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# Evaluation of effect of eggshell extract coated and cissus quadrangularis coated titanium discs on osteoblast cells: An invitro study.

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**Conflicts of Interest:** Nil

## Abstract

**AIM**: The aim of the study was to evaluate the osteoblastic activity on titanium discs which are coated with egg shell extract and cissus quadrangularis extract.

**Materials and methods** : In this study 30 Grade 4 commercially pure Titanium discs of diameter 5mm and thickness 2 mm were used. Suitable solvents were used to dissolve egg shell powder and stems of Cissus quadrangularis. The extracts were tested for cytotoxicity using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on UMR106 cells and coated on titanium discs.

**Results:** The MTT Assay showed that Titanium discs which were coated with Egg shell Extract at 80µg/ml has shown cell proliferation up to 26.9% when compared to Cissus quadrangularis Extract coated discs, which showed only 19.82% cell proliferation and uncoated discs which showed 0%proliferation.

**Conclusion:** Within the limitations of the study it was concluded that Titanium discs coated with Egg shell extract showed cell proliferation when compared to the Titanium discs coated with cissus quadrangularis extract. These hydrophilic modifications indicated good cell attachment and compatibility in osteoblast.

**Keywords**: UMR 106 cell line, Titanium discs, Cell proliferation, Egg shell extract, Cissus quadrangularis extract, MTT Assay.

## Introduction

Dental implants have become an essential component of dentistry and have allowed both the functional and aesthetic rehabilitation of edentulous arches.[1] Titanium and titanium alloy are the most commonly used material for fabrication of implant. Titanium is an inert material.

It was accidentally discovered as an ideal implant material by Dr Per-Ingvar Branemark. Titanium alloys have minimal toxicity, high corrosion resistance, high mechanical resistance, and excellent biocompatibility.[2] The ability of titanium to ooseointegrate is its most remarkable quality.

The biocompatibility of titanium implants is attributed to the stable oxide layer that spontaneously forms when titanium is exposed to oxygen. This reaction prevents the formation of fibrous tissue around the implant and creates direct contact to osseous tissue. By generating a coating onto a titanium surface that mimics the organic and inorganic components of living bone tissue, a physiological transition between the non-physiological titanium surface and surrounding bone tissue can be established. The localised organic and inorganic osteogenic coatings were applied to the surfaces of implants to increase their surface activity and osteopromotive activity.[3] Studies have been conducted to better the implant surface by making it hydrophilic, to further enhance osseointegration.[4]

Here, we are choosing ayurvedic materials like extracts of Egg shell and cissus quadrangularis as a possible medium to enhance osseoinduction and osseoconduction in human jawbone following placement of dental implants

Ayurveda has always been an enigma to the western allopathic medicines. The word Ayurveda is derived from ‘ayu’ meaning ‘life’ and ‘vedha’ meaning ‘knowledge’. Ayurveda literally means the science that imparts all the knowledge of life.[5] Several Ayurvedic medicines have been exploiting for treatment and management of various diseases in human beings. Herbal remedies have been successfully used as antiseptics, antioxidants, and analgesics in dentistry. Several naturally occuring materials have the potency and ability to enhance osteoblastic activity in humans.[6]

Rasashastra is one of the part of Ayurveda that deals with metals/nonmetals/herbomineral preparations called as Bhasma. Bhasma, literally means ‘ash’ are inorganic preparations produced by alchemic process, which converts a metal or mineral into its compounds such as carbonates and oxides.[7] In Kukkutanda Tvak Bhasma (KTB) the calcium is in the form of calcium carbonate which has the highest percentage of elemental calcium among the calcium salts used to treat leukorrhea, urinary tract infections.[8]

Cissus Quadrangularis is known as Asthsrinkhala in Sanskrit, means which saves the bone from their destruction. It has been extensively used as bone setter for both external application (fracture management) and Internal medication (to be taken with milk in case osteoporosis). The anabolic steroidal principles of Cissus showed a noticeable effect in rate of fracture healing by influencing early regeneration of all connective tissues involved in healing and Quicker mineralisation of callus. Thus it helps in building up the bone density.[9]

The cellular metabolism is measured by a colorimetric assay called MTT Assay. The biochemical mechanism behind the MTT assay involves NADPH -dependent cellular oxidoreductase enzyme that converts the Yellow tetrazolium MTT into Insoluble formazan. Formazan can be dissolved with dimethyl Sulfoxide to give purple colour with characteristic absorption of 540 nm. Intensity of purple colour is directly proportional to the Cell number and thus indicating cell viability.[10]

The aim of this study was to evaluate the osseointegrative activity of eggshell and Cissus quadrangularis extracts when they are coated on Titanium discs.

