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Hydroalcoholic Extract of Cuscuta chinensis Suppresses Angiogenesis and Enhances Paclitaxel Activity in the Chick Chorioallantoic Membrane (CAM) Model

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ABSTRACT

Background: With the growing resistance of cancers to conventional therapies, there is increasing interest in identifying natural compounds with potent anticancer properties. *Cuscuta chinensis* (*C. chinensis*), a valuable herb in traditional medicine, has attracted attention for its potential therapeutic effects. Given the critical role of angiogenesis in tumor growth and metastasis, the discovery of effective anti-angiogenic agents is essential. The chick chorioallantoic membrane (CAM) model is a well-established system for studying angiogenesis. This study aimed to evaluate the anti-angiogenic effects of hydroalcoholic extract of *C. chinensis* on the CAM model.

Methods: This case-control experimental study included six groups of fertilized eggs: control groups and treatment groups receiving paclitaxel (40 µg/kg), *C. chinensis* extract (100 and 400 mg/ml), and a combination of paclitaxel and extract. Treatments were administered on day 8 of incubation. Angiogenesis was assessed using morphological parameters (number and thickness of major and minor vascular branches measured by ImageJ software) and molecular analysis of VEGF gene expression using real-time PCR. Statistical analysis was performed using SPSS.

Results: The extract significantly reduced angiogenic parameters in a dose-dependent manner ($p < 0.05$). Paclitaxel showed stronger inhibition of major vessel formation and enhanced the extract's effects. Both treatments significantly decreased VEGF expression, with the combination producing greater inhibition.

Conclusion: *C. chinensis* extract exhibits significant anti-angiogenic activity in the CAM model and may potentiate the effects of paclitaxel.

INTRODUCTION

Herbal compounds and medicines are inseparable parts of almost all ancient cultures. Over half of the approved drugs in the world are either natural compounds or modified derivatives of them.[1] Plants have been important resources in traditional medicine. Among the major advantages of natural bioactive molecules are mild side effects compared to chemically synthesized drugs. Due to the several problems of applying chemotherapeutic agents for cancer treatment (such as drug resistance and severe side effects), the development of new anticancer drugs derived from herbal compounds has been a focus of attention.[2] Herbal drugs are rich sources of natural anticancer agents and, as a result, they are

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appropriate candidates for producing anticancer and antiangiogenic drugs. *Cuscuta* extract is one of the effective natural products with anticancer, antiangiogenic, and immune-stimulatory effects.[3]

Genus *Cuscuta* belongs to the family Cuscutaceae, which includes about 100 to 170 known species throughout the world. Among the members of this family, only the genus *Cuscuta* is parasitic with the highest diversity in tropical and subtropical regions.[4] Previous studies have found that *Cuscuta* might have anticancer and immune-stimulatory activities. Chemical compounds of *Cuscuta* mainly include flavonols like quercetin, kaempferol, and other glycosides.[3] Among these effects are antioxidant, anti-inflammatory, hepatoprotective, antimicrobial, and anti-anxiety activities.[4,5,6]

Cuscuta species are rich in bioactive compounds with a broad spectrum of therapeutic activities. The plant *Cuscuta chinensis Lam.* (*Cuscuta chinensis*; Convolvulaceae) is a parasitic plant with several clinical effects such as antioxidant activity[5,7.8.9], hepato-protective[10] and nephroprotective activities[11], anti-aging properties[6], anti-depression effects[12], anti-osteoporotic effects[13], anti-diabetic activities[14,15], anti-inflammatory effects[6], anti-tumor activities[16,17], anti-mutagenic properties[18], and anti-angiogenic[19] properties.

Currently, the identification of therapeutic strategies to fight against angiogenesis is expanding, and natural products are still important resources for developing new drugs. Many scholars have suggested the application of herbal medicines as therapeutic combinations to inhibit the angiogenesis process.[1,20,21] Numerous plants with anti-angiogenic properties have been identified in recent years. Currently, various research models are used to screen plants that have anti-angiogenic activities.[22] One of these efficient models is the chick chorioallantoic membrane (CAM) model. This is an available and inexpensive screening tool for studying angiogenic properties.[23]

Due to the importance of identifying and using natural compounds with anticancer effects (particularly anti-angiogenic effects as a major pathway of sustaining cancer cells), the present investigation aimed at studying the anti-angiogenic properties of hydroalcoholic extracts of *C. chinensis* on CAM in comparison to the chemotherapeutic drug paclitaxel.

