"TO ASSESS THE EFFECT OF AQUEOUS STEM EXTRACT OF *TINOSPORA CORDIFOLIA* ON THE PROLIFERATION AND MIGRATION OF PERIODONTAL LIGAMENT FIBROBLASTS: AN IN VITRO STUDY"

By

REG. NO. IK0219002

Dissertation

Submitted to

KLE Academy of Higher Education and Research,

Belagavi, Karnataka

In partial fulfilment

of the requirements for the degree of

MASTER OF DENTAL SURGERY
in
PERIODONTICS
(Branch II)

DEPARTMENT OF PERIODONTICS

KAHER's KLE VISHWANATH KATTI INSTITUTE OF DENTAL SCIENCES

KAHER, BELAGAVI-590010, KARNATAKA

2019-2022

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Dr. VINAYAK KUMBHOJKAR M.D.S
Professor & Head,
Department of Periodontics,
KAHER's KLE V. K. Institute of
Dental Sciences, Belagavi

Professor and Head
Department of Periodontics
KLE V. K. Institute of Dental Sciences
Belagavi

Date: 05/01/2022
Place: Belagavi

Dr. ALKA KALE M.D.S, Ph.D.
Principal,
KAHER's KLE V. K. Institute of
Dental Sciences, Belagavi

PRINCIPAL
KLE V. K. Institute of Dental Sciences.
Nehru Nagar, Belagavi

Date: 5/1/22
Place: Belagavi

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ABSTRACT

Background

Periodontal ligament is considered as an important factor for the restoration of diseased periodontium. Hence the proliferation and migration of periodontal ligament fibroblasts is necessary for the maintenance of tissue integrity. *T. cordifolia*, a medicinal plant, is proved to have various therapeutic effects such as anti-inflammatory, antibacterial with a rich source of micronutrients such as zinc, magnesium, iron etc.

Hence the present study was conducted to assess the effect of aqueous stem extract of *T. cordifolia* in proliferation and migration of PDL fibroblasts.

Materials and Methods

The current in vitro study included four concentrations of the prepared *T cordifolia* extract (0.5mg, 1.0mg, 1.5mg and 2.0mg) and one control group to which the PDL fibroblasts were exposed. The teeth extracted for orthodontic purposes were used for the study. The proliferation and migration were done using MTT assay and in vitro scratch test respectively. The proliferation rate was assessed using spectrophotometer and proliferation rate was assessed by visual method.

Results

In the present study, at 24 hours, the mean optical density was found to be 0.44150 ± 0.023864, 0.50683 ± 0.032664, 0.55133 ± 0.043675, 0.55500 ± 0.070359 and 0.57733 ± 0.078724 for Group 1, Group 2, Group3, Group 4 and Group 5 respectively. The mean wound closure happened, at 24 hours, in group 1, group2, group3, group 4 and group 5 were found to be 46766.67 ± 1833.939, 24766.67 ±

21946.830, 53633.33 ± 1517.674, 43566.67 ± 2809.508 and 46766.67 ± 1833.939 respectively.

Conclusion

From the results obtained in the current study it can be concluded that, the aqueous stem extract of *T. cordifolia* can be used as an effective agent or adjunct in the restoration of the lost periodontal structures and maintenance of the integrity of the tissues.

LIST OF ABBREVIATIONS

T.f	*Tinospora cordifolia*
PDL	Periodontal ligament
DMEM	Dulbecco's modified Eagle's media
DMSO	Dimethylsulphoxide
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
ELISA	Enzyme linked immunosorbent assay
mg	Milligram
µl	Microliter
ECM	Extracellular matrix
nm	Nanometer
ANOVA	Analysis of variance
CO_2	Carbon dioxide
EDTA	Ethylene diaminetetraacetic acid
IL	Interleukin
TNF	Tumour necrosis factor
NSAID	Non-steroidal anti-inflammatory drug
(O_2^-),	Superoxide anion
(NO)	Nitric oxide radical
$(ONOO^-)$	Peroxynitrite anion
(OH)	Hydroxyl radicals

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INTRODUCTION

Periodontium involves the structures which surround and support the maxillary and mandibular teeth which includes gingiva, periodontal ligament, cementum and alveolar bone. In health the periodontium will be intact and in case of disease there will be inflammatory changes seen in periodontal tissues which include gingival erythema, loss of gingival stippling, clinical attachment loss, pathological deepening of gingival sulcus, alveolar bone loss etc. which can eventually result in tooth loss.

Periodontal ligament is present in the space between the cementum and alveolar bone which act as the connection between the two. It is attached to the alveolar bone and root cementum by the sharpeys fibers. [(1)] During inflammation, widening or loss of periodontal ligament is the first sign that makes the periodontium unstable and initiate the destructive phase of disease. Hence, the repair of periodontal ligament is necessary for the restoration of the diseased periodontium.

Periodontitis is a diseased condition of the periodontium characterized by irreversible loss of connective tissue attachment and supporting alveolar bone. Hence, it is necessary to provide timely treatment in these cases to preserve the teeth in their healthy condition. Periodontal therapy aims at restoring the lost periodontium and to prevent or control the further progression of the inflammatory disease. [(2)]

The primary etiologic factor of periodontal disease is considered to be bacterial plaque. The three main factors involved in the pathogenesis of periodontitis are bacterial challenge, inflammatory response and host response. Host response refers to the changes occurring in the host tissues in response to bacteria present in the

dental plaque which result in the release of inflammatory mediators. Once there is a change from symbiotic state to dysbiosis state, imbalance in the level of pro inflammatory and anti-inflammatory mediators takes place that result in the alveolar bone loss and destruction of periodontal tissues. [(1)] The increased microbial load can also result in the release of increased reactive oxygen species, produced during microbial killing by neutrophils. Antioxidants will be unable to neutralize the increased release of reactive oxygen species and these excess reactive oxygen species also result in tissue damage via oxidation process. [(3)] Host modulation refers to the change or alteration in the response to a stimulus which is also considered as the key factor in the periodontal treatment.

After injury, healing occurs in a sequential manner resulting in the wound closure. The wound healing response mainly includes three basic steps; **blood coagulation and inflammation**, followed by **nascent tissue formation** and **tissue remodeling** [(4)]

During the healing phase, there will be activation of resident cells which will result in increased gene expression and in turn result in proliferation, differentiation and migration of precursor cells to the site of injury. [(4)] The activation of these resident cells is mediated by cytokines, chemokines and growth factors. Fibroblasts are the cells which play an important role in the healing process during the regeneration phase. They synthesize and secrete collagen fibers from gingiva which will interconnect it to the periodontal ligament, cementum and alveolar bone. The formation of nascent tissue which is mediated by fibroblasts present in lamina propria of gingiva and periodontal ligament is also important for the restoration of a healthy periodontium.

The primary and basic step in the periodontal therapy is the scaling and root planning. Pathogenic microorganisms tend to invade the periodontal tissues and mechanical debridement alone will be ineffective in reducing the microbial load in the tissues. In such cases, it is preferred to give medications along with mechanical debridement to improve the treatment outcome and for better prognosis. Adjunctive therapy can be done by administration of antimicrobials, anti-inflammatory, antioxidants which result in microbial killing, decrease in inflammation and oxidative stress or decrease the release of reactive oxygen species respectively. Adjunctive therapy can be in the form of either systemic or local administration. Systemic administration of drugs is more successful but locally delivered agents are more preferred because of the less adverse effects and they attain high concentration at low doses in the gingival sulcus and periodontal pockets. Some of the locally delivered agents include chlorhexidine chip, doxycycline, minocycline, tetracycline, metronidazole etc. and systemic administration include NSAIDs, chemically modified tetracyclines and so on.

In recent days there is a rise in the occurrence of infectious diseases in people because of increased stress, adverse food habits and changing life style. In modern medicine there is a tremendous use of antimicrobials in the treatment of such diseases. Replacement of the use of these antimicrobials is necessary because of increased antimicrobial resistance, food safety issues, increased side effects, higher cost and decreasing potency of the drug. [(5)] Researchers are now trying to find substitutes for these and some of the upcoming therapeutics includes bacteriophages, immunomodulators, egg yolk antibodies and antibiotics which have proved effective in treating various disorders. [(5)]

Herbal medicine is an upcoming most acceptable form of therapy by many researchers because of its diverse medicinal properties with fewer side effects. It is as ancient as human civilization and it has been incorporated in various traditional medical systems such as Ayurveda, Siddha, European, Tibetan and Unani. [(6)] About 75-80% of cases in developing countries are using this traditional way for primary health care as it is more acceptable and compatible with the human body with less side effects. [(6)] Hence, plants with medicinal properties are gaining much popularity among the researchers to explore the different therapeutic uses so that they can be used as substitutes to antibiotics and antimicrobial agents.

Various studies have been done on plant products and their anti-inflammatory and antioxidant properties have been proved. *Tinospora cordifolia* is a plant that comes under the family *Menispermaceae*, on which researchers have an emerging interest because of its different beneficial medicinal and therapeutic properties. *Tinospora cordifolia* is a deciduous, perennial climber found in India and China with glabrous leaves and fleshy stem. The leaves, stem and root of the plant have been proved for its various medicinal properties among which stem is more therapeutically important.

Researchers have found various properties of *T. cordifolia* which include antimicrobial, anti-inflammatory, antioxidant, immunomodulation, antidiabetic, anti-osteoporotic and anti-stress activity. It upregulates the anti-inflammatory cytokines such as IL-6, IL-1 and downregulation of the pro inflammatory cytokines such as IL -1β, TNF- α and IL -17. [(5)] It is also nutritionally beneficial as it is rich in many minerals such as copper, calcium, zinc, phosphorous in its stem. [(5)] It has been proved that the plant has neither mutagenic effect on bone marrow erythrocytes nor DNA

damage in peripheral blood lymphocytes. The zinc present in the plant stem have the property of maintaining cellular homeostasis, regulating expression of gene [7] and promote proliferation and migration of periodontal ligament fibroblasts.

Therefore, the current study has been undertaken to assess the effect of aqueous stem extract of *Tinospora cordifolia* on the proliferation and migration of periodontal ligament fibroblasts.

AIM AND OBJECTIVES

AIM:

To assess the effect of aqueous stem extract of *Tinospora cordifolia* on the proliferation and migration of periodontal ligament fibroblasts.

OBJECTIVES:

1. To confirm the proliferation and migration of fibroblasts at different concentrations of aqueous stem extract of *Tinospora cordifolia*.
2. To assess the effect of 0.5mg, 1.0mg, 1.5mg and 2.0mg aqueous stem extract of *Tinospora cordifolia* on periodontal ligament fibroblast cell migration.
3. To assess the effect of 0.5mg, 1.0mg, 1.5mg and 2.0mg aqueous stem extract of *Tinospora cordifolia* on periodontal ligament fibroblast cell proliferation.
4. To compare the effect of 0.5mg, 1.0mg, 1.5mg and 2.0mg aqueous stem extract of *Tinospora cordifolia* on periodontal ligament cell proliferation and migration.