**Materials and methods:** The following experiment was done in Skanda life sciences Pvt. Ltd, Bangalore associated with Rajarajeswari Dental College. The procedures were sequentially followed as elaborated below.

**Cell lines and culture medium**: Cytotoxicity of the extracts were evaluated using UMR 106 cells, as these cells were suitable for study related to osteoblast physiology and bone formation. The cell lines were procured from ATCC[American Type Culture Collection] and stock cells were cultured. The cells were dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells were checked and centrifuged. Further, desired number of cells was seeded in culture plates and incubated for 24 hrs at 37oC, 5 % CO2 incubator.

**Extraction of Egg shell and Cissus quadrangularis extract using suitable solvents:** Egg shell powder[figure1] and Cissus quadrangularis stems[figure2] were obtained from Sri Venkateswara Ayurveda College, Tirupati, Andhra Pradesh, India.

20g of dried Sample powder was dissolved in 100ml of D.H2O for Egg shell and Methanol for Cissus quadrangularis in 500ml beaker with aluminium foil covered on it [figure 3]. Then the beaker was kept on hot water bath at 50ºC for 4hours. After incubation period the extract was filtered with Whatmann filter paper and the filtrate was collected in 50ml beaker[figure 4]. Residue present over the filter paper was discarded and filtrate was taken for further use. Then the filtrate was kept at 50ºC for few hours until the extract got completely dried[figure5] and turned into semisolid form.[figure 6] . This semi solid sample was weighed and the yield was noted.[Table1]

Fig 1: Egg shell powder

Fig 2: Cissus quadrangularis stems

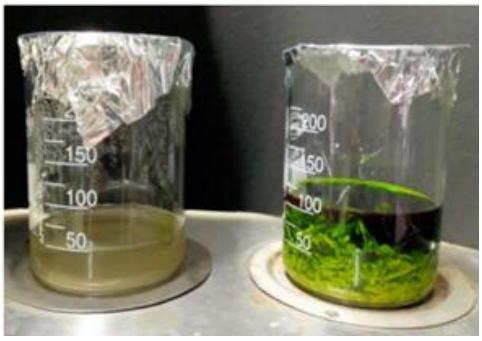



Fig 3: Extraction by solvents

Fig 4: Sample filtration

Fig 5: Extract after drying

Fig 6: Total yield of extracts

Table 1: Yield summary after Sample extraction

| Sample | Solvent | Sample taken for extraction | Yield after extraction |
|---|---|---|---|
| Egg shell Extract | D.H2O | 20g | 1254mg |
| Cissus quadrangularis Extract | MeOH | 20g | 695mg |

**Cytotoxicity test of extracts against UMR106 cells by MTT assay:** The monolayer cell culture was trypsinized and the cell count was adjusted to 1 x $10^5$ cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000 cells/well) was added. After 24hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at $37^0$C for 48hrs in 5% CO2 atmosphere. After incubation the test solutions in the wells were discarded and 100µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4h at $37^0$C in 5% CO2 atmosphere. The supernatant was removed and 100µl of DMSO [**Dimethyl sulfoxide]** was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590nm.The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% ($IC_{50}$) values is generated from the dose-response curves for each cell line [Table 2].

Table 2:Cytotoxicity of control and Egg shell extract against UMR106

| Sample name | Conc. µg/ml | OD at 590nm | % Inhibition |
|---|---|---|---|
| control | 0 | 0.696 | 0.00 |
| Egg shell extract | 10 | 0.668 | 4.02 |
| | 20 | 0.652 | 6.32 |
| | 40 | 0.615 | 11.64 |
| | 80 | 0.572 | 17.82 |
| | 160 | 0.554 | 20.40 |
| | 320 | 0.544 | 21.84 |

Table 3: Cytotoxicity of control and Cissus quadrangularis extract against UMR106 cells

| Sample name | Conc. µg/ml | OD at 590nm | % Inhibition |
|---|---|---|---|
| control | 0 | 0.696 | 0.00 |
| Cissus quadrangularis | 10 | 0.637 | 8.48 |
| | 20 | 0.623 | 10.49 |
| | 40 | 0.605 | 13.07 |
| | 80 | 0.581 | 16.52 |
| | 160 | 0.548 | 21.26 |
| | 320 | 0.498 | 28.45 |

