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Investigating the effect of *Cynodon dactylon* on the secretion of growth factors from Dental pulp stem cells and angiogenic potential in chick embryo model

Research Article

Jai Patil[1], Shital Rasane[2], Avinash Kharat[3*], Ramesh Bhonde[4], Avinash Sanap[3], Pranjali Potdar[5]

1. PG Scholar, 2. Professor, Department of Ayurved Samhita evum Siddhant,
Dr. D. Y. Patil College of Ayurved and Research Centre, Pimpri, Pune.
3. Scientist C, 4. Emeritus Scientist, 5. Research Fellow, Regenerative Medicine Laboratory,
Dr. DY Patil Dental College and Hospital, Dr DY Patil Vidyapeeth, Pimpri, Pune. India.

Abstract

Background: *Durva* (*Cynodon dactylon)* is an abundantly available plant in India with great medicinal properties. It is used for the treatment of various cardiovascular diseases such as heart failure and atherosclerosis. It is a one of the drugs that maintain and stabilise pregnancy. We investigated the effects of the aqueous extract prepared from the rhizomes of C. dactylon on stem cells growth factor secretion and angiogenesis in chick embryo model. Methods: MTT assay was performed to assess cytotoxicity of aqueous extract of *Cynodon dactylon* at various concentrations (2, 5, 10, 15, 20, 25 and 50 µg/ml). Human dental pulp stem cells (hDPSCs) were treated with non-toxic aqueous extract concertation of *Cynodon dactylon* to collect the preconditioned media. Chicken Yolk Sac models model was deployed to observe angiogenic the effect of aqueous extract. Further growth factor analysis of pre-treated hDPSCs condition media analysed for the angiogenic growth factors that affect the angiogenic process. Results: *Cynodon dactylon* does not show cytotoxic effects at any concentration tested. At 10 µg/ml aqueous extract show good proliferation rate compare with other concentration. Preconditioned media of *Cynodon dactylon* showed high amount of growth factors secretion than that of the control conditioned media. The chick embryos treated with 10 µg/ml aqueous extract of *Cynodon dactylon,* showed highest growth of blood vessels followed by the preconditioned media as compared to the control groups. Conclusion: Our study concludes that *Cynodon dactylon* promotes the process of angiogenesis by stimulating secretion of essential growth factors and potential angiogenic properties.

Keywords: Angiogenesis*, Cynodon dactylon, Durva,* Growth factor, Human dental pulp stem cells, YSM model.

Introduction

Process of Angiogenesis is crucial wherever repair of tissue is required. It is also essential in growth and development of the foetus and maintenance of pregnancy.(1) Its expression is essential in conditions like spontaneous abortions (2), non-healing ulcers (3), Intrauterine growth restriction (IUGR) (4), ischemic heart disease (5) etc. This process includes various signalling proteins called as the growth factors. These growth factors include Vascular endothelial growth factor (VEGF), Fibroblast growth factor (FGF), Platelet endothelial cell adhesion molecule (PECAM), Epidermal growth factor (EGF), etc. They contribute in the process of angiogenesis by initiating the process, cell proliferation, cell to cell adhesion, building tubular structure of the vessel, migration and some factors also are responsible for inhibition of the process like the Tumour necrosis factor alpha (TNF-α) and Angiopoietin 2. Over expression of these factors can lead to conditions like diabetic retinopathy or can contribute in growth of malignant tumours and metastasis of the cells (6,7) . Therefore, controlled expression of these growth factors holds utmost importance considering both the situations.

Growth factor therapy is currently popular for treating hair loss and wrinkles, scar marks on skin. They are being used as a therapy in extracted recombinant form as topical treatment or injections in diabetic foot ulcers (8). But they cause uncontrolled expression of angiogenesis and hyperplasia(9). Hence, stimulating the secretions of these growth factors from the bodily cells is much safer. Therefore, identifying the drugs that stimulate the secretion of growth factors and angiogenesis have gained importance.

