

The Anti-Angiogenic Properties of *Morinda citrifolia* L. (Mengkudu) Leaves using Chicken Chorioallantoic Membrane (CAM) Assay

Zulhabri Othman^{1,2*}, Hamimi Rasyiqah Hassan Khalep¹, Azrina Zainal Abidin¹, Halijah Hassan¹, Santosh Fattepur³

Zulhabri Othman^{1,2*},
Hamimi Rasyiqah Hassan
Khalep¹, Azrina Zainal
Abidin¹, Halijah Hassan¹,
Santosh Fattepur³

¹Department of Diagnostic and Allied Health Science, Faculty of Health and Life Sciences, Management and Science University, 40100 Shah Alam, Selangor, MALAYSIA.

²School of Graduate Studies, Post-Graduate Centre, Management and Science University, MALAYSIA.

³School of Pharmacy, Management and Science University, 40100 Shah Alam, Selangor, MALAYSIA.

Correspondence

Dr. Zulhabri Othman

Department of Diagnostic and Allied Health Science, Faculty of Health and Life Sciences, Management and Science University, 40100 Shah Alam, Selangor, MALAYSIA.

Phone no : +60-03-5521 6454

E-mail: zulhabri_othman@yahoo.com

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ABSTRACT

Background: Anti-angiogenesis or inhibition of blood vessel formation is the best way to prevent the growth and metastasis of tumors. The use *Morinda citrifolia* L. extracts have been reported to exhibit a broad range of therapeutic effects, including antibacterial and antitumor.

Objective: This study aims to investigate the anti-angiogenic properties of *Morinda citrifolia* L. leaves extracts using Chicken Chorioallantoic Membrane (CAM) assay.

Materials and Methods: The Fertile White Leghorn eggs were divided into five groups which were control, Bevacizumab drug and treatment groups with 25%, 50% and 75% of *Morinda citrifolia* L. leaves extracts respectively. The reduction of the blood vessel was counted after 12 h and 24 h post-treatment. **Results:** Analysis have shown significant differences ($P < 0.05$) in the reduction of the blood vessel between each group at 24 h post-treatment. The group with 75% extracts showed 37.1% reductions of blood vessel compared to groups 50% and 25% extracts which showed 4% and 12.8% respectively. The phytochemical screening has showed that *Morinda citrifolia* L. leaves extracts was positive for flavonoid, phenols and phytosterols.

Conclusion: *Morinda citrifolia* L. leaves extracts consist of the phytochemical compounds that have the ability to inhibit the angiogenesis.

Key words: Anti-angiogenesis, *Morinda citrifolia* L. leaves, Phytochemical, White Leghorn eggs, Chicken Chorioallantoic Membrane (CAM) assay.

INTRODUCTION

Cancer has the ability to spread to adjacent or distant organs, which makes it life threatening. Tumor cells can penetrate blood or lymphatic vessels, circulate through the blood system and then proliferate at another site which is called metastasis.¹ Angiogenesis is a multistep process leading to the formation of new capillaries emerging from pre-existing blood vessel systems. Recruitment of new blood vessels plays an important role in tumor survival and growth.² Abnormal blood vessel growth, either excessive or insufficient, is now recognized as a “common denominator” underlying many deadly and debilitating conditions, including cancer.³

Morinda citrifolia L. has been heavily promoted for a wide range of uses, including arthritis, atherosclerosis, burns and cancer.⁴ Studies have shown that many of these bioactive components possess anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial and anti-viral activities. Several local plants extracts have shown to have phytochemical compounds such as tannins, alkaloid, steroids, flavonoids and saponins that shown to have anti-bacteria activity especially towards *Staphylococcus aureus*.⁵⁻⁷ *Morinda citrifolia* L. extracts also have

shown to decreased inflammation, oxidative stress, cortisol and hexose-6-phosphate dehydrogenase (H6PD) expressions in cancer cells.⁸ The previous phytochemical studies reported that Noni's leaves contain a variety of phytochemical constituents such as terpenoids, phytosterol, flavonoid glycosides and anthraquinone.^{9,10}

This study aims to investigate the phytochemical properties and anti-angiogenic activity in *Morinda citrifolia* L. leaves extract in chicken chorioallantoic membrane (CAM) assay. Besides the study would emphasize on the identification of the phytochemical compound of *Morinda citrifolia* L. leaves extracts. Furthermore, to examine the anti-angiogenic properties of *Morinda citrifolia* L. leaves extraction using CAM assay and thus to compare the effect of *Morinda citrifolia* L. leaves extraction with Bevacizumab drug.

