Antiangiogenic Activity of *Pithecellobium Dulce* Methanolic Leaf Extract Using Hen Cam Method

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Abstract

Switching from the avascular phase to the vascular phase is a necessary process for tumor growth but none of the conventional therapies targets the inhibition of cancer angiogenisis. The present study aims at the extraction of the flavonoid from the leaves of Pithecellobiumdulce plant using methanol and determining the antiangiogenic activity of the methanolic extract using the innovative HEN-CAM model. Materials and Methods: The PithecellobiumdulceMethanolic leaf extract (PDME) was subjected to the phytochemical analysis. Thirty eggs were procured and divided into five groups(n=6). Group I, II III, IV and V are treated with Distilled Water, Hydrocortisone, PDME 10 µg/ml, 100 µg/ml and 1000 µg/ml respectively. The HEN- CAM Method was used to determine antiangiogenic effect of PDME using Antiangiogenic score, Branching Points and CAM Vascularity of eggs. The final results of our study had shown that the Hydrocortisone is having more angiogenic score (2), followed by the 1000 µg/ml PDME (1.89). Branching points of Distilled Water is 83.00, Hydrocortisone is 48.00, 1000 µg/ml PDME is 46.00. Based on the computed data using the formula for CAM Vascularity, Hydrocortisone and 1000 µg/ml PDME had shown the highest inhibition. The present study findings, conclude that the PDME containing phytochemicals like flavonoids, can act directly on the tumor blood vessels, inhibit the origin of the tumour at their molecular level and produces a strong antiangiogenic effect. In future the anticancer theraphy should also include the use of cheap, less toxic, easily available antiangiogenic plant extracts like PDME, to inhibit the progression of cancer.

Keywords: Antiangiogenic, Pithecellobium Dulce, Methanolic, Leaf Extract, Hen Cam

Introduction

Angiogenesis is the physiological process through which new blood vessels are formed from the pre-existing vessels.Various proangiogenic factors include VEGF, Granulocyte Colony-Stimulating Factor **(G-CSF),** Interleukin-8 **(IL-8),** Transforming Growth Factors alpha and beta (**TGF-α** and **TGF-β**)[1,2].Switching from the avascular phase to the vascular phase is a necessary process for tumor growth but the conventional cancer theraphies focuses only on cytotoxic effect of the cancer cells and none of them targets cancer angiogenesis[3,4,5]. Therefore, the present study focuses on determining the antiangiogenic activity of flavonoids present in the *Pithecellobium dulce* methanolic leaf extract (PDMLE) using HEN-CAM method. The use of common available plant extracts containing flavonoids like kaempferol, quercitin can alter the expression of HIF and VEGF in the cancer cells[6,7]. If these angiogenesis inhibitors under trails are prove to be both safe and effective in treating human cancer, they may be approved by the FDA.

***Pithecellobium Dulce* PlantProfile**

Pithecellobium dulce(Roxb.) Benth. (FamilyLeguminosae, is one of 100-200 species in this genus. A large, nearly evergreen tree that grows up to 20 m or more in height. At the base of each leaf is normally found a pair of short, sharp spines, though some specimens are spineless.It contains the flavanoids, tannins, fixed oil, olein. Leaves yield quercitin, kaemferol, dulcitol and afezilin.Reported activities include Astringent, emollient, abortifacient, antidiabetic [8], antioxidant, antibacterial potential [9], CNS depression action [10]and gastroprotective effect [11]. Though*Pithecellobium dulce* extracts were proved to have many biological activities, none of the previous studies focused on the antiangiogenic activity.

Materials and Methods

Reagents and Chemicals

Absolute methanol (Research lab fine chem industries, Mumbai), Hydrocortisone (Himedialaboratories Pvt. Ltd., Mumbai), Silica Gel (Finar Chemicals Ltd., Ahmedabad), Ninhydrin Reagent (Finar Chemicals Ltd., Ahmedabad).

Animals/Experimental models

Fertilised eggs of native chick obtained from the poultry farm in Vijayawada, maintained at ambient temperature. This project was approved by the Institutional Ethical Committee for Animal Experimentation. Eggs weighing<60 or >70 g, more than 7 days after release were excluded. Eggs were incubated in incubator at 37°C and 60% relative humidity (RH).