Durva (Cynodon dactylon) is included in the *Prajasthapak Mahakashay* (substances which helps sustenance of foetus) and is also mentioned as *Vranaropak* (substances facilitating wound healing) (10). An animal study was conducted on albino wistar rats using gel prepared with alcoholic and aqueous extract of *Cynodon dactylon* on excision as well as incision wounds which showed significant increase in healing rate in comparison to paraffin wax as the control and povidone iodine as the standard control (11). Studies have also revealed its properties like anti-

*** Corresponding Author:**
Avinash Kharat
Scientist C, Regenerative Medicine Laboratory,
Dr. DY Patil Dental College and Hospital,
Dr DY Patil Vidyapeeth, Pimpri, Pune. India.
Email Id: avinashkharat25@gmail.com

inflammatory(12) , anti-oxidant(13), anti-diabetic(14), immunomodulatory and DNA protective(15) , Although its wound healing activity is established, its effect on the growth factors and angiogenesis was unclear. By assessing its effect on the growth factors and evaluating its angiogenic potential, it is possible to widen its use in several conditions other than wound healing. Hence, this study was designed to assess the effect of aqueous extract of *Cynodon dactylon* on the secretion of growth factors in hDPSCs-CM and angiogenic potential in the Yolk sac membrane (YSM) model(16).

This study aims to investigate the effects of *Cynodon dactylon* extract on DPSC proliferation and growth factor secretion in vitro and on angiogenesis in the chick embryo model. The study will explore whether *Cynodon dactylon* extract can enhance DPSC behaviour and promote tissue regeneration, paving the way for its potential incorporation into DPSC-based regenerative therapy

Materials and Methods

Ethical approval

The present study was approved by The Institutional committee for Stem Cell Research (IC-SCR) of Dr. D. Y. Patil Dental College and Hospital, Pimpri, Pune (IC-SCR/RM/26/23)

Sample Collection

Cynodon dactylon powder was purchased from a GMP certified pharmacy and the drug sample was standardized and TLC was carried out in Dr. D. Y. Patil College of Ayurved at the Sudhatattva Pharmacy (Table: 1)

Table 1: Analytical test of *Cynodon dactylon* extract

Sr. No.	PARAMETERS	TEST OBSERVATION
1	Description	Colour – Yellowish Green Odour – Characteristic Taste - Bitter
2	Average weight content	50 gm
3	Colour of aqueous solution (10%)	Faint Greenish
4	pH of Aqueous Solution (10%)	7.1
5	Foreign Matter	NIL
6	Loss on Drying	3.49%
7	Total Ash	7.89%
8	Acid Insoluble Ash	2.21%
9	Water Soluble Extractive	9.81%
10	Alcohol Soluble Extractive	4.86%

Preparation of extract

The standardized *Durva* powder, 50 gm powder of was packed in the extractor of the Soxhlet apparatus. The flask was filled with 450 ml of distilled water and temperature of the hot plate was set at 60 degrees. The process was continued for about 26 hours. The obtained extract was filtered and kept in a petri dish for evaporation on water bath at the temperature of 80 degrees. Once the extract was completely dried, it was scraped and stored in a tube.

Preparation of stock from extract

Extract was weighed 10 mg and dissolved in 1 ml distilled water as stock solution. 15µg/ml concentration of aqueous extract of *Cynodon dactylon* was prepared for the of analysis using analytical test. 1.5µl of stock prepared earlier was added in 998.5µl of DMEM to prepare 1ml of 15µg/ml concentrated extract.

MTT assay for Cytotoxicity

MTT assay was performed to evaluate the effect of aqueous extract of *Cynodon dactylon* on proliferation of human dental pulp stem cells (hDPSCs). The cells were seeded in a 96 well plate and incubated for 24 hrs. After incubation the cells were treated with different concentrations of the extract (2 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, 50 µg/ml). After incubating for 48 hours, 50 µl of MTT solution (5mg/ml in PBS) was added and again incubated for 3 hours at 37^0C. 100 µl of MTT solvent was added to each well after discarding the supernatant. To completely dissolve the formazan salt in MTT, the plate was wrapped in an Aluminium foil and shacked on orbital shaker for 5 mins. The absorbance was read at OD-590 nm (optical density at 590 nm).