MATERIALS AND METHODS

Collection of Plant Material

The samples were collected locally at Kampung Kenangan Puchong and the identification species was at Forest Research of Institute Malaysia (FRIM),

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Kepong, Selangor, Malaysia. The specimen identification of *Morinda citrifolia* L. leaves was obtained from the Herbarium of Forest Research Institute Malaysia (FRIM).

Preparation of Plant Extracts

The samples were wash, cut into small pieces and dried in an oven at 37°C for 72 h and then ground into a fine powder with electrical grinder. 200 g of *Morinda citrifolia* L. powder was soaked into 95% methanol for 24 h and filtered with Whatman no. 1 filtered paper and concentrated via rotary evaporator.

Phytochemical Screening

The *Morinda citrifolia* L. leaves extracts was then proceeding to phytochemical screening for flavanoid, phenols and phytosterol.

Flavanoids

The lead acetate test was used to determine the presence of flavanoids in a given sample. Dried samples (5 mg) were mixed with a few drops of lead acetate solution. The formation of yellow colour precipitate indicates the presence of flavanoids.¹¹

Phenols

The ferric chloride test was used to determine the presence or absence of phenols in a given sample. The dried sample (5 mg) were added with 3-4 drops of ferric chloride solution. The formation of bluish black colour indicates the presences of phenols.¹¹

Phytosterols

Salkowski's test was used to determine the presence or absence of phytosterol (plant sterol) in a given sample. Extracts (5 mg) were mixed with 2 ml of chloroform and filtered. The filtrates were added with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden brown colour indicates the presences of phytosterols.¹¹

Ex-ovo Chicken Chorioallantoic Membrane Assay (CAM)

The White Leghorn eggs were incubated for three days at 40°C and 72±2% of relative humidity. On the third day, the eggs were cracked and the eggs content was transferred to the Petri dish followed with incubation for another three days. All the eggs were divided into five groups (control, Bevacizumab drug, treatment groups with 25%, 50% and 75% extracts). Each group was done triplicate.

The extracts were diluted with 100 ml of distilled water and 100µl of DMSO. Two pieces of Whatman no. 1 filtered papers with the size of 5mm were put on the top of a fine blood vessel. Each filtered paper was added with extracts and PBS (phosphate buffer saline). PBS function was to moist the embryo and avoid it from dry. To maintain the survival of the embryo, PBS were applied every 4-5 times daily since day 6. The extracts were added on the day 6. The image of the blood vessel was captured using camera after 12 h and 24 h post-treatment.

Data and Sample Analysis

The data was analyzed for the statistical image of identification and also the comparison of *Ex-ovo*. Chorioallantoic Membrane (CAM) Assay. The results of CAM assay were recorded and analyzed with one-way ANOVA (Analysis of Variance) using SPSS™ (Statistical Package for Social Science) version 20. The data were collected at two different time points which were at 12 h and 24 h post-treatment. The significance reduction of the blood vessel reported with $p < 0.05$.

RESULTS

Phytochemical Screening

The phytochemical screening shows positives, indicates for the presence of flavanoids, phenols and phytosterols (Table 1) in the *Morinda*

Table 1: Phytochemical screening of flavonoid, phenols, phytosterols shows positive results for the *Morinda citrifolia* L. extract.

Phytochemical test	Results
Flavonoids	+
Phenols	+
Phytosterols	+

citrifolia L. extracts. For flavonoid shows the appearance of yellow colour, meanwhile for phenols shows the formation of bluish black colour and for phytosterols shows the appearances of golden brown or yellow colour. The phytochemical screening of *Morinda citrifolia* L. leaves extract shows the presences of all main phytochemical compounds such as flavanoid, phenols and phytosterols.