METHODS

The present investigation was a case-control study with 26 fertilized eggs of the Ross breed. Paclitaxel (PTX) was used as a standard chemotherapy drug with known anti-angiogenic properties. After removing the perivascular tissues and washing with PBS, RNX-Plus solution for total RNA isolation was purchased from Cinnagen (Iran) and the cDNA synthesis kit was supplied by Add Bio (South Korea) Company. RealQ-Plus Master Mix was provided by Ampliqon (Denmark).

Preparation of C. chinensis Extract

The dry powder of the aerial organs of the plant (20% w/v) was soaked in 70% ethanol solution for 72 hours. The herbal mixture was then passed through a strainer and the strained solution was centrifuged at 1000 rpm for 10 minutes. The supernatant was transferred to a sterile container and freeze-dried.

Studied Groups and Treatments

In this study, 26 eggs were randomly divided into six groups: control (treated with normal saline), experimental group 1 (100 mg/kg *C. chinensis* extract), experimental group 2 (400 mg/kg *C. chinensis* extract), experimental group 3 (40 µg/kg PTX), experimental group 4 (100 mg/kg *C. chinensis* + 40 µg/kg PTX), and experimental group 5 (400 mg/kg *C. chinensis* + 40 µg/kg PTX).

After randomized grouping and disinfecting the eggshells, the eggs were put into an incubator with a temperature of 38 ºC and humidity of 55-60%. After making sure that the eggs were healthy and fertilized on the eighth day of incubation (through the candling method), a small window was opened on the eggshell, and a gelatinous sponge (0.03 g agar and egg white in 5 mL of normal saline supplemented with penicillin and streptomycin) was put on the CAM. After treating the eggs, the window was sealed with a coverslip and sterile paraffin.[24,25] On the twelfth day, all the eggs were removed from the incubator. The windows were reopened and the eggs were photographed in the area of the sponge by a research photostereomicroscope (Zeiss, Germany). CAM vessels were isolated to investigate changes in *VEGF* gene expression.

Investigation of CAM Angiogenesis

The photographs taken from the windows on the twelfth day of treatment were inspected by ImageJ software to evaluate the length and number of major and minor vessel branches. Real-time PCR was used to assess the changes in *VEGF* gene expression as an angiogenesis marker. The specific primers of the *VEGF* gene were designed by Allele ID Software (Table 1).

Each group included three independent repeats. After removing the perivascular tissues and washing them with PBS, the total RNA was extracted by RNX Plus Solution (Cinnagen, Iran) according to the manufacturer's instructions. The quality and quantity of the extracted total RNA were evaluated by a Nano-drop apparatus, and the cDNA was synthesized using

PrimeScript RT reagent Kit (AddBio, Iran). The changes in relative levels of *VEGF* expression were calculated by the equation $2^{-\Delta\Delta ct}$.[26]

Statistical Analysis

Data analysis was carried out by the SPSS Software, Version 21. One-way analysis of variance (ANOVA) with Duncan's post-hoc test was used for the statistical analysis. Data are expressed as the mean ± standard deviation (SD). Each group included three independent repeats.

RESULTS

On the twelfth day of incubation, the eggs were reopened under the aseptic conditions of a laminar hood to inspect angiogenesis molecularly and morphologically. Weighing the eggs during the treatment period showed that the average weight of an egg was 59 ± 8 g.

Investigation of Morphological Angiogenic Changes in the Treated CAMs

The photographs taken from the CAM region were inspected by ImageJ Software in terms of the angiogenic indices such as the number of major and minor branches and their diameter (Figure 1 and Table 2).

Table 1. Sequence of primers used in the study

Primers	Gene	Primer sequence	Primer length (bp)	Fragment length (bp)
VEGF-gallus F	NM_205042	TGCCTCTGATGAGATGTG	18	121
VEGF-gallus R		GCTATGTGCTGACTCTGA	18	
GAPDH-gallus F	NM_204305	CAGCCATTCCTCCACCTT	18	119
GAPDH-gallus R		GACCATCAAGTCCACAACAC	20	