**Calculating Inhibition**

% Inhibition = ((OD Of control – OD of sample)/OD of Control) x 100

Fig 7: Egg shell Extract at 10µg/ml

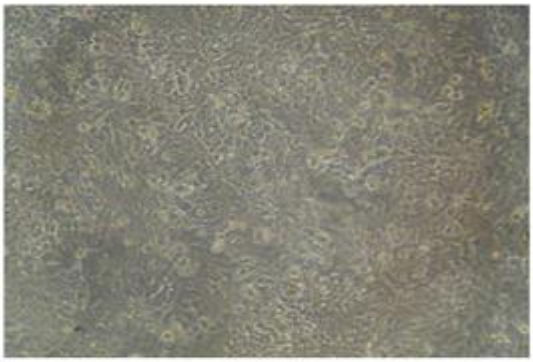


Fig 8: Egg shell Extract at 320µg/ml

Fig 9: Cissus quadrangularis Extract at10µg/ml

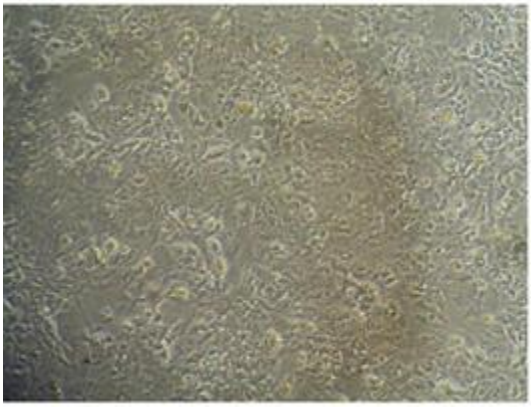


Fig 10: Cissus quadrangularis Extract_320µg/ml

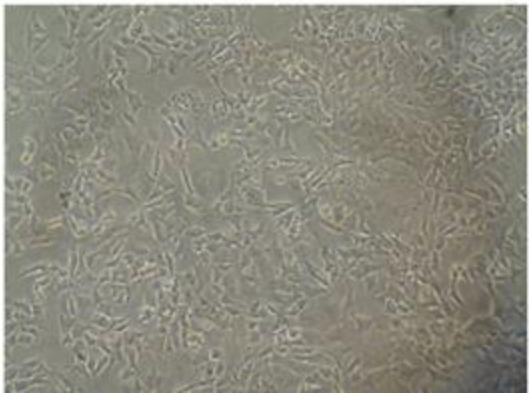
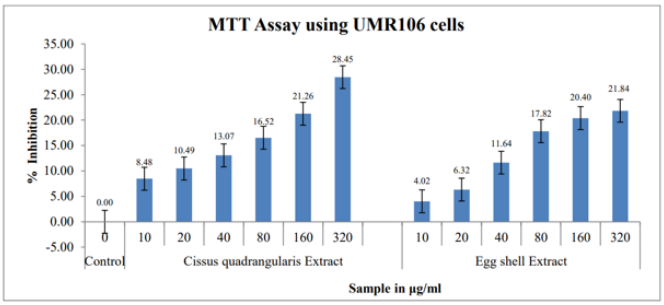


Fig 11: Control Untreated cells




Graph 1:Cytotoxicity of sample extract against UMR106 Cells.

- Sample Egg shell Extract and Cissus quadrangularis Extract did not show significant cytotoxicity up to 80µg/ml with % inhibition less than 20% in UMR106 cells.[graph 1]
- Hence, for coating the titanium discs, 80µg/ml can be used for biocompatibility testing for both the samples.

Grade 4(ASTM F67) commercially pure titanium discs of diameter 5 mm and thickness 2 mm were used in the study.