REVIEW OF LITERATURE

Tinospora cordifolia is found throughout India and China, with diverse medicinal properties. Alkaloids, steroids, phenols, aliphatic compounds and polysaccharides are some of the biologically important active components that have been isolated from different parts of the plant. These compounds are well known for their biological actions so that these can be used for the treatment of various ailments. [8] The potent antibacterial effect of *Tinospora cordifolia* have been reported with its ability in bacterial clearance and improved phagocytic and intracellular bactericidal capacities of neutrophils. [8] The plant is also known for its antioxidant property which is due to the presence of an arabinogalactan polysaccharide and phenolic compound (epicatechin). [5] The plant extract has been reported to have the free radical scavenging properties against superoxide anion (O_2^-), nitric oxide radical (NO), peroxynitrite anion ($ONOO^-$) and hydroxyl radicals (OH). [8] The plant is also well known for its analgesic and anti-inflammatory properties. The analgesic effect is produced by acting via both peripheral and centrally mediated mechanisms and the anti-inflammatory action is by downregulating the proinflammatory mediators such as IL -1β, TNF- α and IL- 17. It also possesses other beneficial effects such as immunomodulatory property, neuroprotective property, osteoprotective property, anti-diabetic property and anti-stress activity.

a) A study was done by **Sankalp Agarwal, Priyadarshini H Ramamurthy, Bennete Fernandes, Avita Rath and Preena Sidhu (2019)**; to assess the antimicrobial activity of different concentrations of commercially available *Tinospora cordifolia* against *Streptococcus mutans*. The extract was obtained by using 100% ethanol by maceration. The test was done using seven different

concentrations against *S. mutans* grown in brain-heart infusion agar medium. Then the agar plates were incubated at 37°C for 48 hours, aerobically and zone of inhibition measured using Vernier calipers. 0.2% Chlorhexidine was used as positive control and dimethylformamide served as negative control in the study. The maximum antibacterial activity was seen at a volume of 40µl at 2% concentration with a zone of inhibition of 19mm. Hence the study concluded that the plant extract has significant antibacterial activity against *S. mutans* but the author recommends to confirm the results with further *in vivo* studies.[6]

b) A study was done **by Mohamed F Alajmi, Ramzi A Mothana, Adnan J Al-Rehaily and Jamal M Khaled (2018)**; where they investigated the antibacterial activity of different extracts of *Alpinia galanga* and *Tinospora cordifolia*. The safety profile assessment of both the plants was done through *in vivo* acute and chronic toxicity studies in animal model. H37Rv INH sensitive and resistant strains of *Mycobacterium tuberculosis* were used to test the dichloromethane and ethanolic extracts of the plants. The extracts of both the plants showed significant inhibitory effect, which was dose dependent, with maximum effect of 18-32% at 50µg/ml against both strains of *M. tuberculosis*. Hence the study concluded that both the plants have considerable antimycobacterial activity but can be toxic to heart, liver and kidney after long term administration. [9]

c) A study was done by **Kiran Kumar reddi and Sarada D Tetali (2018)** to study the antioxidant and anti-inflammatory effect of alcoholic and water extracts of T. cordifolia leaves in activated human monocytic THP-1 cells. Arachidonic acid (AA)- and lipopolysaccharide (LPS)-activated human monocytic (THP-1) cells as experimental models were used to study the antioxidant and anti-inflammatory activities of the plant extracts. The extracts showed significant amounts of

phenolic and flavonoid content, and both types of extracts weakened AA-induced ROS generation by enhancing activity of catalase enzyme in THP-1 cells. Real time PCR and ELISA experiments demonstrated that elevated levels of LPS-induced TNF-α were reduced in THP-1 cells which were pre-treated with T. cordifolia extracts. Western blot and confocal microscopy showed that alcoholic extract's anti-inflammatory activity is by attenuating NFκB translocation into the nucleus in LPS-activated THP-1 cells by the inhibition of degradation of IκB in the cytosol. Hence the study was able to prove that the plant extracts show significant antioxidant and anti-inflammatory activity through the upregulation of antioxidant enzymes and reduction of NF- κB nuclear translocation in activated human monocytic (THP-1) cells. [(10)]

d) A study was done by **Sheena Philip, Greeshma Tom and Asha V Vasumathi (2018)**; focussed on the anti-inflammatory effect of chloroform extract of T. cordifolia on macrophages stimulated with LPS. The results showed that it helped in reducing the upregulated level of proinflammatory biomarkers without affecting COX-1. The authors also observed retained levels of NF-κB in the cytoplasm that lead to a reduction in the level of phosphorylated p38 MAPK levels.[(11)]

e) A review was done by **Areeful Haque, Ibrahim Jantan, Syed Nasir Abbas Bukhari (2017)** on *Tinospora* species to critically analyse the immunomodulatory effect of *Tinospora* species using the already available literature. The results suggested that *T. sagittate, T.bakis, T. smilacina* and *T. sinensis* have reported effective immunomodulatory activity. The authors observed that most of the studies were done on the crude extracts of the plant and the bioactive compounds responsible for the activity remained unknown. Hence

more detailed studies were required to isolate and identify the bioactive compounds. [12]

f) A study done by **L. Hussain, Muhammad S H Akash, Noor Ul Ain, Kanwal Rehman and Muhammad Ibrahim (2015)** checked the analgesic and anti-inflammatory effect of T. cordifolia and the results proved that the product have showed significant analgesic effects on the three pain models used for the study. It also helped in reducing the inflammation in the carrageenan-induced inflammation test and showed significant antipyretic effects in the brewer's yeast-induced pyrexia test.[13]

g) A study conducted by **Archa Vermani, Navneet and Shiv Shanker Gautam (2013)** screened the antibacterial potential of *T. cordifolia* stem extracts against dental pathogens. Crude extracts of *Tinospora cordifolia* was checked for the ability to inhibit the growth of dental pathogens such as *Staphylococcus aureus, Streptococcus mutans, Streptococcus salivarius, Lactobacillus acidophilus, Streptococcus sanguinis* and their isolates. The stems were collected, washed dried, crushed and the extract preparation was done in petroleum ether, chloroform, methanol and aqueous using Soxhlet apparatus. The antimicrobial activity was determined using agar well diffusion method and reported that methanolic extract to be most effective and better antibacterial activity was observed against *S. sanguinis* and lowest against *S. salivarius*. The study concluded that the stems of *Tinospora cordifolia* have good antibacterial property and can be used against specific dental pathogens. [14]

h) An in vitro study done by **Nagaprashanthi. Ch, Rafi Khan. P, Gopichand K, Aleemuddin MA, R. Begum. G (2012)** evaluated the antibacterial and antifungal properties of hydroalcoholic extract of *T. cordifolia* grown on

Azadirachta indica tree (TC_1) in comparison to the *T. cordifolia* grown on fencing (TC_2). Microorganisms including *S. aureus, E. coli, Klebseilla pneumonia, Pseudomonas sp., Aspergillus niger, Aspergillus fumigates, Pencillium* and *mucor species* were selected for the study to check antibacterial and antifungal activity of the plant. The results suggested that TC_1 showed potential antimicrobial activity against all the microorganisms whereas TC_2 showed inhibition zone on limited species. Hence the study concluded that *Tinospora cordifolia* can be used effectively as therapeutic agents in the treatment of infections due to its antimicrobial activity. [(15)]

i) A study was conducted by **S.M. S Miguel, L.A Opperman, Edward P Allen, Jan Zeilinski, Kathy K.H (2011)** to provide data regarding the effects of bioactive AO mixtures on cytotoxicity, proliferation and migration of human oral fibroblasts. Fibroblasts were obtained from gingival and periodontal tissues and cultured separately in different concentrations; resveratrol, ferulic acid, phloretin and tetrahydrocurcuminoids. The results reported an increase and decrease, at higher and lower concentrations respectively, on both gingival and periodontal ligament cells. Also, they found an increased DNA synthesis on PDL cells after a period of 48 hours.

Hence, they concluded that high and low concentrations of these antioxidant mixtures may have an effect on the proliferation and migration of gingival and PDL fibroblast and hence may play a beneficial role in the gingival healing or periodontal repair.[(16)]

j) A study was done by **Ramya Premanath and N. Lakshmidevi (2010)** to check the antioxidant activity of *T.cordifolia* where they used dried and powdered leaves of *T. cordifolia* and the extract preparation was done with hexane,

chloroform, methanol, ethanol and water. Total phenolic and flavonoid contents were examined and the highest content was found with ethanol extracts with a value of 5.1 ± 0.25 mg/g and 0.52 ± 0.02 mg/g respectively. Antioxidant assays were performed and ethanol extract showed highest total antioxidant activity of 41.4 ± 0.45 µM Fe (II)/g. The results suggest that the active antioxidant compounds are better extracted in ethanolic extract and there is a positive relation between the extracted total polyphenols and its anti-oxidant activity. [(17)]

k) **Emil Rudolf and Miroslav Cervinka (2010)** did a study to see the changes happening in the motility, morphology and proliferation of gingival and periodontal fibroblast which was grown in a zinc deficient environment or medium. There was reduced motility, derangement in the actin and myosin filament, alteration in the cell morphology, changes in the cell cycle and expression of certain biochemical markers and increased oxidative stress. They also found that after supplementation of the medium with exogenous zinc these changes were reversed i.e, there was decreased oxidative stress, restoration of cell morphology and stimulated fibroblast proliferation.[(18)]

l) A study on **A high-throughput cell migration assay using scratch wound healing, a comparison of image - based readout method** (2004) by Justin C Yarrow, Z.E Perlman, Nicholas J.W, Timothy J.M

It is difficult to perform current migration assays, used for screening small molecules and other perturbations, at the scale required to screen large libraries. The commonly used scratch wound healing assay was adapted to a 384 well plate format, by mechanically scratching the cell substrate with a pin array. It was found that imaging using an automated fluorescence microscope allowed

to differentiate the perturbations affecting cell migration, morphology and division.

They concluded that this assay can be used for screening greater than 10,000 perturbations per day with a quantitative, high-content readout, and to characterize small numbers of perturbations in detail.[19]

m) **R. Jeyachandran, T. Francis Xavier and S.P Anand (2003)** studied the antibacterial effect of ethanolic, chloroform and aqueous extract of *Tinospora cordif*olia stems using the disc diffusion method. *E. coli, Salmonella typhi, P. vulgaris, E. faecalis, Serratia marcesenses* and *S. aureus* were selected for the study. The results suggested that all three extracts exhibited antibacterial activity among which ethanolic extracts showed greater potential. It was assumed that the difference might be due to the more solubility of chemical compounds, which are responsible for the antibacterial activity, in ethanol. So, the study concluded that *Tinospora cordifolia* stems have the potential to inhibit the bacterial activity thereby can be used in the treatment of infections.[20]

n) A study was done by **Isaka J, Ohazama A, Kobayashi M, Nagashima C, Takiguchi T, Kawasaki H, Hasegawa K (2001)** to determine whether PDL cells have a dominant role in alveolar bone repair during the course of periodontal regeneration. Here, roots of mandibular third premolars of canines were taken and divided into a PDL(+) group, where the PDL was preserved, and a PDL(-) group, where the PDL was removed.