The Reduction of Blood Vessel

The blood vessel has shown reduction with different concentrations of *Morinda citrifolia* L. leaves extraction (25%, 50% and 75%) after 12 h and 24 h post-treatment on *Ex-ovo* chicken chorioallantoic membrane assay (CAM) (Figure 1). Results have shown significant differences ($P < 0.05$) in the reduction of the blood vessel between each group at 24 h post-treatment. At 75% extraction, after 12 h the blood vessel showed prevalent reduction to about ±21.7% and after 24 h the reduction of the blood vessel reduces to ±37.1%. Meanwhile, at 50% extraction, 12 h have showed that reduction of the blood vessel increasingly reduce to ±9.5% and 24 h to ±12.8%. However, at 25% extraction, for 12 h only a few reductions occur about ±2% and 24 h about ±4%. For group with Bevacizumab drugs after 12 h the reduction of blood vessel reduces greatly compared to other extractions. It reduced about ±38.8% and after 24 h the blood vessel reduces to ±49.2% (Figure 2). The results of control groups for 12 h and 24 h do not show any reduction of the blood vessel.

The overall phase shows the results of the reduction of the blood vessel of *Morinda citrifolia* L. leaves extract after 12 h and 24 h. There were significant differences on the percentages of blood vessel reduction between control group and with treatment groups. For the control group, there was no reduction of blood vessel occur, meanwhile for the lowest percentage was 25% extraction about 2% reductions and 4% reduction after 12 h and 24 h respectively. However, the highest percentage of the reduction was Bevacizumab drugs about 39% and 49% after 12 h and 24 h respectively.

The overall phase shows the result of the reduction of blood vessel with *Morinda citrifolia* L. leaves extract treatment after 24 h. The bar chart was performed the reduction of blood vessel from the baseline 12 h. The control doesn't show any reduction of blood vessel. Somehow, 25% extraction shows reduction to 144 of blood vessel and was the lowest reduction after 24 h. Meanwhile, the greater reduction of blood vessel was shows at 50% extraction compared to 25% extraction. On the other hand, at 75% extraction the blood vessel reduces to 95 blood vessels. However, for Bevacizumab drugs the blood vessel shows increasingly reduces to 77 blood vessels.

The overall phase shows that, there was a significant reduction of the blood vessel in different concentration after 12 h and 24 h. In this direction, the plant is being actively explored as a source of new chemical substances that can inhibit angiogenesis. Independent of this effect in this study, it is clearly elucidated that anti-angiogenic activity of *Morinda citrifolia* L. leaves extract by performing *ex-ovo* anti-angiogenesis assay. It has been observed that *Morinda citrifolia* L. significantly inhibits the development of capillary networks in CAM.

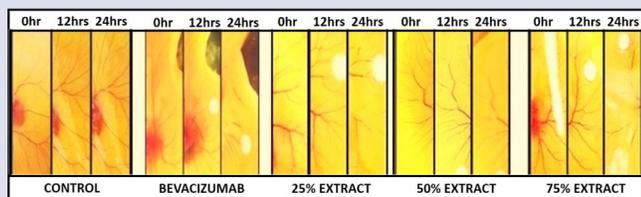


Figure 1: The reduction of the blood vessel with different concentrations of *Morinda citrifolia* L. leaves extraction (25%, 50% and 75%) after 12 h and 24 h post-treatment on *Ex-ovo* chicken chorioallantoic membrane assay (CAM).

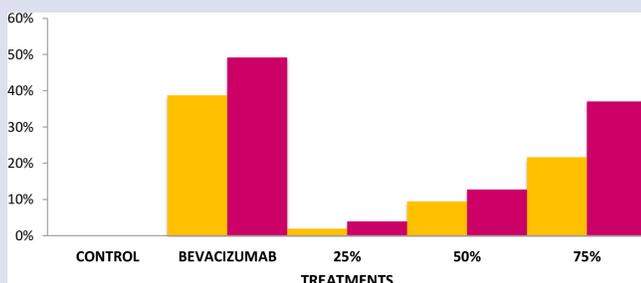


Figure 2: Percentage of the reduction of blood vessel. The higher percentage of blood vessel reduction was at 75% extracts of *Morinda citrifolia* L. compared to 50% and 25% extracts.