Equipments

Incubator, Microscope, Centrifuge, Thin-layer Chromatogram.

Experimental Methodology

Extraction[12]

Leaves of the *Pithecellobium dulce* were collected from the local areas of Vijayawada and the plant was authenticated by the Department of Pharmacognosy, Vijaya Institute of Pharmaceutical Sciences for Women. The leaves were shade dried, finely powdered sieved, and subjected to extraction using soxhlets apparatus using Methanol.

Phytochemical Analysis[13]

The phytochemical analysis of the methanolic extract was performed as mentioned by M. Yadav *et al*., 2014. Tests for detection of flavonoids like Shinoda test, Sodium hydroxide testwas performed.

Antiangiogenic effect of *Pithecellobium Dulce* methanolic leaf extract using HEN- CAM Method[14]

Fresh, intact chicken eggs less than one week after laying are collected and divided into five groups(n=6) that is Group I, II, III, IV, and V. The Group I(Negative control) receives DistilledWater, Group II (Positive control) receives Hydrocortisone. Group III, IV and V receives the PDMLE10 µg/ml, 100 µg/ml and 1000 µg/ml respectively.All eggs are incubated for nine days, their blunt ends are then (on day 10) illuminated with a candling lamp. During candling, the air space outlined at the blunt end of the eggs is marked[15]. The eggs are opened at the marked end and cellulose discs impregnated with drug solutions were dried and placed carefully in the corresponding groups. The opened egg ends are closed

with the sterile cellophane tape and are replaced in the incubator. The eggs are removed from the incubator on the 12th day and the occurrence of haemorrhage, lysis of blood vessels and coagulation was observed and compared among different groups using Photomicrography[16].

Antiangiogenic scoring of PDMLE[17]

The inhibitory effects of the drugs on angiogenesis in chorioallantoic membrane were evaluated under a microscope and assessed according to the scoring system used previously in several studies. In this scoring system, the change in the density of the capillaries around the pellet and the extent of the effect are evaluated. In the initial scoring of each subject, a score of 0 indicated the absence of any demonstrable antiangiogenic effect (normal embryo and no difference in surrounding capillaries); 0.5 indicated a very weak antiangiogenic effect (no capillary-free area, but an area with reduced density of capillaries not larger than the pellet area); 1 indicated a weak moderate antiangiogenic effect (a small capillary-free area or a small area with significantly decreased density of capillaries, less than double the size of the pellet); and 2 indicated a strong antiangiogenic effect (a capillary-free area around the pellet equal to or more than double the size of the pellet). The equation used for the determination of the average score was as follows:

Average score = [Number of eggs (Score 2) × 2 + Egg Number (Score 1) × 1] /
[Total number of eggs (Score 0, 1, 2)].

Statistical Analysis

The angiogenesis scores were compared with a Kruskal-Wallis ANOVA test and a Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

Branching points of eggs treated with PDMLE[18]

The number of branching points of the blood vessels in the disc region of different groups of experimental models was determined by using Angiotool Software. The analysis was based on the state and intensity of the bloodvessels in the photograph.

CAM vascularity of PDMLE[19, 20]

The CAM Vascularity inhibition was expressed as % of the control:

No. of branching points(treated) - No. of branching points(negative control)×100/ No.of branching points(negative control)

Results and Discussion

The percentage yield of PDMLEwas found to be 86%. The phytochemical analysis shows that PDMLE consists of Phenols, Flavonoids, Saponins, Alkaloids and Glycosides. Table-01 shows the major constituents present in the PDMLE, may contribute to the antiangiogenic effect.

Antiangiogenic Image Analysis

There are many *in vitro*, *in vivo* and *in ovo* angiogenic assays but the most difficult challenges in the angiogenesis studies is the selection of an appropriate assay for its evaluation and quantification. Among all the methods, HEN–CAM method is a simple and reliable method. The antiangiogenic effect of PDMLE was included in the Figure-01, 02, 03,04, and 05. Leaves of the *Pithecellobium dulce* of Leguminosae family was collected from the local areas of Vijayawada. The

leaves were shade dried, finely powdered and are subjected to soxhlets extraction using methanol as solvent. The percentage yield of the final PDMLE was found to be 86.5%.