Preparation of Pre - Conditioned media

The cells were seeded in all the wells of a 6 well cell culture plate and were allowed to achieve 80 – 90 % confluency. Then to the first row of wells labelled as control, MEM along with 1% antibiotic, antimycotic solution was added without serum. To the second row of wells, 15µg/ml concentrated *Cynodon dactylon* extract was added along with 1% antibiotic, antimycotic solution without serum. The plate was then incubated for 48 hours. After incubation the conditioned media was collected and stored in Eppendorf tubes for further analysis.

Yolk Sac Membrane (YSM) assay to assess effect on Angiogenesis

Yolk sac membrane assay was performed to assess efficacy of *Cynodon dactylon* extract and its pre – conditioned media on Angiogenesis. Chick eggs of Zero hours were purchased from Central Hatchery, Khadki, Pune. They were cleaned and incubated for 48 hours at 37^0C. After incubation a window of about 2 cm diameter was created at the blunt side of each egg and about 4-5 ml albumin was removed from each egg. For the control groups, DMEM and Conditioned media was used and treatment groups were 15 µg/ml concentrated *Cynodon dactylon* extract and pre-conditioned media prepared earlier. 100 µl of each was added on surface of yolk sac membrane of each egg in respective groups. The openings were covered with a transparent plastic tape and incubated in an incubator at 37^0C maintaining the humidity of the incubator for 24 hours. The openings were then widened and pictures of the developed embryo along with formed network of Blood

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vessels were taken and quantitative assessment was done on online software tool (Wimasis – WimCam).

Growth Factor analysis

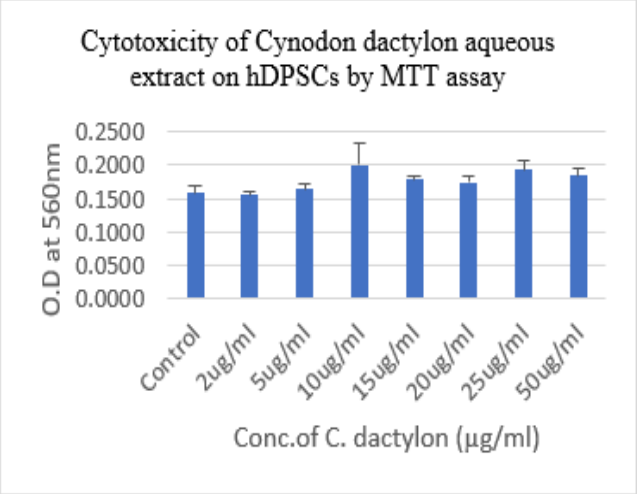
The growth factor analysis assay was performed using LEGENDplex Multi-Analyte Flow Assay Kit. To 25 µL of each sample, 25 µL of mixed beads were added and incubated for 2 hours. After incubation, the samples were centrifuged at 250rpm for 5 minutes and washing steps were followed. After performing the washing steps twice, 25 µL of detection antibodies were added to each sample and incubated for 1 hour and 25 µL of SA-PE was added in each sample. The samples were then centrifuged for 30 mins and washing steps were repeated. The samples were tested using a flow cytometer and results were analysed.

Result

MTT assay for assessment of cytotoxicity of aqueous extract of *Durva (Cynodon dactylon)*

Cytotoxicity of the drug was assessed using MTT assay. There was no cell death at any concentration hence the drug proved to be non - toxic at all given concentrations.