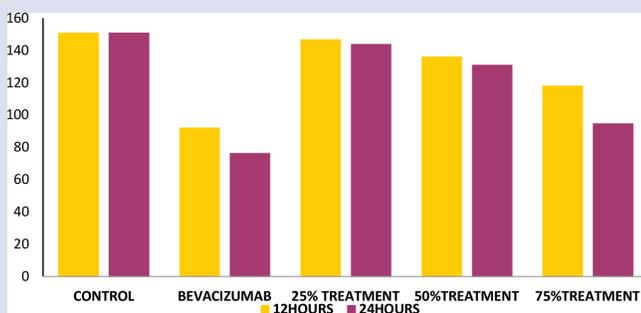


Figure 3: The comparison reduction of blood vessel after 12 h and 24 h. For both 12 h and 24 h 75% extracts of *Morinda citrifolia* L. shows the higher reduction of blood vessels compared to 50% and 25% extracts groups.

Comparison Reduction of Blood Vessel between 12 h and 24 h

Assay with 75% extracts shows the highest reduction in the blood vessel formation after 24 h compared to 25% and 50% extracts groups (Figure 3). Thus, the 75% extracts shows decreasing numbers of blood vessel almost likely as Bevacizumab drug. Control group didn't show any reduction of the blood vessels.

DISCUSSION

A previous study by Saad *et al.* has suggested that *Morinda citrifolia* L. serve as a potent source of natural anti-oxidant and exhibit high of flavonoid, tannins and anthraquinones.¹² Flavanoid and phenols are antioxidants compound which have role in cancer prevention.¹³ Mean-

while, phytosterol has shown to inhibit angiogenesis.¹⁴ *Morinda citrifolia* L. extracts have shown to have a strong correlation between carotenoids and ascorbic acid, tannin, flavonoids and phenol. The genotypes showed wide range of antioxidant capacity which showed positive correlation with carotenoids, flavonoids and Cu, Mn and Mg.¹⁵ Study by Adnan *et al.* on photophysical properties of five natural anthraquinone as one of active compound from *Morinda citrifolia* L. extracts that may have medicinal value.¹⁶

This study suggests that *Morinda citrifolia* L. leaves extract at 75%, strongly exhibit the anti-angiogenic activity. This study showed that the concentration of 75% extraction able to inhibit the formation of blood vessel, were more prominent compared to the concentration of 25% extraction after 12 h and 24 h of the extraction after treatment. These finding have in agreement with a report by Beh *et al.* that *Morinda citrifolia* L. extracts possess anti-angiogenic activity using CAM assay.¹⁷ In addition, it also has almost same ability to inhibit the blood vessel like Bevacizumab drugs. A previous study by Piaru *et al.* has showed that *Morinda citrifolia* L. oil have a tendency to reduce the growth of the blood vessel in anti-angiogenic effect using *ex-vivo* on rat aortic rings.¹⁸ Thus, it may have a potential to be useful deactivator of numerous serious disease characterized by angiogenesis. The virgin coconut oil has shown to possesses anti-atherosclerotic activity by decreases in atherogenic index and improve lipid profile while increase HDL level in rats.¹⁹

CONCLUSION

Morinda citrifolia L. leaves extracts have shown to contain phytochemical compounds and has the ability to inhibit the formation or growth of blood vessel. This study shows that the use of *Morinda citrifolia* L. leaves extract has a potential of anti-angiogenesis properties. The average branch points or blood vessel in the White Leghorn eggs using CAM assay was decreased with higher treatment of *Morinda citrifolia* L. leaves extract. The study has been concluded that *Morinda citrifolia* L. leaves extract has promising anti-angiogenic properties and a possible source of chemotherapeutic agent against cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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GRAPHICAL ABSTRACT



SUMMARY

- This study aims to investigate the anti-angiogenic properties of *Morinda citrifolia* L. leaves extracts using Chicken Chorioallantoic Membrane (CAM) assay. The Fertile White Leghorn eggs were divided into five groups which were control, Bevacizumab drug and treatment groups with 25%, 50% and 75% of *Morinda citrifolia* L. leaves extracts respectively. Results have shown significant differences ($P < 0.05$) in the reduction of the blood vessel between each group at 24 h post-treatment. The group with 75% extracts showed 37.1% reductions of blood vessel compared to groups 50% and 25% extracts which showed 4% and 12.8% respectively. As a conclusion *Morinda citrifolia* L. leaves extracts consist of the phytochemical compounds that have the ability to inhibit the angiogenesis.

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