Table 01: Phytochemical Analysis of PDMLE

S. No	Chemical Constituents	Result
1	Flavonoids	+
2	Phenols	+
3	Saponins	+
4	Alkaloids	+
5	Tannins	_
6	Glycosides	_
7	Lipid	_
8	Terpenoids	_

The phytochemical analysis was performed as mentioned by M. Yadav *et al*., 2014 and the extract had shown positive results for the phytochemicals like flavonoids, phenols, saponins, alkaloids and glycosides. The extract was also analysed for the flavonoid specific tests and had shown positive result, there by confirms the qualitative presence of flavanoids in the PDMLE.All the three concentrations of PDMLE have comparable anti-angiogenic effects. They were able to lower down the number of blood vessels like the positive control when compared to the negative control.

Figure 01: Group I (Negative control, Distilled Water treated)

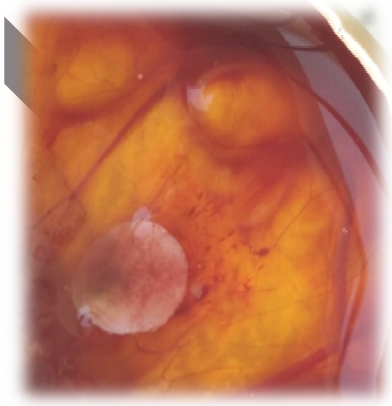

Figure 02: Group II (Positive control, Hydrocortisone treated)

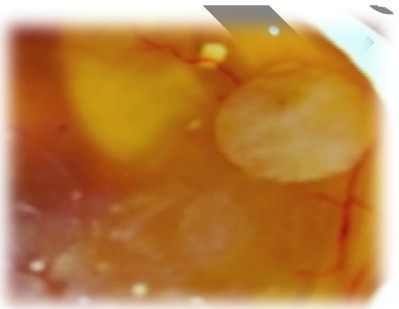

Figure 03: Group III (10ug/ml PDMLE treated)

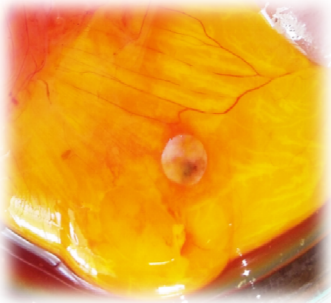

Figure 04: Group IV (100ug/ml PDMLE)

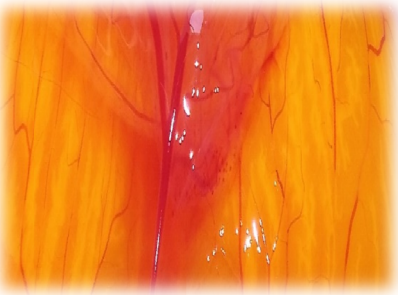

Figure 05: GROUP V (1000ug/ml PDMLE)

Angiogenic score indicates the growth of the blood vessels[17]. More the scoring value, more will be the inhibitory effect on the blood vessel[21,22]. The positive control is having more angiogenic score with the value of 2, followed by the 1000 µg/ml PDMLE with the score value of 1.89, followed by the 100 µg/ml PDMLE with the score value of 1.66, followed by the 10 µg/ml PDMLE with the score value of 0.83 and the negative control with the score value of 0 is not having any inhibitory effect on angiogenesis(Figure 06).

