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

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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, anticarcinogenic, 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 to compare the effect of Morinda citrifolia L. leaves extraction with Bevazicumab 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 were 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 added 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. 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 egg contents 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 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 ±9.3% 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.

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

<table>
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<tr>
<th>Phytochemical test</th>
<th>Results</th>
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<tr>
<td>Flavonoids</td>
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<td>Phenols</td>
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that antioxidants compound which have role in cancer prevention, flavonoid, tannins and anthraquinones. A previous study by Saad et al. has suggested that 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),

Thus, the 75% extracts shows decreasing numbers of blood vessel almost similar as Bevacizumab drug. 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. 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.

**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.

**REFERENCES**

8. Shalan N, Mustapha NM, Mohamed S. Morinda citrifolia leaf enhanced perfor-

**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.