agents compared to the established anti-angiogenic drug Avastin. Further studies with various in-ovo and in-vivo (animal) models are recommended to more deeply study the anti-angiogenic effect of MOL extract.

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