Osteoblast phenotype was exhibited by both cultured dog periodontal ligament cell and dog bone cell (DBC). The group where PDL was preserved only showed new connective tissue attachment. However, alveolar bone was almost completely restored to the original bone height in both the groups but the

amount of new bone formation was not significantly different between the 2 groups.[21]

o) A study done by **James J.A, Sayers N.M, Drucker D.B, Hull P.S** evaluated the effects of nicotine and cotinine on the growth and attachment of periodontal ligament fibroblasts. Cell cultures were obtained from extracted premolar teeth. The cells were used at high and low passages and the cell numbers were analysed at the end of 14 days.

The results suggested that both nicotine and cotinine had inhibitory effect on the growth and attachment of both gingival and periodontal ligament fibroblasts, confirming the role of these substances in the progression of periodontitis and inhibitory role in the regenerative therapy.[22]

p) A study conducted by **S. Takayama, S. Murakami, Y. Miki, K. Ikezawa, S. Tasaka, A. Terashma, T. Asano, H. Okado (1997)** observed how basic fibroblast growth factor (bFGF) regulated the mechanism of periodontal regeneration and its effect on proliferation of, activity of alkaline phosphatase, extracellular matrix alkaline phosphatase activity and mineralized nodule formation by periodontal ligament cells. The results suggested that bFGF may play a role in wound healing by reducing cytodifferentiation of PDL cells into mineralized tissue forming cells. This meant that immature PDL cells were included in wound healing and that in turn accelerated periodontal regeneration.[23]

q) **Kuzuya. M, Nainto M, Funaki C, Hayashi T, Yamada K, Asai K, Kuzuya F (1991)** studied the effect of antioxidants, especially probucol in the proliferation of endothelial cells. Probucol, a lipid-lowering drug which has antioxidant properties, was added to the growth medium and found that it stimulated the

proliferation of endothelial cells. They also concluded that the stimulatory effect is not due to the prevention of free radical reactions but due to the uptake of antioxidants by endothelial cells.[24]

r) A **Comparative study of human periodontal ligament cells and gingival fibroblasts** was done by **Somerman M.J, Archer S.Y, G.R Imm and Foster R.A (1988)** for a clear understanding regarding the roles of periodontal ligament and gingival tissue in periodontal regeneration. Hence, they compared periodontal ligament fibroblast and gingival fibroblast, derived from the same patient, in vitro and found that protein and collagen production and alkaline phosphatase levels were high in periodontal ligament cells when compared to that of gingival fibroblasts.[25]

s) A study conducted by **Gottlow J, Nyman S, Karring T, Lindhe J (1984)** aimed to check whether the new attachment formation occurs in the absence of oral epithelium and gingival connective tissue during healing period following the treatment. Three monkeys were taken and four roots from each monkey were selected for the study and the contralateral teeth served as controls. After exposing the coronal portion of the buccal root surface surgically, plaque was allowed to accumulate for a period of 6 months. After which the fap was raised and the scaling of the root surfaces and root planning was done carefully. The crowns were resected for both groups and sutured back ensuring proper root coverage. In test groups they placed Millipore filter membranes before suturing to avoid the contact between soft tissue and root surfaces. All 3 monkeys were sacrificed after 3 months and the jaws were removed and histological sections of the experiment teeth was obtained. The results of the study concluded that the test group was having more new tissue attachment than the control group proving

the fact that periodontal ligament cells are capable of inducing connective tissue attachment.[26]

t) A study was done by **Nyman S et al (1982)** on **The Regenerative Potential of the Periodontal Ligament,** to evaluate the formation of new cementum and new attachment during the wound healing period in such a way that the repopulation of the periodontal ligament cells occurs around the wound area adjacent to the root surface which has been deprived of the periodontal ligament and cementum layer surgically. Maxillary lateral incisors and mandibular canines were selected for the study. Mucoperiosteal flap was elevated followed by which buccal and proximal alveolar bone was removed in an area extending from the mid-root level to a point 2mm apical to the marginal bone crest. Then, curettage of the root surfaces was done to remove the cementum after which they prepared notches on the root surface to define the denuded root portion. Before flap repositioning, a Millipore filter was placed to avoid the contact between connective tissue and surface of root during healing time. All the animals were sacrificed after 6 months of the surgery and their jaw bones were removed to obtain the histological sections of experimental teeth and surrounding tissue. The results suggested that the healing was not consistent along the entire root length. The coronal surface was characterized by the connective tissue adhesion without the formation of cementum or fibrous attachment. Hence the study concluded that PDL cells have the capacity for the reestablishment of connective tissue attachment.[27]

METHODOLOGY

ARMAMENTARIUM

- *Tinospora cordifolia aqueous* stem extract
- Whatman filter paper
- 15ml sterile bottle
- Kidney tray
- Surgical gloves
- BP blade no.22 and handle
- 5ml of phosphate buffer saline
- Vertical laminar air flow
- Glass petri dish
- Dulbecco's modified Eagles media
- Micropipettes of all range
- 24 well culture plate
- CO_2 incubator
- 0.25% trypsin in EDTA buffer
- Neubauer's counting chamber
- 96 wells microtiter plate
- Micropipette tip
- Inverted microscope
- MTT reagent
- Aluminium foil
- Dimethyl sulfoxide
- Spectrophotometer

CHEMICAL REAGENTS

Chemical reagents used for the study are :-

SR.NO	CHEMICAL	COMPANY
1.	Dulbecco's Modified Eagle's Media	HIMEDIA
2.	0-25% trypsin	HIMEDIA
3.	Antibiotic + Antimycotic 100X	Gibco
4.	Fetal Bovine Serum	HIMEDIA
5.	Phosphate Buffer Saline	HIMEDIA
6.	MTT Reagent	HIMEDIA
7.	Alizarin Red S Dye	HIMEDIA
8.	Dimethyl Sulfoxide (DMSO)	Fischer Scientific (Qualigens)

Fig. 1: CHEMICAL REAGENTS

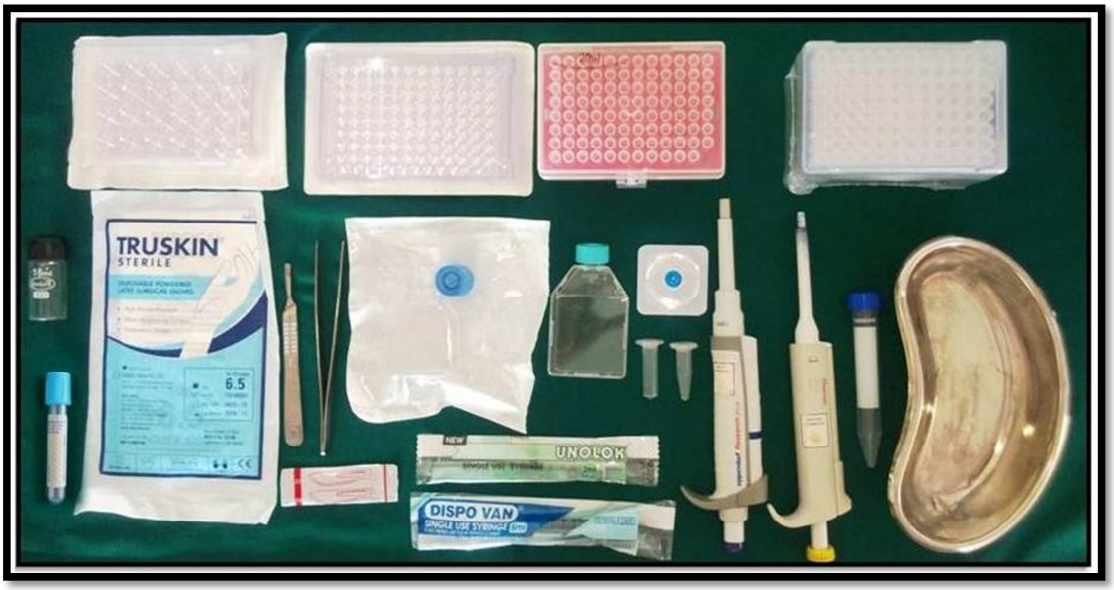


Fig.2 : ARMAMENTARIUM

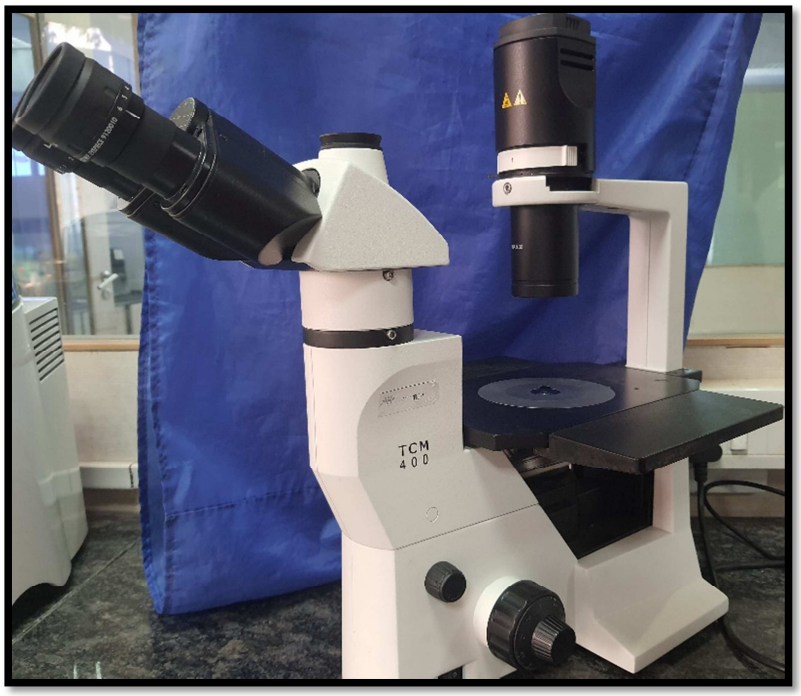


Fig.3: MICROSCOPE

Fig.4: LAMINAR AIR FLOW

Fig.5: CO2 INCUBATOR

Fig.6: SPECTROPHOTOMETER

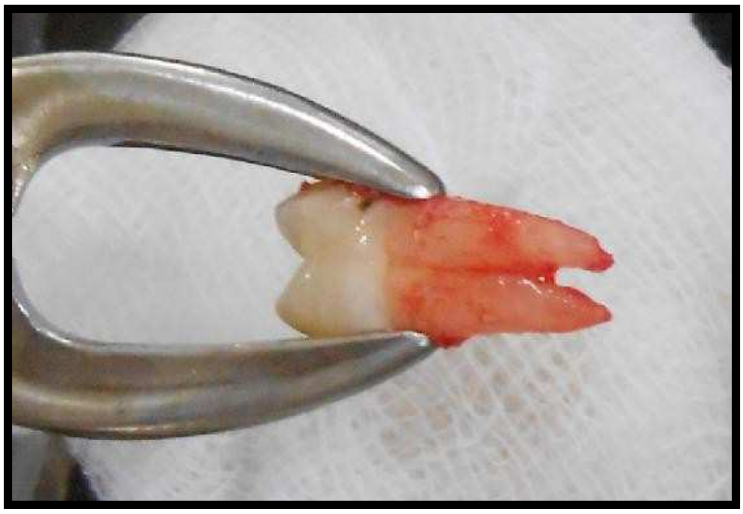
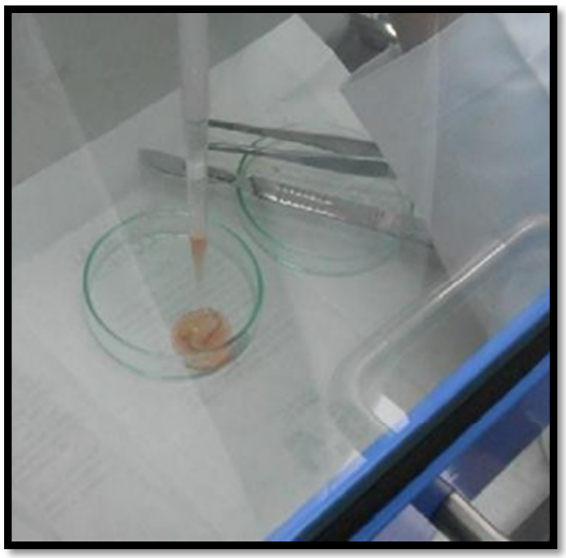