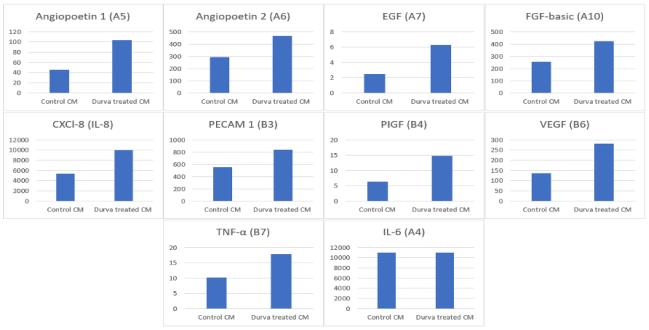
Figure 1: Cytotoxicity of *Cynodon dactylon* aqueous extract on hDPSCs by MTT assay at various concentrations.



Growth factor analysis of Preconditioned media treated with Aqueous extract of *Cynodon dactylon:*

The effect of Aqueous extract of *Cynodon dactylon* was assessed using the LEGENDplex Multi-Analyte Flow Assay Kit by flowcytometry. The conditioned media derived from shed was used as the control. The preconditioned media showed higher secretion of Angiopoetin 1 and 2, EGF, FGF, PECAM 1, PIGF, VEGF and TNF-α as compared to the control ($p<0.05$). The Interleukin 6 levels were above the tracable levels in both the control as well as the treated CM. Interleukin 6 was above traceable levels in both CM and Interleukin 8 was above traceable level in the treated CM but was significantly high as compared to the control CM.

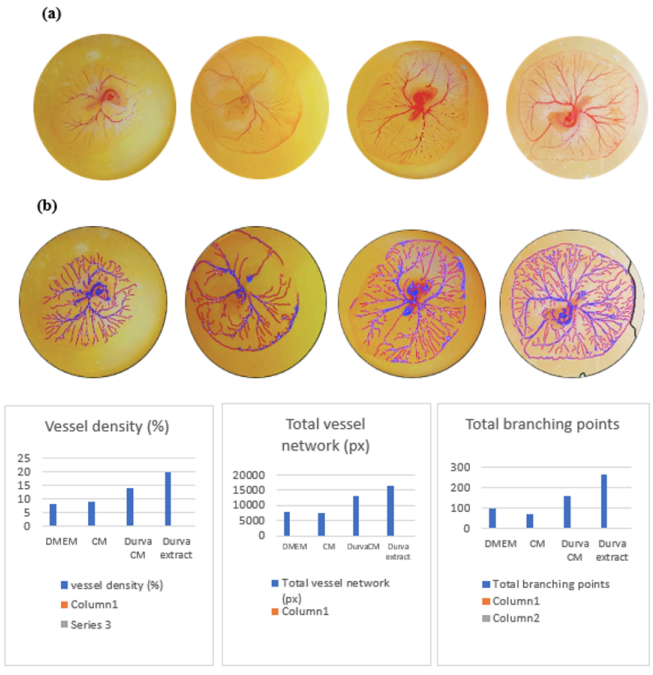
Figure 2: Effect of *Cynodon dactylon* aqueous extract on various growth factors; Angiopoetin 1, Angiopoetin 2, Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Interleukin 8 (cxcl-8), Platelet endothelial cell adhesion molecule (PECAM 1), Placental growth factor (PIGF), Vascular endothelial growth factor (VEGF), Tumour necrosis factor alpha (TNF-α), Interleukin 6. Culture medium derived from the shed was kept as control.



Assessment of angiogenic potential of *Cynodon dactylon* using YSM model

Angiogenic potential of *Cynodon dactylon* extract along with pre conditioned media was assessed in comparison with DMEM and Conditioned media derived from the shed as control groups. Quantitative assessment of the pictures taken was done using online software tool WimCam (Wimasis). The assessment showed pro angiogenic activity of *Cynodon dactylon* extract as well as its preconditioned media in comparison to the control groups. *Durva* extract showed highest vessel density, total vessel network as well as total branching points.