- Samples: 1. Group 1: Uncoated discs
  2. Group 2: Egg shell extract coated discs
  3.Group 3: Cissus quadrangularis extract coated discs

Fig12: Titanium discs 5mm in diameter and 2mm in width used in the study

**C. Coating of titanium discs using Extracts with established non-toxic concentration**:Titanium discs are cleaned with 70% alcohol and autoclaved before the procedure. Coating of titanium discs was performed by incubating Samples with the prepared solution overnight at 2-8°C and later air-drying. Dried coated discs are sterilized by exposure to UV light in a sterile biosafety cabinet

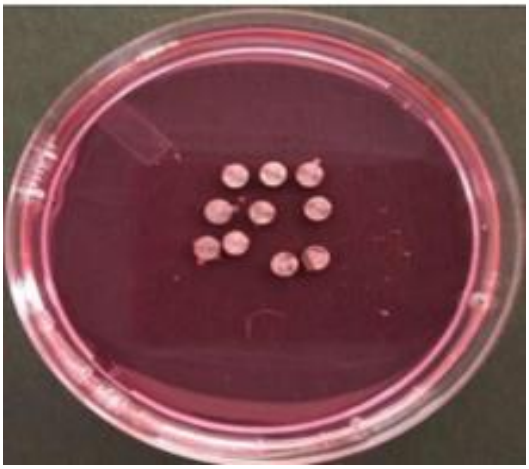


Fig 13: Coating discs with respective extracts

**d. Biocompatibility of coated titanium discs against UMR106 cells:**

The monolayer cell culture was trypsinized and the cell count was adjusted to 1 x $10^5$ cells/ml using respective media containing 10% FBS. Titanium discs were placed in the 12 well plates and presoaked with DMEM complete media[figure14]. To each well of the 12 well plate, 1000µl of the diluted cell suspension (1,00,000cells/well) was added on the coated and uncoated titanium sample discs and incubated for 48hrs at $37^0$C in 5% CO2 atmosphere undisturbed. After 48hrs incubation the test solutions in the wells were discarded and 500µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well [figure 15]. The plates were incubated for 4hrs at $37^0$C in 5% CO2 atmosphere. The supernatant was removed and 500µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan [figure 16]. Later 100µl of the coloured solution was transferred to a 96 well plate. The absorbance was measured using a microplate reader at a wavelength of 590nm. The percentage growth inhibition was calculated using the formula and concentration of test drug needed to inhibit cell growth by 50% ($IC_{50}$) values is generated from the dose-response curves for each cell line.

**Calculating Inhibition**

% Proliferation = [(OD of sample/OD of Control) x 100]-100

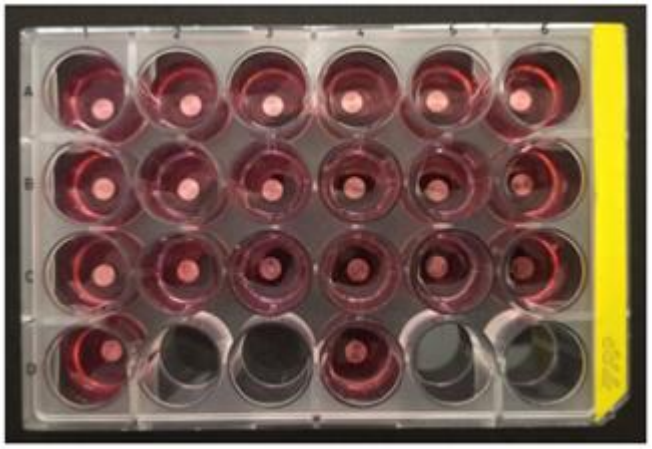


Fig 14: Biocompatibility of coated titanium discs

Fig 15: Addition of MTT reagent after treatment

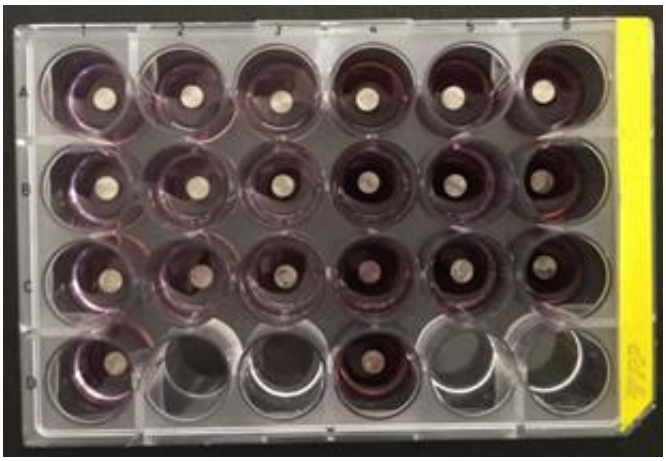


Fig 16: Formazan crystals dissolved in DMSO read at 590nm

**Results**

MTT Assay: The viability of cells were tested with MTT Assay and it was found that 80µg/ml can be used for biocompatibility testing for both the samples. MTT Assay biocompatibility analysis of 10 uncoated discs with UMR106 cells was performed and the mean value obtained was 0.640 and standard deviation obtained was 0.058.[Table 4]. Biocompatibility evaluation of Egg shell extract coated discs with UMR106 cells by MTT Assay was performed and mean value obtained was 0.813 and standard deviation obtained was 0.062[Table 5]. Ten discs coated with Cissus quadrangularis extract were subjected to an MTT Assay biocompatibility analysis against UMR106 cells; the mean value obtained was 0.767, and the standard deviation obtained was 0.070.[Table 6].