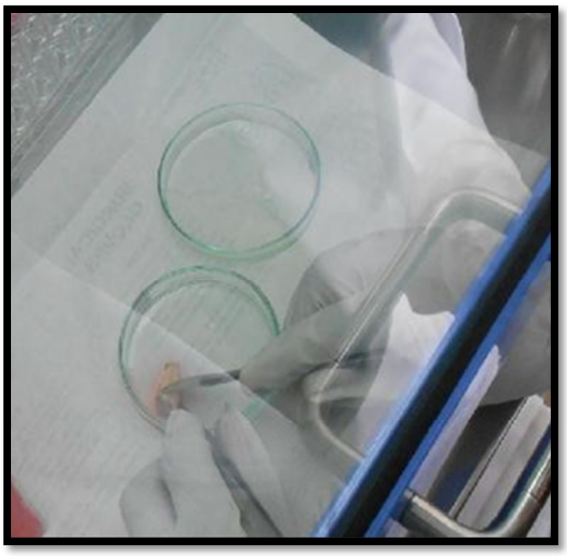
Fig.7: FRESHLY EXTRACTED TOOTH

Fig.8: TOOTH COLLECTED IN FBS

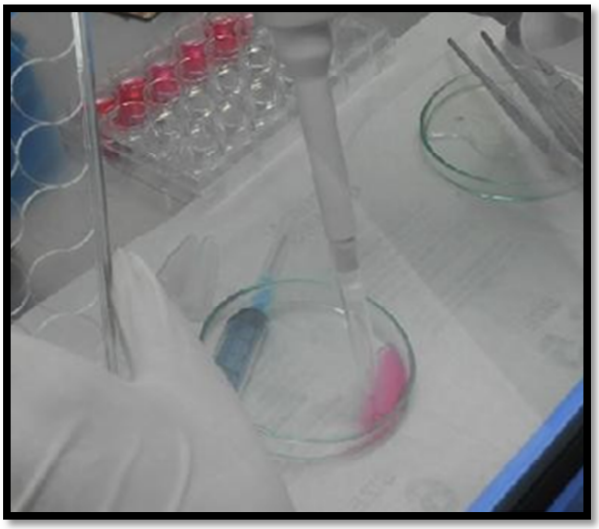
Fig.9: HARVESTING OF PERIODONTAL LIGAMENT FIBROBLASTS



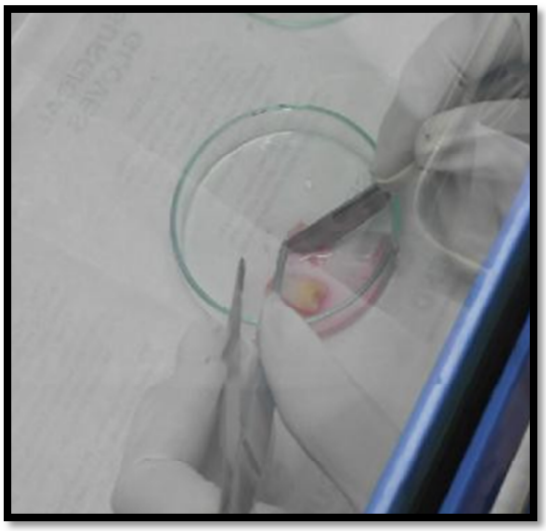
Tooth Sample Taken into a Petri-dish and 2ml DMEM added into it



Periodontal ligament fibroblasts harvested by scraping the mid-root section of the teeth



Tissue Minced into small pieces



Tissue along with media aspirated and transferred to 24 well plate using micropippette

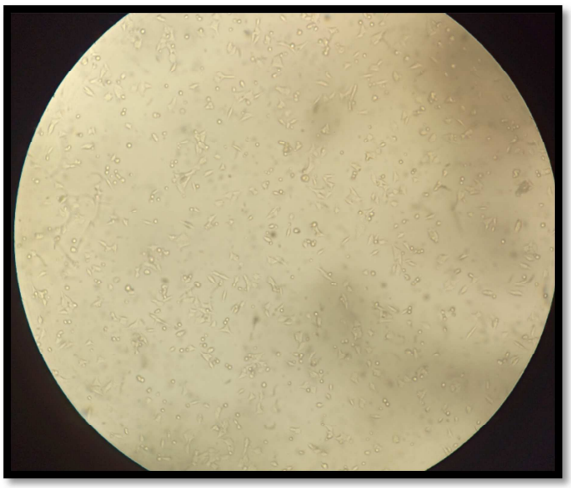


Fig.10: Cultured PDL Fibroblast

Fig.11: *Tinospora cordifolia*

Fig.12: Preparation of Extract

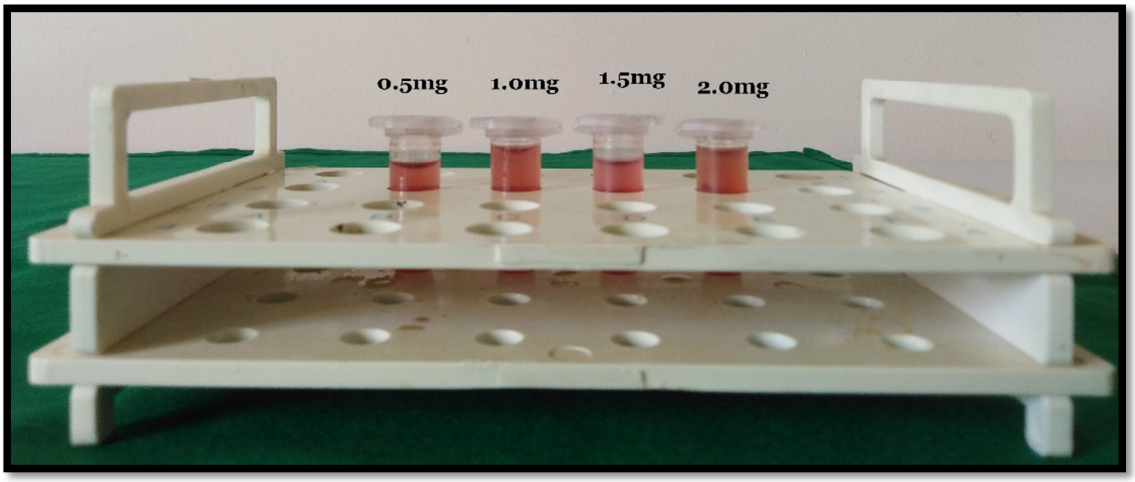


Fig.13: Different Concentrations of aqueous extract of T. cordifolia (0.5mg, 1.0mg, 1.5mg, 2.0mg)

SOURCE OF DATA

All experimental procedures were approved by the Research and Ethical Committee of KAHER's KLE V K Institute of Dental Sciences, Belagavi.

This study was conducted in the Department of Periodontics, KAHER's KLE V K Institute of Dental Sciences, Belagavi utilizing the outpatient department facilities.

The laboratory procedure was undertaken at KAHER's Dr. Prabhakar Kore Basic Science Research Center (BSRC), Belagavi.

Extracted teeth of the patients who reported to the Department of Oral and Maxillofacial Surgery for orthodontic reasons were collected.

The dried stem of *Tinospora cordifolia* was procured and authenticated from KAHER's Shri B M Kankanwadi Ayurveda Mahavidyalaya, Belagavi.

The preparation of aqueous stem extract of *Tinospora cordifolia* was done at KAHER's Dr. Prabhakar Kore Basic Science Research Center (BSRC), Belagavi.

Primary cultures of periodontal ligament fibroblast cells were prepared from the teeth extracted for orthodontic reasons. Sufficient numbers of cells were obtained by subsequent propagation. The study was carried out on periodontal ligament fibroblast, from third to fifth passages. The experiments for cell migration and proliferation included a total of 45 wells, which were divided into five groups:

Group 1 (Control group): PDL fibroblast without any treatment.

Group 2 (Test group): PDL fibroblast treated with 0.5mg of *T. cordifolia* extract

Group 3 (Test group): PDL fibroblast treated with 1.0mg of *T. cordifolia* extract

Group 4 (Test group): PDL fibroblast treated with 1.5mg of *T. cordifolia* extract

Group 5 (Test group): PDL fibroblast treated with 2.0mg of *T. cordifolia* extract

CRITERIA FOR SELECTION

Inclusion Criteria:

1. Systemically healthy patients between the age of 18-30 years.
2. Teeth indicated for extraction for orthodontic reasons.
3. Teeth without any pathology like caries, periapical lesions etc.

Exclusion Criteria:

1. Patients who have taken antibiotics in the past 3 months before getting the tooth extracted.
2. Smokers and patients consuming tobacco in any form.
3. Pregnant women and lactating mothers.

METHOD OF COLLECTION OF DATA

1. Preparation of Aqueous Stem Extract of *Tinospora cordifolia*

Dried stems of *Tinospora cordifolia* were collected and authenticated from KAHER's Shri B M Kankanwadi Ayurveda Mahavidyalaya, Belagavi. These dried stems were powdered into fine particles in the laboratory of KAHER's Dr. Prabhakar Kore Basic Science Research Center (BSRC), Belagavi. 25 grams of this powder was soaked in 250ml of distilled water for 48 hours. Followed by the filtration of the solution was done using Whatman filter paper No.1 and dried using water bath. After evaporation, the extracted was collected into a sterile Eppendorf tube, sealed and stored in -4°C.

2. Periodontal Ligament Isolation and Cell Culture

Periodontal ligament fibroblast cells were obtained from the teeth extracted for orthodontic reasons. The patients before undergoing extraction were given a pre-procedural rinse with 0.2% chlorhexidine mouthwash, after which the extraction was carried out as atraumatically as possible. The extracted teeth were immediately transferred to a 15ml sterile bottle containing the transport media. The transport media used was 5ml of Phosphate buffer saline (PBS). Extracted teeth were then sent to the Dr. Prabhakar Kore Basic Science Research Center for further procedures. Under the vertical laminar air flow, teeth were removed from the bottle and transferred to a sterile petri dish. They were first washed with Phosphate buffer solution (PBS) in the petri dish and then transferred to another petri dish to which 2ml of Dulbecco's Modified eagle's Medium (DMEM) was added. Following which the tissue over the

mid root surface of the teeth were scraped with a BP blade No. 22 attached to its handle and macerated into smaller portions.