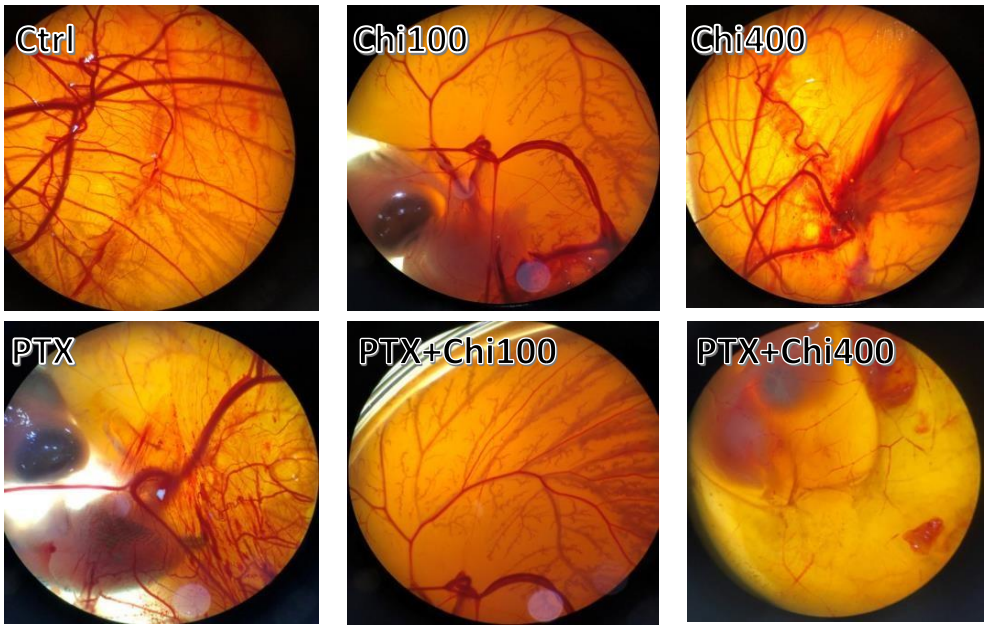

Figure 1. The photographs of control and case groups; Ctrl: the control group; Chi: *C. chinensis* extract; PTX: paclitaxel.

Table 2. Investigation of morphological indices of angiogenesis in the treated CAMs

Angiogenesis indicators Treatment	Major branches No.	Minor branches No.	Thickness
Control	1	1	1
PTX (40 µg/kg)	0.348	0.479	0.880
Chi (100 mg/kg)	0.696	0.892	0.815
Chi (400 mg/kg)	0.739	0.677	0.742
PTX40 + Chi100	0.652	0.809	0.485
PTX40 + Chi400	0.217	0.148	0.213

The results show that paclitaxel significantly ($p < 0.05$) decreases the number of major and minor branches and their diameter. This is consistent with the known anti-angiogenic effects of paclitaxel.

The results indicate that *C. chinensis* extract significantly decreases the angiogenic indices in a dose-dependent manner. Although paclitaxel affects the major branches more, it can enhance the effects of *C. chinensis* extract and strongly inhibits angiogenesis. This inhibitory effect, which is caused by the combination treatments, is higher than each of the single treatments and suggests an enhanced inhibitory effect when combined (paclitaxel and *C. chinensis*).