The results suggested that titanium discs which were coated with Egg shell Extract at 80µg/ml has shown cell proliferation up to 26.9% when compared to Cissus quadrangularis Extract coated discs, which showed only 19.82% cell proliferation [Table 7] [Graph 2]. These modifications indicated good cell attachment and compatibility in osteoblasts.

Table 4: Biocompatibility evaluation of uncoated discs with UMR106 cells byMTT Assay.

| Group Details | Trials | OD at 590 nm |
|---|---|---|
| Group 1 Uncoated Discs | n=1 | 0.658 |
| | n=2 | 0.662 |
| | n=3 | 0.698 |
| | n=4 | 0.636 |
| | n=5 | 0.501 |
| | n=6 | 0.689 |
| | n=7 | 0.591 |
| | n=8 | 0.625 |
| | n=9 | 0.678 |
| | n=10 | 0.665 |
| | Mean | 0.640 |
| | SD | 0.058 |

Table 5: Biocompatibility evaluation of Egg shell extract coated discs withUMR106 cells by MTT Assay.

| Group Details | Trials | OD at 590 nm |
|---|---|---|
| Group 2 Egg shell extract coated Discs | n=1 | 0.864 |
| | n=2 | 0.762 |
| | n=3 | 0.817 |
| | n=4 | 0.695 |
| | n=5 | 0.834 |
| | n=6 | 0.912 |
| | n=7 | 0.758 |
| | n=8 | 0.834 |
| | n=9 | 0.877 |
| | n=10 | 0.813 |
| | Mean | 0.813 |
| | SD | 0.062 |

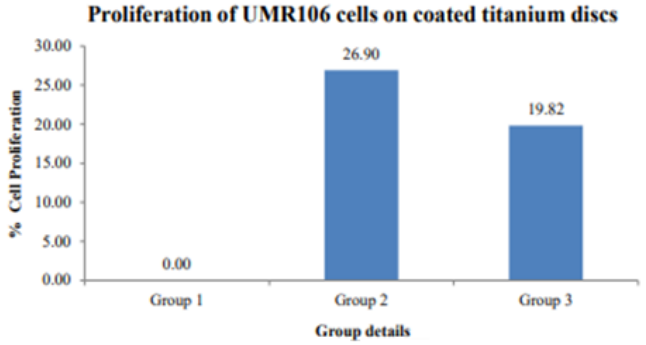
Table 6: Biocompatibility evaluation of Cissus quadrangularis extract coated discs with UMR106 cells by MTT Assay

| Group Details | Trials | OD at 590 nm |
|---|---|---|
| Group 3: Cissus quadrangularis extract coated Discs | n=1 | 0.698 |
| | n=2 | 0.852 |
| | n=3 | 0.736 |
| | n=4 | 0.715 |
| | n=5 | 0.892 |
| | n=6 | 0.702 |
| | n=7 | 0.699 |
| | n=8 | 0.824 |
| | n=9 | 0.765 |
| | n=10 | 0.789 |
| | Mean | 0.767 |
| | SD | 0.070 |

Table 7 :UMR 106 Cell proliferation on titanium discs coated with samples.

| Group details | OD at 590 nm | % cell proliferation |
|---|---|---|
| Group 1 | 0.640 | 0.00 |
| Group 2 | 0.813 | 26.90 |
| Group 3 | 0.767 | 19.82 |

Graph 2:UMR 106 cell proliferation on Titanium discs coated with samples




## Discussion

Osseointegration is a time-dependent healing process that results in the stiff attachment of alloplastic substances to bone while they are functionally loaded and are clinically asymptomatic.[11] Bone healing around implants involves a cascade of cellular and extracellular biological events that take place at the bone-implant interface until the implant surface appears finally covered with a newly formed bone. This cascade of biological events is regulated by growth and differentiation factors released by the activated blood cells at the bone-implant interface.[12]