The obtained tissue was then seeded with a micropipette into a 24 well plate to which Dulbecco's modified Eagle's Medium (DMEM) supplemented with antibiotic + antimycotic 100 X and 10% fetal bovine serum (FBS) was added. The plate was then placed at 37°C in a 5% CO_2 humidified atmosphere and allowed to incubate. The media was changed every 2 to 3 days and the cells were sub-cultured on acquiring sufficient confluency.

By 4-6 weeks, the cell growth becomes 60 – 70% confluent and it was subjected to the process of trypsinisation. The medium was removed from the wells completely and the cell layer was washed with Phosphate buffer solution (PBS).

To allow the cells to detach from the plate, 0.25% trypsin in EDTA buffer was added to the wells and was allowed to stand for 2-3 minutes. The cell count was done, after staining with tryphan blue stain, using Neubauer's counting chamber.

Dilutions were prepared to get the required cell density. Cells were then seeded in 96 well plates and were allowed to attach to the plate overnight. These periodontal ligament fibroblast cells were then subjected to test compounds to perform the proliferation and migration assays.[28]

3. Cell Migration

In vitro scratch test was performed to detect the migration of the periodontal ligament fibroblast.

Principle

This in vitro test is based on the observation that, on a confluent cell monolayer, by creating a new artificial gap, (so called "scratch"), the cells from the edge of the newly created gap will move toward the opening to close the "scratch" till new cell to cell contacts are formed again. Followed by, recording of images at the beginning and at regular intervals during cell migration to close the scratch, will be recorded and comparison of the images will be done to evaluate the rate of cell migration.[29]

Procedure

The cells were seeded at a concentration of 1 x 10^5 cell/well in 96 wells microtiter plate and were allowed to attach overnight. Using a sterile micropipette tip, a scratch was made in each well. For the control group, DMEM was added into the wells. Separate wells were treated with test compounds. The scratch was then observed under microscope and a picture was taken at 0^{th} hour. The cell culture plate was placed at 37°C in a 5% CO_2 humidified atmosphere and allowed to incubate. Pictures of the same were taken after 24, 48 and 72 hours. The width of the wound was measured using the imageJ software.

The distance between the edges of scratch was calculated (in µm), at three points (upper, middle and lower), for all pictures using imageJ software. The mean of these three points were calculated and taken as the mean migration rate of each group. [30]

4. Cell Proliferation

Proliferation of the periodontal ligament fibroblast cells as determined by MTT assay.

Principle of MTT assay

MTT assay is a calorimetric assay which determines cell proliferation. The reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase is evaluated in this assay. MTT that enters the cells is taken up by the mitochondria and is reduced to an insoluble, coloured (dark purple) formazan product. The crystals formed are then solubilized using an organic solvent (i.e. DMSO, SDS or Isopropanol). The solubilized formazan is measured spectrophotometrically at 570nm.

Reduction of MTT takes place only in metabolically active cells. The amount of colour produced and the number of viable cells are directly proportional to each other.[28]

Procedure

The cells were seeded at a concentration of 1×10^5 cells/well in 96 well microtiter plate and were allowed to attach overnight. In the test group, 100µl of different concentrations of extract, which was dissolved in DMSO (100µl of DMSO and 900µl of extract), were added and in the control group, the cells were treated with only DMEM media. The plate was then kept for incubation for 5 days in CO_2 incubator at 37°C in a humidified atmosphere containing 5% CO_2. 20µl of 5mg/ml MTT reagent was added to the wells after 2 days, wrapped in an aluminium foil (since MTT reagent is photosensitive) and the plate was kept at room temperature for incubation in a dark place for 4 hours. Without disturbing the precipitated Formazan

crystals, the supernatant was carefully removed and 200µl of DMSO was added to dissolve the formed crystals. The optical density was measured using spectrophotometer at a wavelength of 570nm. The proliferation rate of aqueous stem extract of *T. cordifolia* was assessed by determination of colour change in spectrophotometer occurred due to conversion of MTT into "Formazan blue" by living cells.

STATISTICAL ANALYSIS

1. Comparison of proliferation and migration between the 5 groups was analysed by ANOVA (analysis of variance).
2. The significant, pairwise comparison between groups was done by Tukey post hoc test.
3. Probability value of less than 0.05 was considered as statistically significant.

RESULTS

1. PROLIFERATION ASSAY

Table1: Mean and Standard Deviation of the proliferation rate for each groups of different concentrations (0.00, 0.50, 1.00, 1.50, 2.00)

Proliferation	N	Mean	Std. Deviation
0.50	6	0.50683	0.032664
1.00	6	0.55133	0.043675
1.50	6	0.55500	0.070359
2.00	6	0.57733	0.078724
Total	30	0.52640	0.070272

Table2: Intergroup Comparison using ANOVA

There is a statistically significant difference (p = 0.002) in the mean of proliferation rate between the different concentrations.

Proliferation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.070	4	0.017	5.934	0.002
Within Groups	0.073	25	0.003		
Total	0.143	29			

Table3: Tukey post hoc test for intergroup comparisons of proliferation rate

The results show a statistically significant difference in the rate of proliferation between the control group and Group 3 (p = 0.014), Group 4 (p = 0.010), Group 5 (p = 0.002). However, no statistically significant differences were found between the Group 1 (control group) and Group 2 (p = 0.257)

Tukey post hoc test						
					95% Confidence Interval	
(I) Concentration		**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**Lower Bound**	**Upper Bound**
0.00	0.50	-0.065333	0.031296	0.257	-0.15725	0.02658
	1.00	-.109833*	0.031296	0.014	-0.20175	-0.01792
	1.50	-.113500*	0.031296	0.010	-0.20541	-0.02159
	2.00	-.135833*	0.031296	0.002	-0.22775	-0.04392
0.50	0.00	0.065333	0.031296	0.257	-0.02658	0.15725
	1.00	-0.044500	0.031296	0.620	-0.13641	0.04741
	1.50	-0.048167	0.031296	0.548	-0.14008	0.04375
	2.00	-0.070500	0.031296	0.194	-0.16241	0.02141
1.00	0.00	.109833*	0.031296	0.014	0.01792	0.20175
	0.50	0.044500	0.031296	0.620	-0.04741	0.13641
	1.50	-0.003667	0.031296	1.000	-0.09558	0.08825
	2.00	-0.026000	0.031296	0.918	-0.11791	0.06591

1.50	.00	.113500*	0.031296	0.010	0.02159	0.20541
	.50	0.048167	0.031296	0.548	-0.04375	0.14008
	1.00	0.003667	0.031296	1.000	-0.08825	0.09558
	2.00	-0.022333	0.031296	0.951	-0.11425	0.06958
2.00	.00	.135833*	0.031296	0.002	0.04392	0.22775
	.50	0.070500	0.031296	0.194	-0.02141	0.16241
	1.00	0.026000	0.031296	0.918	-0.06591	0.11791
	1.50	0.022333	0.031296	0.951	-0.06958	0.11425

*. The mean difference is significant at the 0.05 level.

2. MIGRATION ASSAY

Table 4: Mean and Standard Deviation of Each Group at Different Time Intervals

Groups	N	Time	Mean	Std. Deviation
Group 2 (0.5mg)	3	0 to 2 hours	22866.67	2779.089
	3	2 to 4 hours	14733.33	3962.743
	3	4 to 6 hours	733.33	642.910
	3	6 to 24 hours	24766.67	21946.830
	12	Total	15775.00	13772.312
Group 3 (1.0mg)	3	0 to 2 hours	1233.33	907.377
	3	2 to 4 hours	13400.00	4190.465
	3	4 to 6 hours	2266.67	1401.190
	3	6 to 24 hours	53633.33	1517.674

	12	Total	17633.33	22365.450
Group 4 (1.5mg)	3	0 to 2 hours	3933.33	1320.353
	3	2 to 4 hours	20233.33	7092.484
	3	4 to 6 hours	4266.67	5071.818
	3	6 to 24 hours	43566.67	2809.508
	12	Total	18000.00	17338.030
Group 5 (2.0mg)	3	0 to 2 hours	7300.00	2443.358
	3	2 to 4 hours	20900.00	3269.557
	3	4 to 6 hours	5300.00	1873.499
	3	6 to 24 hours	63033.33	945.163
	12	Total	24133.33	24359.591
Control	3	0 to 2 hours	10733.33	3271.595
	3	2 to 4 hours	12433.33	808.290
	3	4 to 6 hours	900.00	100.000
	3	6 to 24 hours	46766.67	1833.939
	12	Total	17708.33	18189.930
Total	15	0 to 2 hours	9213.33	8043.442
	15	2 to 4 hours	16340.00	5190.211
	15	4 to 6 hours	2693.33	2838.125
	15	6 to 24 hours	46353.33	15601.413
	60	Total	18650.00	19111.834

Table 5: Intergroup comparison between different concentrations of compounds, different time intervals and between compound and time interval

Statistically significant difference was seen between the different concentrations, different time intervals and between compounds and time interval.

Dependent Variable: Migration					
Source	**Type III Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
Corrected Model	20251250000.000[a]	19	1065855263.158	32.815	0.000
Intercept	20869350000.000	1	20869350000.000	642.519	0.000
Compound	488105000.000	4	122026250.000	3.757	0.011
Time	16747150000.000	3	5582383333.333	171.869	0.000
Compound * Time	3015995000.000	12	251332916.667	7.738	0.000
Error	1299220000.000	40	32480500.000		
Total	42419820000.000	60			
Corrected Total	21550470000.000	59			