Fig 3: (a) Assessment of angiogenic potential of *Cynodon dactylon* aqueous extract and preconditioned media; DMEM and control conditioned media were the control groups. (b)The images obtained in the assay were analysed using online software tool (Wimasis-wimcam)



Discussion

Several studies have shown involvement of growth factors in angiogenesis which stimulate the proliferation of endothelial cells. VEGF and FGF are responsible for essential steps of angiogenesis like tube formation, sprouting, EC migration and mitogenesis. C. dactylon is a haemostatic drug(17). The FGF contributes in angiogenic process by building of the blood vessel wall due to its ability to proliferate the fibroblasts which also leads to formation of the granulation tissue. The PECAM 1 contributes by enabling cell to cell adhesion. Considering these facts, it can be said that *Cynodon dactylon* promotes the secretion of growth factors required for the process of angiogenesis and hence act as haemostatic by building back the walls of damaged blood vessels which is the prime cause of haemorrhage.

The Tumour necrosis factor alpha (TNF-α) was also raised in the *Cynodon dactylon* treated CM. It has shown both anti-angiogenic as well pro-angiogenic stimulation at higher and lower concentration respectively(18) . Due to the presence of this growth factor, *Cynodon dactylon* does not cause uncontrolled tissue proliferation unlike in extracted forms of growth factors. The Angiopoietin 2 also is responsible for inhibition of angiogenic process but its role is altered in presence of the VEGF (19). In the *Cynodon dactylon* treated CM both the Ang 2 and VEGF are raised concluding the effect of Ang 2. The Ang 1 is responsible for early development of heart and the circulatory system in the foetus(20,21). Placental growth factor is responsible for vasculogenesis and development of larger blood vessels for providing nutrition to the foetus hence is considered as an important marker in cases of IUGR(22). *Cynodon dactylon* have stimulated secretion of both Ang 1 and PlGF supporting its claim of being a drug that helps in sustenance of the foetus(10).

The pre-conditioned media was assessed for growth factors (VEGF, EGF, FGF, Angiopoietins, Interleukins and PECAM). Secretion of these factors was found significantly high in the *Cynodon dactylon* treated pre-conditioned media as compared to the control conditioned media.

The YSM assay, allows to visualize the growth of blood vessels in a chick embryo. It is an in-ovo model developed for measuring angiogenic and anti-angiogenic potential of drug(23). In the current study this model was used to assess angiogenic potential of aqueous extract of *Cynodon dactylon* and its pre-conditioned media. The embryos that were treated with aqueous extract of *Cynodon dactylon* and the pre-conditioned media showed significant increase in vessel density, total vessel network length and total branching points as compared to the control groups.

This whole study supports the theory of *Cynodon dactylon* being one of the Prajasthapak group of drugs, as the growth factors assessed play a significant role in conception, maintenance of pregnancy and foetal growth. Angiogenesis is crucial for adhesion of placenta and implantation. This study establishes *Cynodon dactylon*as a potent pro-angiogenic drug and expression of TNF-α and Ang 2, assures controlled proliferation of the endothelial cells. Hence, *Cynodon dactylon* can be effectively used in non-healing ulcers as it also shows anti-inflammatory and anti-diabetic action along with being pro-angiogenic. It can also be employed in early pregnancy pertaining to its potential to increase the secretion of growth factors and anti-oxidant property. Its effect on osteogenic differentiation is worth exploring as it stimulates the secretion of EGF which has shown enhancement of osteogenic differentiation in stem cells(24). Furthermore the drugs can also be tested in animal models for fractures as bone is a highly vascularised tissue.

The absence of significant toxicity in both in vitro and in vivo models is encouraging, suggesting that *Cynodon dactylon* extract is relatively safe for use in biological applications. However, further studies are needed to assess its long-term toxicity and biocompatibility.

Conclusion

This study demonstrates that *Cynodon dactylon* extract possesses promising regenerative potential by promoting DPSC proliferation and growth factor secretion in vitro and enhancing angiogenesis in the chick embryo model. These findings suggest that *Cynodon dactylon* may be a valuable natural agent for wound healing and angiogenesis. Further research is warranted to fully elucidate the mechanisms of action and to develop *Cynodon dactylon* ased regenerative therapies.

Conflict of interest: Author declares no conflict of interest.

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