Bone in contact with the implant surface undergoes morphological remodeling as adaptation to stress and mechanical loading. The turnover of peri-implant mature bone in osseointegrated implants is confirmed by the presence of medullary or marrow spaces containing osteoclasts, osteoblasts, mesenchymal cells and lymphatic/blood vessels next to the implant surface. During the remodeling of the peri-implant bone, new osteons circle around the implant with their long axes parallel to the implant surface and perpendicular to the long axis of the implants. Osteoid tissue is produced by osteoblasts suggesting that osteogenesis is underway. The remodelled bone can extend up to 1 mm from the implant surface.[13]

To enhance the patient compliance toward implant treatment and to reduce the osseointegration period various modifications are done to the implant. Topography, coatings, and wettability are surface characteristics of implants that interact with the host osteoblasts and aid in bone production during osseointegration. In the early stages of osseointegration as well as in long-term bone remodelling, surface topography is essential for osteoblast adherence and differentiation to implant surface.[14]

Hicklin et al, in his clinical trial placed hydrophilic implants and loaded early. Based on his findings, he concluded that the hydrophilic implants can be used for early functional loading. Wettability or hydrophilicity of the implant surface has shown that the osseointegration is faster and stability of the implants is maintained.[15]

Pachimalla et al in their preliminary study on rabbits considered Grade 4 Commercially pure Titanium discs of diameter 5mm and thickness 2mm for the evaluation of hydrophilic gel made from Acemannan and moringa oleifera in enhancing osseointegration of dental implants. The same dimensions were used in this study for evaluating the effect of extracts.[1]

Literature indicates that dentistry was included in Ayurveda's Shalakya Tantra.[16] The bhasma known as Kukkutanda Twak Bhasma, made from the kukkutanda twak (hen egg shell), has been utilised for various therapeutic purposes. Kukkutanda twak (egg shell) mentioned under Sudha varga group.[17]

The organic form of calcium found in Kukkutanda Twak is superior to the inorganic form because it is more bioavailable. Since it is a good source of calcium and is therefore utilised in conditions like arthritis and osteoporosis, it is helpful for enhancing bone density. With the frequent usage of eggshell calcium, no serious negative effects have been documented (KTB). It is therefore safe to assume that regular, long-term use is involved.[8]

Cissus quadrangularis is an herb found in the dry and hotter parts of the Indian subcontinent and parts of Africa. It is also known as the Veld grape. The shrub Cissus quadrangularis has a thin, fleshy, fibrous, smooth stem and four winged internodes. The stem and roots of this plant have antibacterial and antioxidant effects.[18]The taxonomy is as follows:

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Vitale

Family : Vitaceae

Genus : Cissus

Species : quadrangularis[19]

Cissus quadrangularis contains significant amounts of anabolic steroids, calcium, and phosphorus. The stem extracts of this plant have been used for centuries to treat a variety of ailments, including gout, back pain, irregular menstruation, and fractures. The rate of callus mineralization and fracture healing is significantly influenced by the steroidal constituents of Cissus quadrangularis.[18] Recently, Cissus quadrangularis has been widely used in mandibular fractures repair which reduces fracture healing time through down regulate the inflammation and its associated pain.[20] Calcium and minerals are abundant in C. quadrangularis, which may encourage osteoprogenitor cells to deposit bone over the titanium surfaces.[21]

One of the many ways to coat a substrate on the surface of an implant is by dip coating. Many scientists have experimented with dip coating titanium surfaces with various gentamicin gel antibiotics, titanium salts in

polymers, or silica PEG hydrogel hybrids in an effort to increase the titanium surface's bioactivity. The same dip coating technique is used in this study to boost the bioactivity of implants.[21]

Pachimalla et al performed MTT Assay in their study to test the viability of the cells similarly MTT Assay in the present study was done to evaluate the cell viability on the discs which are coated with eggshell extract and cissus quadrangularis extract.[1]

Our research shows that discs treated with eggshell and cissus quadrangularis extracts exhibited cell growth. However, when these two were compared, the discs coated with eggshell extract showed greater proliferation of cells.

**Conclusion**

Based on the limitations of the study we can conclude that titanium discs which were coated with Egg shell Extract at 80µg/ml has shown cell proliferation up to 26.9% when compared to Cissus quadrangularis Extract coated discs, which showed only 19.82% cell proliferation.

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