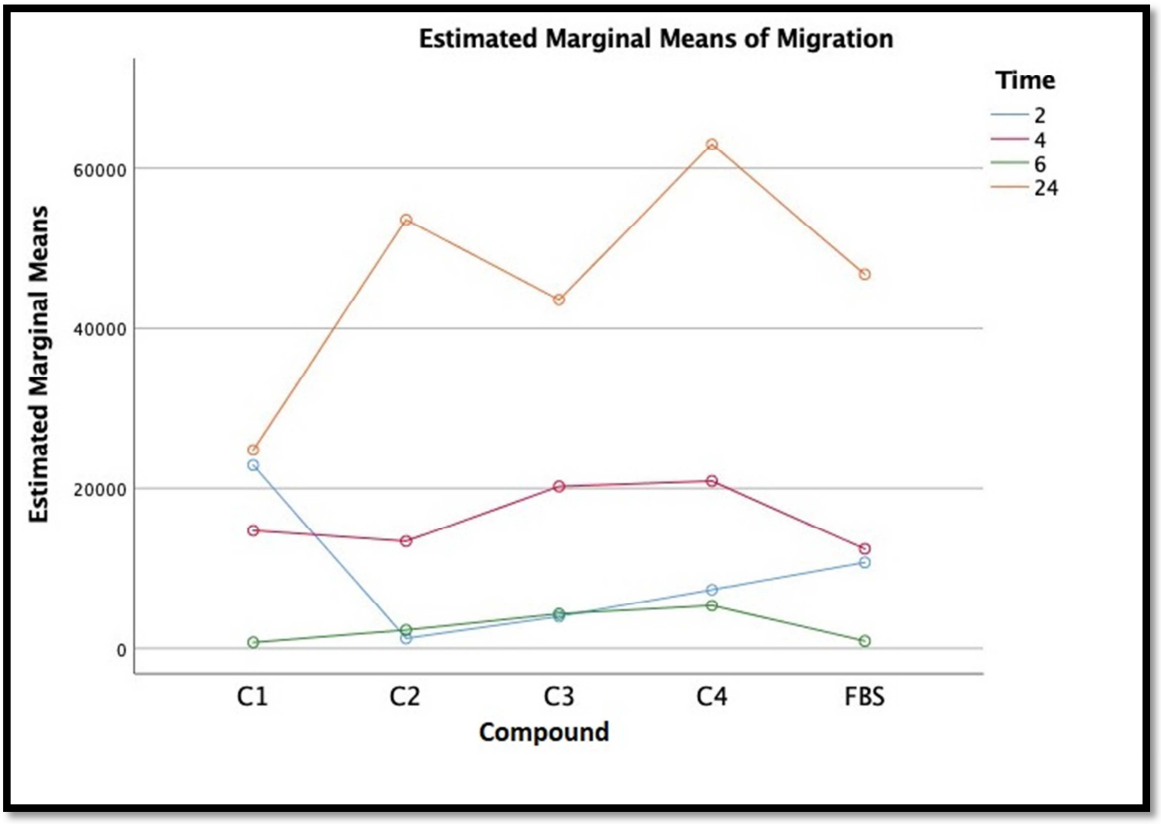
a. R Squared = .940 (Adjusted R Squared = .911)

Table 6: Tukey Post Hoc Tests done for the intergroup comparison between different concentrations of the compound and time.

Tukey HSD		Migration				
					95% Confidence Interval	
(I) Compound		Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Group 2 (0.5mg)	Group 3	-1858.33	2326.675	0.929	-8503.53	4786.86
	Group 4	-2225.00	2326.675	0.873	-8870.20	4420.20
	Group 5	-8358.33*	2326.675	0.007	-15003.53	-1713.14
	Control	-1933.33	2326.675	0.919	-8578.53	4711.86
Group 3 (1.0mg)	Group 2	1858.33	2326.675	0.929	-4786.86	8503.53
	Group 4	-366.67	2326.675	1.000	-7011.86	6278.53
	Group 5	-6500.00	2326.675	0.058	-13145.20	145.20
	Control	-75.00	2326.675	1.000	-6720.20	6570.20
Group 4 (1.5mg)	Group 2	2225.00	2326.675	0.873	-4420.20	8870.20
	Group 3	366.67	2326.675	1.000	-6278.53	7011.86
	Group 5	-6133.33	2326.675	0.083	-12778.53	511.86
	Control	291.67	2326.675	1.000	-6353.53	6936.86
Group 5 (2.0mg)	Group 2	8358.33*	2326.675	0.007	1713.14	15003.53
	Group 3	6500.00	2326.675	0.058	-145.20	13145.20
	Group 4	6133.33	2326.675	0.083	-511.86	12778.53
	Control	6425.00	2326.675	0.062	-220.20	13070.20
Control	Group 2	1933.33	2326.675	0.919	-4711.86	8578.53
	Group 3	75.00	2326.675	1.000	-6570.20	6720.20
	Group 4	-291.67	2326.675	1.000	-6936.86	6353.53
	Group 5	-6425.00	2326.675	0.062	-13070.20	220.20

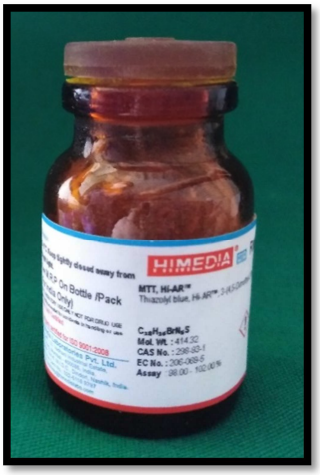
Graph 1: Estimated Marginal Means of Migration with Respect to Time

The plot of the mean " Migration " value for each combination of groups with respect to "Concentrations" and "Time".

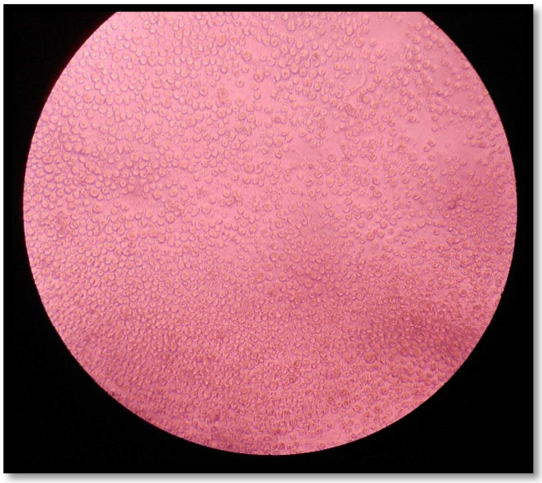


Results of two-way ANOVA test showed Group 5 was having statistically significant difference in the rate of migration than Group 2 at 2, 4, 6 and 24 hours intervals ($p = .002$). However, there were no statistically significant differences between the other concentrations at different time intervals.

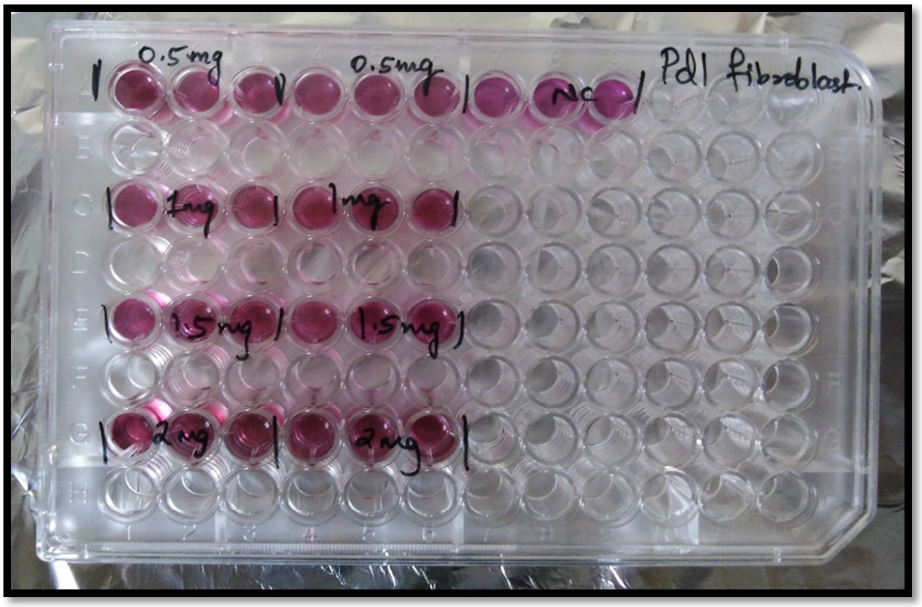
Fig.14: PROLIFERATION ASSAY



MTT Reagent



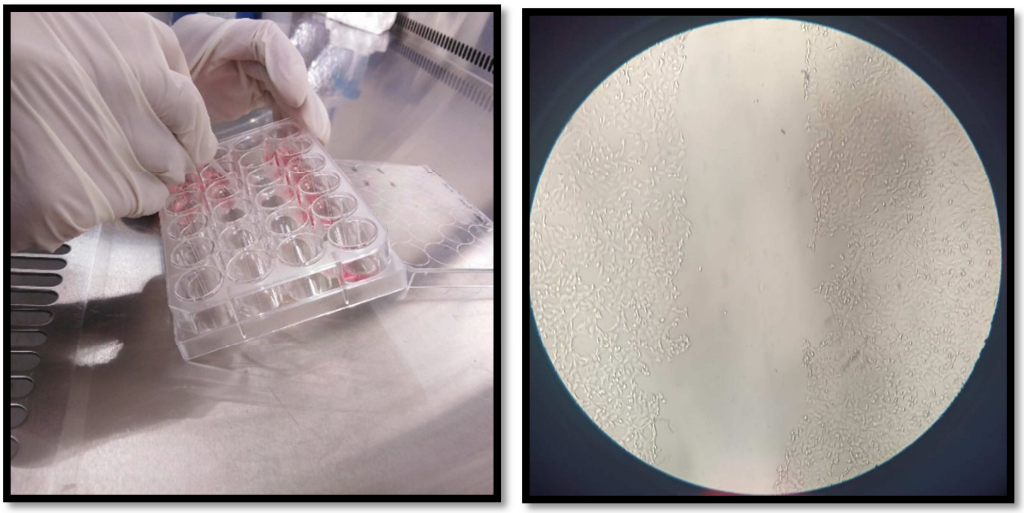
Spectrophotometer



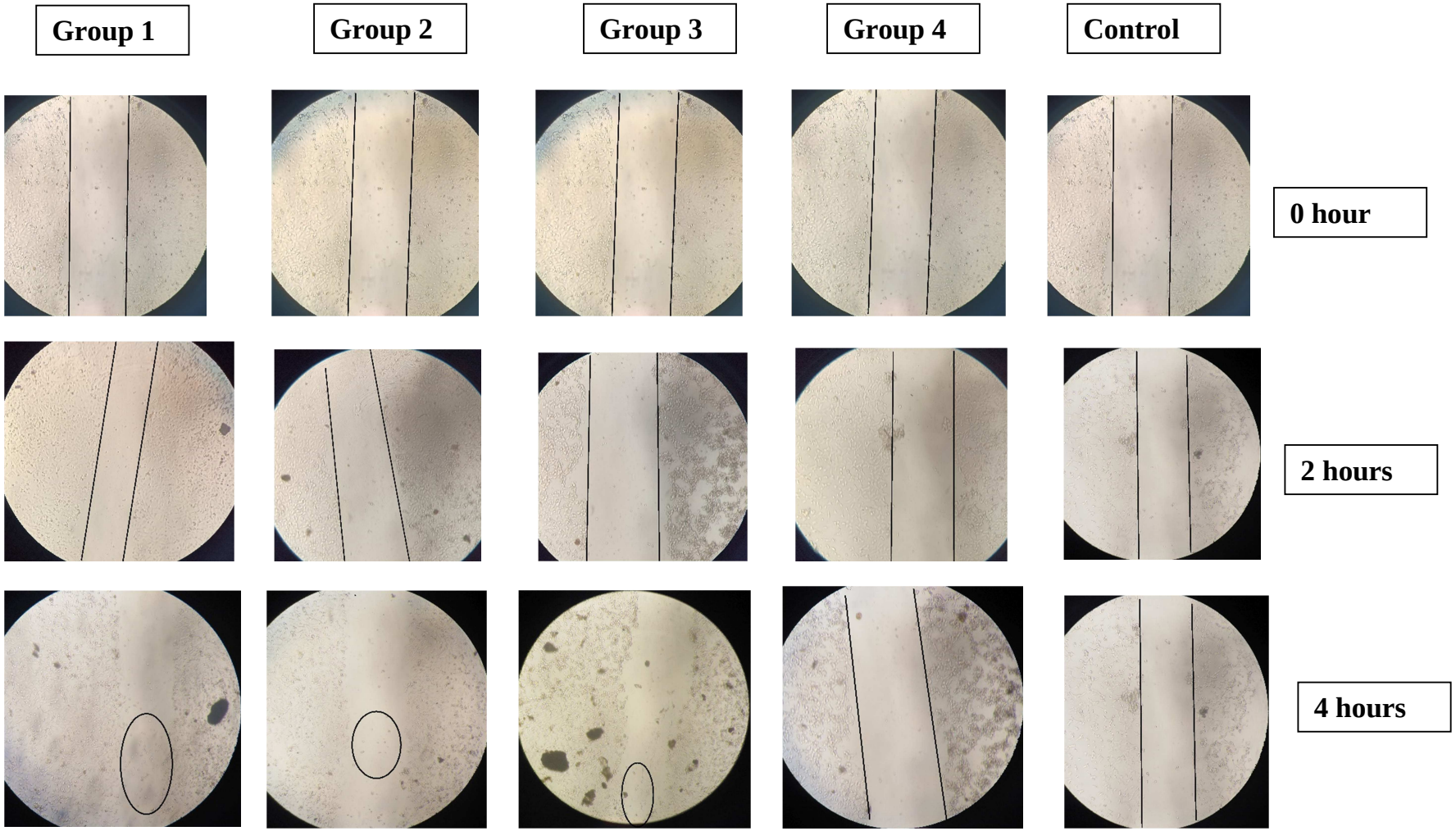
Color changes observed due to reduction of MTT reagent in all four groups