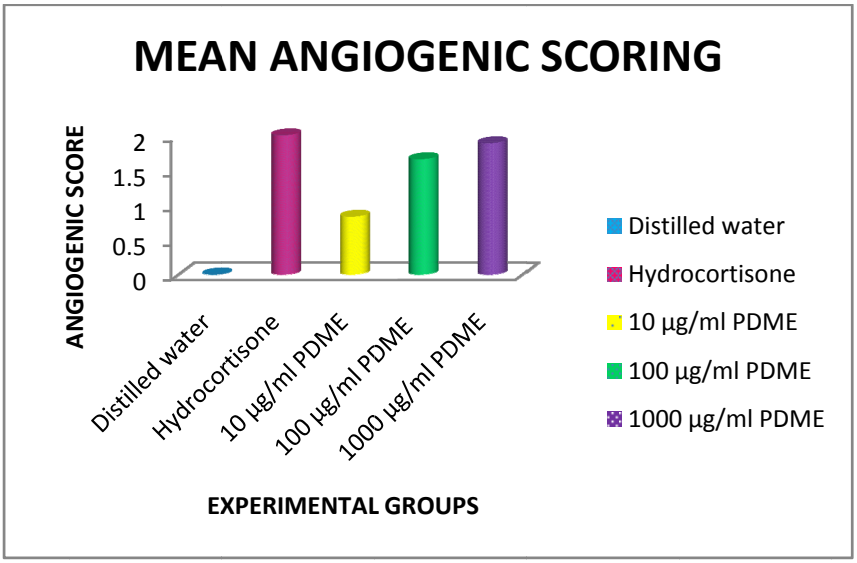

Figure 06: Angiogenic Score of different experimental groups are determined.
Data was analysed using Kruskal-Wallis ANOVA test and a Mann-Whitney U test.
*$p < 0.05$ was considered statistically significant.

This indicates that the PDMLE is having good anti-angiogenic potential. The phenolic compounds seen on the phytochemical test conducted in the study may support the inhibition of angiogenesis in the CAM. The averagemean number of branching points of different groups was assessed for the CAM Vascularity.

Branching points of eggs treated with PDMLE

The HEN- CAM image was analysed using Angiotool software for determining branching points in treated eggs CAM[18].The mean number of thebranching points of different groups are seen in the above table. Based on statistical analysis, there is a significant difference in the mean number of branching points of each group.

CAM Vascularity of the PDMLE

The averagemean number of branching points of different groups were assessed for CAM Vascularity[20,27,30]. Based on the computed data, using the formula for CAM Vascularity the results obtained are as follow. CAM Vascularity for the Group II, positive control is -49.39%, for Group III is -14.45%, for Group IV is -26.17 %, for Group V is -45.02%. The 1000µg/ml PDMLE had shown the highest inhibition of CAM vascularity. This shows that the PDMLE is having good antiangiogenic activity.

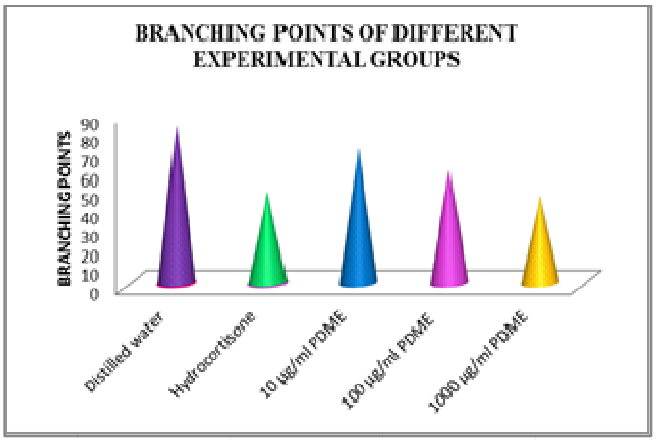

Figure 07: Branching points of different experimental groups.
Data was analysed usingKruskal-Wallis ANOVA test and a Mann-Whitney U test.
*$p < 0.05$ was considered statistically significant.

The phytochemical analysis, we proved the qualitative presence of the plant metabolites, flavonoids in the PDMLE. Further investigations using the innovative HEN-CAM Method, like Antiangiogenic score, branching points, CAM vascularity had supported and confirmed the antiangiogenic activity of flavonoid present in PDMLE.

Conclusion

Nourishment and sustainability of cancer cells depends on the blood supply through the newly formed blood vessels of the tumour. Majority of anticancer drugs in use are cytotoxics, but none of the drugs dealts with the tumour antiangiogenisis. The present study, conclude that the PDMLE containing phytochemicals like flavonoids, may act directly on the tumor blood vessels, inhibit the origin of the tumour at their molecular level and produces a strong antiangiogenic effect. So this PDMLE may be used as a natural antiangiogenic agent to prevent not only the tumour progression, but also to prevent the process of cancer metastasis. In future the anticancer theraphy should also include the use of cheap, less toxic, easily available antiangiogenic plant extracts like PDMLE, to inhibit the progression of cancer. The future work on PDMLE are planned to concentrate on the *in-vitro* anticancer studies, and in preparation of the nano-topical formulations, so as to achieve site specific and more prominent action.

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