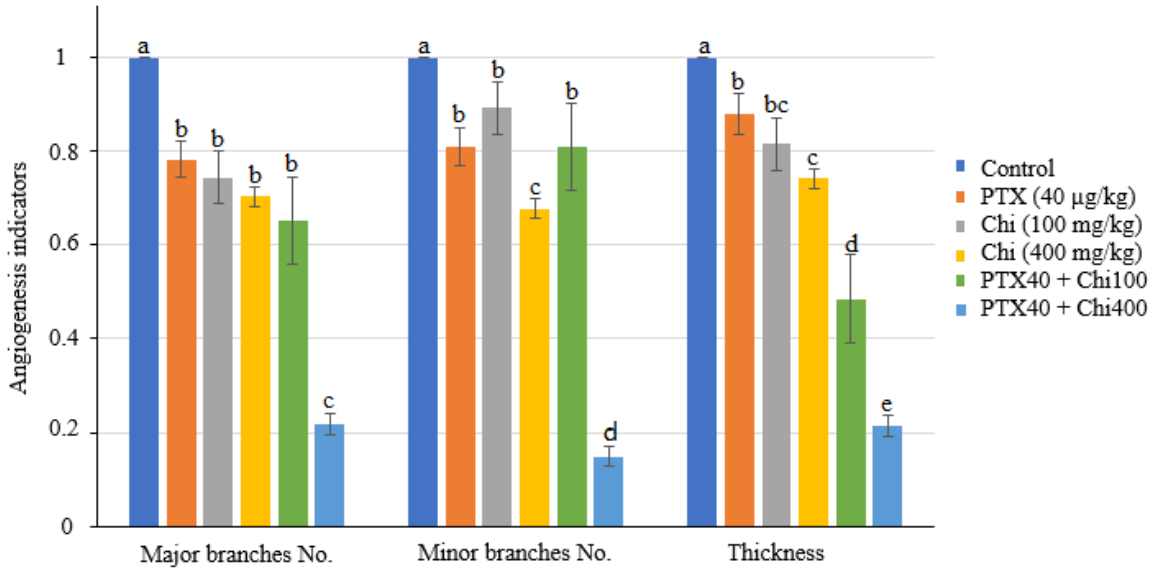

Figure 2. Comparison of the effects of treatments with *C. chinensis* extract and paclitaxel on CAM angiogenesis. The results are the means of three independent experiments. Statistical analysis was performed using one-way ANOVA with Duncan's post-hoc test. In each panel, a similar alphabet does not imply statistical significance.

Investigation of Molecular Angiogenic Changes in the Treated CAMs

To inspect the molecular changes of CAM angiogenesis under the treatments with paclitaxel and *C. chinensis* extract, the changes in the expression of the *VEGF* gene were evaluated by the real-time PCR technique. Calculations of changes in VEGF gene expression in groups treated with paclitaxel or *C. chinensis* extract suggested inhibition of angiogenesis. Although paclitaxel downregulates VEGF expression, *C. chinensis* extract also produces a significant, dose-dependent downregulation of this gene. The inhibitory effect of paclitaxel could be enhanced in the presence of *C. chinensis* extract.

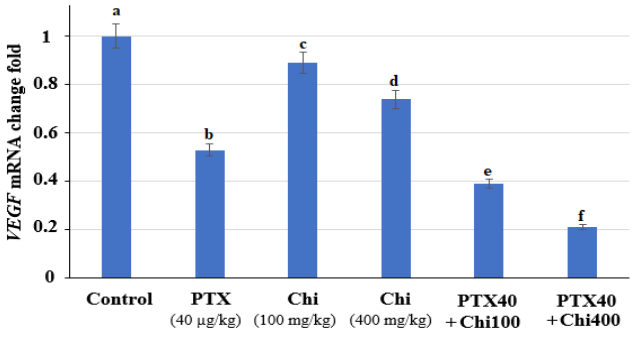

Figure 3. Changes in the expression of the VEGF gene in the groups treated with paclitaxel and *C. chinensis* extract. In each panel, a similar alphabet does not imply statistical significance.

Gene expression analysis further revealed that the combination of *C. chinensis* extract and paclitaxel produced greater inhibition of angiogenesis than either agent alone.

DISCUSSION

Due to the increasing resistance of cancer cells against common treatments like chemotherapy, scholars are seeking new anticancer agents, which increase the sensitivity of cancer cells and resolve the problem of undesirable side effects. Resistance of the cancer cells to chemotherapeutic agents decreases their response to the drug and ends in the failure of therapeutic measures. As a result, it is important to develop more effective drugs with fewer side effects.[27] Due to the importance of angiogenesis in cancer treatment, the treatments inhibiting this important process seem promising in fighting against cancer.

In the present study, an important useful animal model was used to screen the factors activating or inhibiting the angiogenesis process.[20] CAM is a thin respiratory tissue with a dense and highly organized network of blood vessels whose physiological responses can be generalized to the responses of mammalian tissues to similar treatments.[20,25,28] Therefore, it has been widely used to study angiogenesis.

With several therapeutic properties, *C. chinensis* has been the focus of attention in traditional and modern medicine. Previous studies have shown that *C. chinensis* has anticancer activities.[16,17] In the present study, a comparative examination of single and combination treatments of paclitaxel and

hydroalcoholic extract of *C. chinensis* was done on both morphological and molecular levels. The results of the first part of the study show a decreased number of major and minor branches and decreased diameter of CAM vessels under these treatments.