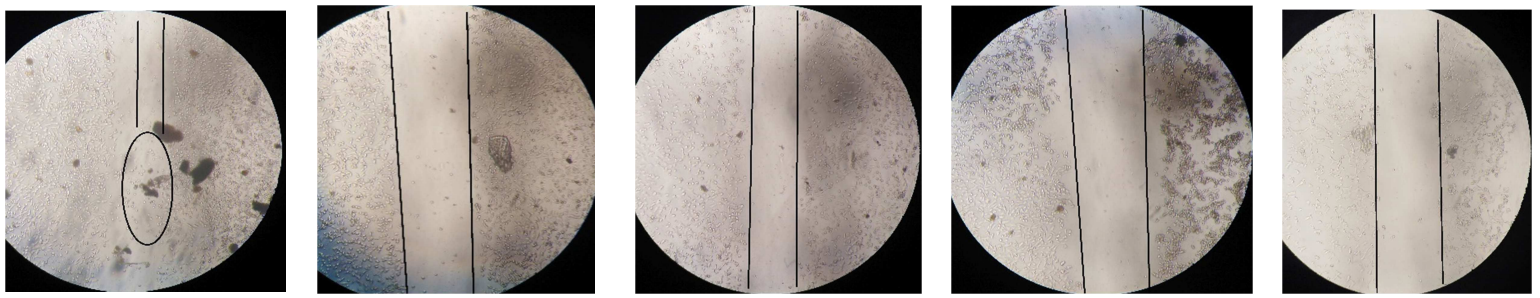
After Addition of MTT Reagent

Fig.15: MIGRATION ASSAY

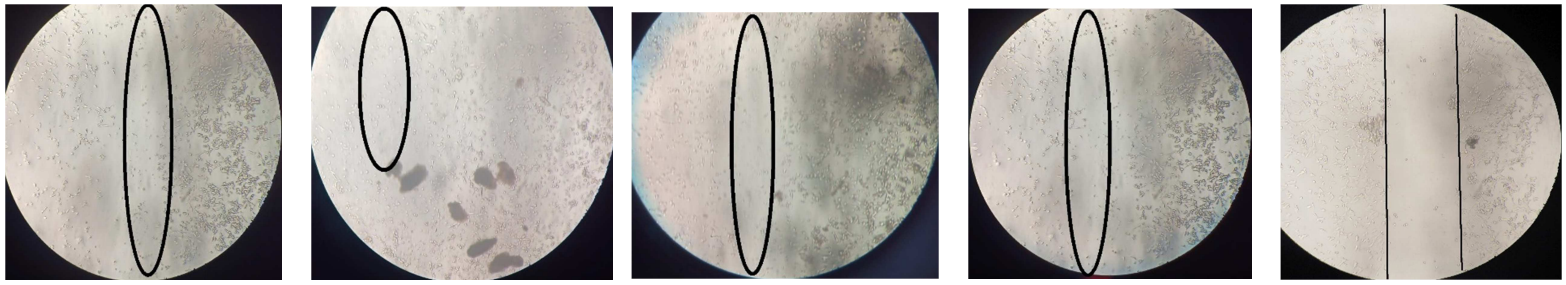


Scratch test


Group 1
Group 2
Group 3
Group 4
Control
0 hour
2 hours
4 hours



6 hours



24 hours

Migration of different groups seen at different time intervals

DISCUSSION

The goals of periodontal therapy include arrest of periodontal disease progression and also the regeneration of structures that are lost due to the disease. Histologically periodontal regeneration is defined as the regeneration of the tooth supporting structures, alveolar bone, periodontal ligament and cementum, over a previously diseased root surface.[31]

In periodontal tissues, wound closure involves restoration of a mucosal seal through the tightly regulated coordination of resident cells in the epithelial and connective tissue compartments. This is essential for protecting the periodontium by preventing microbial invasion and colonization. In the healing phase, the reconstruction of periodontal tissue structures mainly involves fibroblasts, which form and organize collagen fibers that connect alveolar bone and gingiva to the cementum covering the root surface.[4]

Tinospora cordifolia (commonly named as "Guduchi") is a quintessential herb that is genetically diverse in nature and belongs to family *Menispermaceae.* Many active components have been derived from different parts of this plant which are beneficial medicinally.(8) It is a Rasayana which have been used to help improve the immune system and thereby making the body resistant to infections.(6) Studies have proved that the plant have various other properties like antibacterial, immunomodulatory, antioxidant, anti-osteoporotic, antitoxic effects(8) along with the presence of various minerals such as copper, calcium, zinc etc (5)

The immunomodulatory property of *Tinospora cordifolia* is documented as it is reported to boost the phagocytic activity of macrophages, production of reactive

oxygen species and enhances nitric oxide production. [8] The compounds which are responsible for the immunomodulatory activity are 11-hydroxymustakone, N-methyl-2-pyrrolidone, N-formylannonain, cordifolioside A, magnoflorin and syringin.[5]

This plant also has antioxidant property which is due to the presence of arabinogalactan polysaccharide and phenolic compound.[5] In the study conducted by Ramya et al(2010), antioxidant activity of *Tinospora cordifolia* leaves were evaluated and suggested that the active antioxidant compounds are better extracted in ethanol. [17]

Tinospora cordifolia possess potential antimicrobial activity and reported to function in bacterial clearance with improving phagocytic and bactericidal capacity of neutrophils, in mice models.[8] It is also reported to have an effect on the proliferation, differentiation and mineralization of bone like matrix, in vitro, due to the presence of 20-OH-β-Ecd.[8]

The stem of *T. cordifolia* contains zinc which is critical for the biologic functioning of many enzymes, transcription factors, metalloproteins and structural proteins involved in various aspects of cellular metabolism. [32] In addition, zinc contributes to the regulation of gene expression and cell signalling pathways, and helps in the maintenance of cellular homeostasis.[33] Zinc is an anti-inflammatory, anti-oxidant agent that is known to stimulate human gingival and periodontal fibroblast proliferation.[18]

The present in vitro study used periodontal ligament fibroblast cell lines which were obtained from teeth extracted from healthy individuals within the age range of 18-30 years of age. Cellular density differs depending on age, function and animal

species considered.[34] With increase in the age, certain changes occur in the periodontal ligament including progressive thinning of the PDL, decline in mitotic activity of cells, decelerated production inorganic matrix leading to scarcity soluble collagen.

Grant and Bernick (1972) studied specimens obtained from four human male cadavers to evaluate the effect of aging on periodontium. The data suggested that there was an age associated increase in fibrosis and a decrease in cellularity of the PDL tissue.[35]

Krieger et al; 2013 did a study to check age related changes in the density of periodontal ligament tissues and a significant decrease in the fibroblast number with age was seen. It was observed that individuals between the age range of 20-29 years had a significantly higher fibroblast density when compared to other higher age groups (>30 years). Also, the fibroblast density was less in the age range of 20-29 years when compared to 10-19 years of age group. [36]

The amorphous nature of the dental follicle is progressively replaced by the composed formation of the PDL, during the course of root formation. The eruption of premolars occurs at an age of 10-12 years in humans and the root formation is completed by 12-13 years when occlusion is achieved. Harvesting of PDL fibroblasts at different stages of tooth development and under varying situations may influence the behavior of cells in culture. [34]

Therefore, in the current study all the premolars which had completed their root formation were included so as to avoid the bias of including stem cells from the apical papilla of the erupting tooth. This is because of the high proliferation rate of

stem cells which can result in recording false positive results. So, individuals undergoing extraction for orthodontic treatment aged between 18-30 years were included.

To avoid any microbial infection of fibroblasts from apical region or pulp tissue, teeth with caries or any other periapical pathology were excluded.

Liu J et al; 2013 did a study where he treated cultured human periodontal ligament with different concentrations of glucose (5.5, 15, 25 and 35mM) for 24 hours and the results showed that at higher concentrations of glucose, there was a concentration- and caspase-3-dependent increase of apoptosis in cultured human periodontal ligament fibroblasts in vitro.[37]

Nicotine has a proven inhibitory effect on the migration, proliferation, attachment, alkaline phosphatase activity and chemotactic activity of PDL cells in a concentration dependent manner and thus has a detrimental biological effect on PDL cells. [34]

Because of these facts, individuals having diabetes mellitus, habit of tobacco use or chronic smokers were excluded from the study.

Using preserved cell lines may help to establish more consistent results among different studies, but there are advantages in using primary cultures for regeneration experiments. The main advantage of using primary PDL fibroblast cell cultures at early passages is that they retain their phenotypic and functional heterogeneity distinctive of original tissue. When there is an increase in the number of passages (passage 20), cells bring about change in the structure and biological activity when

compared to the cells at early passages (passage 7). [34] Hence, in this study, primary cultures of PDL fibroblast were grown from freshly extracted teeth

Harvesting of PDL fibroblasts was done from the mid root section as there are less chances for contamination of this portion through gingival sulcus or pulp tissue. [38]

Fibroblasts were evaluated because they are the predominant cells of the periodontal ligament constituting approximately 60% of the PDL cell population. The PDL fibroblast plays a significant role in normal turnover, repair and regeneration, development, structure and function of the tooth supporting apparatus. [39]

Many in vitro and in vivo studies have been done to evaluate the potential of PDL cells in periodontal regeneration. Some of the results showed that PDL fibroblast have properties like expressing classic osteoblastic features, function as osteoblast or cementoblast under regenerative conditions and regulation of mineral formation, thereby helping to prevent ankylosis during regeneration. [31]

Thus, early recruitment and restoration of fibroblasts in the periodontal lesion, and accelerated formation of PDL are regarded as critical events for successful periodontal regeneration and hence the effect of the prepared extract was assessed on PDL fibroblasts. [40]

The two important cell functions that were assessed in this study were periodontal ligament fibroblast cell proliferation and migration, which are important for periodontal regeneration.

Cell proliferation is defined as increase in the number of cells as a result of cell growth and cell division. [41]

Traditionally, cell growth is assessed by staining the cells with a vital dye and then counting the number of viable cells. A number of methods have been used like, Trypan blue staining which is a simple process of determining cell membrane integrity (and thus assume cell proliferation or death). However, this method is not sensitive and nor can be adapted for high throughput screening. Other methods used measure the uptake of radioactive substances, usually tritium-labeled thymidine. Although this technique is more precise it also is time consuming and involves handling of radioactive substances. [28]

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A wavelength of 500-600 nm is used to measure the absorbance under spectrophotometer. Active mitochondrial reductase enzymes are responsible for the reduction of MTT and thus measures number of viable (living) cells. With an increase in the number of cells there is increase in the amount of purple formazan formed which results in increased measure of absorbance. The physiological state of cells and the inconsistency in mitochondrial dehydrogenase activity in different cell types can influence the outcomes of MTT assay. [11] Hence MTT assay was used in the present study to evaluate cell proliferation since it follows simple protocol which saves time, is nonradioactive, less costly.

In the present study, at 24 hours, the mean optical density was found to be 0.44150 ± 0.023864 (Group 1), 0.50683 ± 0.032664 (Group 2), 0.55133 ± 0.043675 (Group 3), 0.55500 ± 0.070359 (Group 4) and 0.57733 ± 0.078724 (Group 5).

A statistically significant difference was found between the control group and the different concentrations taken for the study. A statistically significant difference

was found in the rate of proliferation between the group 1(control) and group 3 ($p = 0.014$), group 4 ($p = 0.010$) and group 5 ($p = 0.002$). However, there were no statistically significant differences between group 1 and group 2 ($p = 0.257$).

Cell migration is defined as the orchestrated movement of cells in particular directions to specific locations. It is a central process in formation of tissues during development of the embryo and wound healing. [41]

The various methods used to determine migration include ECIS (electropolation and wound healing) which does develop a chemical gradient but is expensive and requires commercial instruments and plates. Another method used is Boyden chamber technique; it creates a chemical gradient as the chamber is separated by filters of required size. The chambers are easy to set up. However, it requires procurement of Boyden chamber and filter membranes. [29]

In the current study periodontal ligament fibroblasts cell migration was assessed by scratch test. The in vitro scratch assay is a direct and inexpensive method to study cell migration in vitro. The main advantage of this method is that it replicates to some extent migration of cells in vivo. Also, this test is appropriate to study the regulation of cell migration by cell interaction with extracellular matrix (ECM) and cell-cell interactions. It is well suited with microscopy including live cell imaging. It is perhaps the simplest means to study cell migration in vitro and uses no more than the regular and inexpensive materials and instruments commonly available in most cell culturing laboratories.

The drawbacks of in vitro scratch test are that no chemical gradient is established, requires longer time and fairly large number of cells are required.

Nevertheless in vitro scratch test still remains the method of choice to examine cell migration because it is easy to set up, no special equipments are required and most of the materials needed are present in any laboratory that performs cell culture. [29]

At 24 hours, the mean wound closure was found to be 46766.67 ± 1833.939 (Group 1), 24766.67 ± 21946.830 (Group 2), 53633.33 ± 1517.674 (Group 3), 43566.67 ± 2809.508 (Group 4) and 46766.67 ± 1833.939 (Group 5).

A statistically significant difference was seen in the mean wound closure in group 2 and group 5, whereas no statistically significant difference between group1, group 3 and group 4, was seen at different hours.

Within the limitations of the study, it can be concluded that

a) The proliferation rate of fibroblasts was found to be higher in the group 5 (2mg concentration of aqueous extract of T. cordifolia) than compared to group 1 (control group) and the difference was found to be statistically significant.
b) The proliferation rate was found to be better in all concentrations of the prepared extract when compared to the control group.
c) The migration rate of fibroblasts in the test groups was found to be better than the control group, but there was no statistically significant difference seen between the groups. A statistically significant difference was found in the migration rate of Group2 and Group 5 ($p = 0.007$)
d) Hence, it can be inferred that aqueous extract of T. cordifolia can be used as an effective agent to promote the periodontal regeneration. A 2.0mg concentration of the extract can be considered as the most effective concentration which can be used for the periodontal regeneration, in vivo.

SUMMARY & CONCLUSION

The purpose of the current investigation was to evaluate the effect of aqueous stem extract of *Tinospora cordifolia* on the proliferation and migration of periodontal ligament fibroblasts. The study was done in the Department of periodontics, KLE VK Institute of Dental Sciences, Belagavi. The laboratory procedures were performed in Dr. Prabhakar Kore Basic Science Research Institute (BSRC), Belagavi.

Periodontal ligament fibroblasts were cultured from the teeth extracted for orthodontic purpose and were treated with four concentrations of the prepared aqueous extract of *T.cordifolia* and one control group, without the extract, to determine the proliferation and migration. The rate of proliferation and the rate of migration was determined using MTT assay and in vitro scratch test respectively.

The results of the study showed better proliferation with the prepared extract at different concentrations than the control group and the proliferation rate was found to be higher with the 2.0mg concentration. The migration rate was found to be higher in the different concentrations of prepared extract, difference of which was not statistically significant. However, migration rate was found to be better with 2.0mg concentration of the extract.

Hence, within the limitations of the study it can be concluded that the aqueous stem extract of *T.cordifolia* is equally or slightly better than the control group in the proliferation and migration of PDL fibroblasts, thereby can be used as an effective agent in the periodontal regeneration.

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ANNEXURE I :- ETHICAL CLEARANCE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362 Web: http://www.kledental-bgm.edu.in
FAX: 0831-2470640 E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1311

CERTIFICATE

This is to Certify that the synopsis titled

TO ASSESS THE EFFECT OF AQUEOUS STEM EXTRACT OF TINOSPORA CORDIFOLIA ON PROLIFERATION AND MIGRATION OF PERIODONTAL FIBROBLASTS - IN VITRO STUDY *Submitted by*

Dr. REG. NO. IK0219002. *P. G. Student /*

Staff, Guided by [redacted] *from Department of*

PERIODONTICS *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE II :- PLAGIARISM CERTIFICATE

Scientific Correspondence and Review Committee

KLE VK Institute of Dental Sciences
A Constituent Unit of KLE Academy of Higher Education and Research
(Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi - 590 010, Karnataka State
Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)
☎: 0831-2470362 Web: http://www.kledental-bgm.edu.in
FAX: 0831-2470640 E-mail:principal@kledental-bgm.edu.in

Date : 21-12-2021 Serial No. : 074

PLAGIARISM CHECK REPORT

Name of the Applicant : **REG. NO. IK0219002.**
UG / PG / Ph.D / Staff : POST GRADUATE
Batch & Year : 2019-22
Department : PERIODONTICS

The soft copy of Research Work / Manuscript by **REG. NO. IK0219002.** entitled "TO ASSESS THE EFFECT OF AQUEOUS STEM EXTRACT OF TINOSPORA CORDIFOLIA ON THE PROLIFERATION AND MIGRATION OF PERIODONTAL LIGAMENT FIBROBLASTS - AN INVITRO STUDY"

under the guidance of .....has been submitted for Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

The scan has been carried out and the scanned output reveals a Similarity Index of .....6.....%, which is **within** / **not within** the acceptable limits of 10% as per the UGC guidelines.

Member Secretary
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER-Belagavi

Chairman
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER - Belagavi

ANNEXURE III :- BIOSTATISTICIAN CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of **REG. NO. IK0219002.** **Graduate Student**, under the guidance of **Professor, Department of Periodontics**, entitled **"TO ASSESS THE EFFECT OF AQUEOUS STEM EXTRACT OF *TINOSPORA CORDIFOLIA* ON THE PROLIFERATION AND MIGRATION OF PERIODONTAL LIGAMENT FIBROBLASTS - AN IN VITRO STUDY"** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date:

Name & Signature of Biostatistician

DEPARTMENT OF EPIDEMIOLOGY & BIOSTATISTICS, KAHER'S BELAGAVI

ANNEXURE IV :- AUTHENTICATION FORM

SHRI B.M.K. AYURVEDA MAHAVIDYALAYA

A constituent unit KLE Academy of Higher Education & Research
Deemed–to-be-University

Central Research Facility

DRUG AUTHENTICATION REPORT

Submitted By: KLE Ayurveda Pharmacy

Submitted Date: 24/08/2020 **Date of Issue: 28/08/2020**

SN	Sample Name	Scientific Name	Family	Part submitted	CRF Code	Authenticated as			
						Ayurvedic Name	Scientific Name	Family	Part Authenticated
1	Amruta	*Tinospora cordifolia* Willd.	Menispermaceae	Stem	CRF/Auth /Aug/2020 /25	Amruta	*Tinospora cordifolia* Willd.	Menispermaceae	Stem

Signature:
Authentication Expert Name: J
Date: 28/08/2020

Signature of Coordinator
ASU Drug Testing Laboratory

ANNEXURE V :- WAIVER FORM

Department of Periodontics

KAHER V.K Institute of Dental Sciences, Nehru Nagar, Belagavi.

"TO ASSESS THE EFFECT OF AQUEOUS EXTRACT OF *TINOSPORA CORDIFOLIA* STEM ON THE PROLIFERATION AND MIGRATION OF PERIODONTAL LIGAMENT FIBROBLASTS - AN IN-VITRO STUDY"

Waiver form

It is not feasible to obtain individual informed consent of donors of specimens used in this study. However, I assure that confidentiality of the participant information will be ensured and no identifying information related to the study participants will be disclosed in any report/publication arising from the study.

ANNEXURE VI - MASTER CHART

(A) PROLIFERATION

0.5mg	1.0mg	1.5mg	2.0mg	Control
0.542	0.537	0.579	0.658	0.48
0.547	0.584	0.513	0.514	0.454
0.496	0.537	0.494	0.462	0.444
0.46	0.519	0.684	0.659	0.432
0.495	0.508	0.51	0.572	0.428
0.501	0.623	0.55	0.599	0.411

(B) MIGRATION

	C1 (0.5mg)	C2 (1.0mg)	C3 (1.5mg)	C4 (2.0mg)	Control
0 hour	74800	68600	70000	99000	65500
	77600	70000	72000	97600	71200
	76900	73000	74000	93000	75800
2 hour	55100	67000	67500	90000	57800
	53600	68100	66900	93100	60900
	52000	72800	69800	84600	61600
4 hour	40000	57200	53400	67900	45500
	43000	55700	48300	69700	49200
	33500	54800	41800	67400	48300
6 hour	40000	55000	43300	64100	48800
	41800	52000	46500	62300	44700
	32500	53900	40900	62700	47400
0-2 hours	24900	800	4300	8400	14200
2-4 hours	18500	18000	28000	17200	13300
4-6 hours	1000	900	900	4700	900
6-24 hours	32500	53900	40900	62700	47400