Paclitaxel was used as an approved anticancer drug inhibiting angiogenesis to be compared with the effects of *C. chinensis* extract. The results showed that 40 µg/kg of paclitaxel could synergistically and significantly decrease the number of major and minor CAM vessels. It also reduced the number of minor branches by 65%, 52%, and 12%, respectively. These observations agree with the 47% reduction of *VEGF* under paclitaxel treatment.

The first published evidence on the possible antiangiogenic activity of paclitaxel was a brief report about 27 years ago. Dordunoo *et al.* prepared epsilon-caprolactone containing paclitaxel and used this formulation to inhibit angiogenesis in the CAM model.[29] Consistent with our results, Vacca *et al.* showed the anti-angiogenic effects of paclitaxel on CAM. They concluded that 1 nanomolar of this drug (equivalent to 862 µg/kg) could inhibit angiogenesis. The minimum pharmacological dose of paclitaxel in an adult patient is 3 mg/kg. It is noteworthy that the concentration used in the present work was much lower than the pharmacological doses and the concentrations used in some similar studies. Application of these low concentrations in metronomic chemotherapy (i.e., chemotherapy with low continuous doses) seems to be a strategy targeting the angiogenesis process, especially to fight against drug-resistant tumors.[29]

In addition, low-dose chemotherapy drugs are not expected to have acute toxicity and side effects when used as metronomic therapies. As a result, the low-dose paclitaxel chemotherapy has anti-angiogenic effects and deserves further investigation to enhance the anticancer activities along with other low-side effect agents(30).

This study found that *C. chinensis* extract had a higher inhibitory effect on minor branch formation on CAM. This inhibitory effect was dose-dependent (Figure 2). Although both paclitaxel and the herbal extract individually reduced angiogenic indices, their combination enhanced this inhibitory effect. Specifically, combining paclitaxel with 400 mg/kg of *C. chinensis* extract resulted in an average 80% decrease in these indices. These findings suggest that *C. chinensis* extract can enhance the anti-angiogenic effects of paclitaxel. The results of morphological changes in the blood vessels are completely consistent with the results of the *VEGF* gene expression (Figure 3). Paclitaxel decreased VEGF gene expression by 47%. In combination with the extract at doses of 100 and 400 mg/kg, it further reduced expression to 0.39 and 0.21 times that of the control group, respectively. Combination therapy caused a significantly lower VEGF expression when compared to the single treatments.

There have been no reports about the anti-angiogenic activities of *C. chinensis* in CAM. However, according to Nisa *et al.*, different *Cuscuta* species have anti-melanoma effects in albino mice.[17] It has been acknowledged in some reports that *C. chinensis* decreases cell viability in some cancerous cell lines like PC3, MCF7[17], CCRF, and JM.[31] Very few studies have investigated the anti-angiogenic effects of the *C. chinensis* plant. In a recent study by Saadoon *et al.* in 2020, it was established that *C. chinensis* extract significantly decreased the serum levels of MMP3 and VEGF in mice.[32] This was in line with the results of our investigation.

CONCLUSION

In conclusion, the hydroalcoholic extract of Cuscuta chinensis demonstrated anti-angiogenic activity in the CAM model and enhanced the inhibitory effects of paclitaxel on vessel formation and VEGF expression. While these findings are promising, they are limited to an *in ovo* angiogenesis model. Further studies using mammalian tumor models are required to assess the potential of *C. chinensis* extract as an adjunct to paclitaxel in breast cancer therapy.

ETHICAL CONSIDERATIONS

All the experimental procedures in this study were conducted in accordance with the Institutional Animal Care Guidelines of Ardakan University and approved by the Research Ethics Committee of Ardakan University, Ardakan, Iran (ethical code: IR.YAZD.REC.1401.104).

DATA AVAILABILITY

All relevant data can be found within the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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AI DISCLOSURE

The authors declare that no artificial intelligence (AI) tools or software were used in the writing, analysis, or preparation of this manuscript.

AUTHOR CONTRIBUTION

Fatemeh Sadat Ahmadi: Conceptualization, Methodology, Investigation, Writing – Original Draft. Majid Morovati-Sharifabad: Conceptualization, Methodology, Investigation. Gholam Hossein Pourghanbari Marvast: Conceptualization, Methodology, Investigation. Elham Salehi: Methodology, Investigation. Ehsan Zarei MahmoudAbadi: Data Curation, Formal Analysis, Visualization. All authors contributed to Writing – Review & Editing and approved the final manuscript.

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