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In vitro Synthesis of Calcite Crystals from *Ormocarpum cochinchinense* (L.) a Traditional Bone Healing Aid of Southern India

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ABSTRACT

India has a diversity of medicinal plants. Most of them are used by the villagers against various diseases and are not revealed completely because of the secrecy maintained by them. *Ormocarpum cochinchinense* is found to be one among them which potentially shows good effect against bone fracture healing. The level of calcium in the plant is found to be one of the reasons for the bone healing effect. The scientific evidence of minerals present in the plant was important to prove the health effect of such medicinal plant. Since, the availability of *O. cochinchinense* is rare, an approach was made to study under *in vitro* condition. Callus induction was fast and reliable with Murashige and Skoog media containing 3 mg L^{-1} of kinetin and 1 mg L^{-1} of 2 4-D. The extract from leaves and callus was subjected to biomeneralization for calcite growth. The obtained calcite crystals were analysed with scanning electron microscope and the levels of calcium was quantified. Individual crystals were rod shaped and hexagonal in transactional view. When comparing the levels of minerals in the leaf and callus extract, it clearly indicated that the amount was high with leaf extract, but the variation was less. Results proved that the tissue culture of *O. cochinchinense* can be an alternative for biomeneralization of calcite crystals and also it can be used as a source for bone healing aid. The property of the plant in bone healing has to be ascertained by further experiments on animal models.

Key words: Biomeneralization, calcite crystals, elumbotti, *Ormocarpum cochinchinense*, trigonalrhombohedral, vaidyas

INTRODUCTION

Plants have been used as a source of medicines by humans from ancient time to the present day. Most of the medicines in practice were formulated based on plant derived metabolites (Samy *et al*., 2008). India is one of the countries which contain lot of traditional knowledge in terms of medicinal plants and its effect against various diseases (Bisht and Badoni, 2009). The traditional knowledge to scientific evidence was found to be lacking because of the information about the medicinal plants were kept secret by the village vaidyas (Vedavathy, 2001). Revealing such plants will help modern medicine to formulate effective medicines against various diseases.

Among the inorganic minerals present in biological systems, calcium hydroxyapatite, the major component in bones and teeth, is one of the most important calcium-based minerals due to its biocompatible and biodegradable properties. In India, *Cissus quadrangularis* was studied extensively to hasten the process of healing bone fractures and exploited to synthesize calcite crystals (Sanyal *et al*., 2005). The benefits of various minerals and vitamins on fracture healing had been demonstrated in animal models (Yilmaz *et al*., 2001).

Many endemic and rare plant species were used by villagers of Tamil Nadu for curing various diseases. So, adapting tissue culture will help raise the source of biochemicals of the medicinal plant without disturbing the population of such endemic medicinal plants (Amin *et al*., 2003). *O. cochinchinense* (L.) Merr. is one among them which has been traditionally used for healing bone fracture by Tamil Nadu Villagers. *O. cochinchinense* is a leguminous shrub which is extremely efficacious in mending bone fractures, but at present its use is known only to a handful of villagers for healing fractures, hence its Tamil name 'Elumbotti' (bone-knit) was narrowed to the plant. The availability of this plant is less, so, the present study was made to meet the lack of availability of this medicinal plant, *in vitro* development of the plant using plant tissue culture. Calcium crystals obtained from *in vitro* source was analyzed by scanning electron microscope (SEM) and they were compared with leaves of *O. cochinchinense*. The bioavilability of such minerals between plants and its *in vitro* derivative was compared to evidentially support the traditional medicinal plant scientifically.

MATERIALS AND METHODS

Collection of plant: Healthy and disease free *O. cochinchinense* (L.) Merr. Plant was collected from mountain areas of Kancheepuram District, Tamil Nadu. Young leaves were separated and used for the study. This study was conducted at SRM University, Kattankulathur, over a period of one year starting from June 2010 to April 2011.

Establishment of *in vitro* culture: Murashige and Skoog (MS) media supplemented with 3% sucrose and 0.8% agar along with growth hormones were used for the establishment of callus from the cultures (Murashige and Skoog, 1962). After trimming, the leaves were inoculated to culture bottles containing MS medium with 3 mg L^{-1} of NAA and 1 mg L^{-1} of Kinetin. The explants were kept under dark condition at 28°C for callus initiation. The obtained callus was served for the analysis of calcite crystal formation.

Calcium precipitation method: Twenty milliliter of aqueous extract and 1 g of ammonium carbonate were taken separately in two beakers, placed in a desicator and was closed. Then the set up was kept overnight (Chen *et al*., 2010). After overnight incubation the plant extract was taken and observed for calcite crystal formation. A comparative study was made among the plant and callus derived calcite crystals by using Scanning Electron Microscope (SEM).

Mineral analysis: The samples were washed and dried at 28°C and blended into powder. 500 mL of distilled water was added to 20 g of the powder to form slurry. The suspension of the slurry was vigorously shaken to effect dissolution and then suspension was filtered through a filter paper to obtain the extract (Ilondu, 2011) and used for element analysis under Indulgence coupled plasma optical emission spectroscopy for the elements present in the extract.

The SEM and Indulgence coupled plasma optical emission spectroscopy analysis was made at IIT, Madras.

RESULTS AND DISCUSSION

In vitro culture of *Ormocarpum cochinchinense* was done using MS basal medium. Callus was developed from leaf explants on MS media containing 3 mg L^{-1} of NAA and 1 mg L^{-1} of KIN in 45 days. Hence MS media containing 3 mg L^{-1} of kinetin and 1 mg L^{-1} of 2 4-D was found to be an ideal medium to grow *O. cochinchinense* (Lour.) Merr (Fig. 1a). According to Bennaceur and Karray (2002) 2,4-D and kinetin based growth regulator in the medium, produces callus in a shorter period of time. Same result was observed within 20 days time, the callus subculture was made and after 25 days the cultures were used for the extraction.

The plant and callus were extracted and biomeneralization was made to study the calcite growth. The calcite crystals obtained from the callus extract was presented in Fig. 1b. The Ca^{2+} ions in the extracts react with proteins leading to the formation of stable aggregates. The entrapped Ca^{2+} ions then react with CO_2 produced by the cells thereby forming $CaCO_3$ crystals. In biological systems, it is widely accepted that highly anionic, soluble biomacromolecules such as acidic proteins, glycoproteins and polysaccharides play an important role in biomineralization (Lakshminarayanan *et al.*, 2002). The structural analysis of calcite crystals were studied by using SEM and are presented in Fig. 2. Surface of the crystals obtained from callus and the magnified view were presented in Fig. 2a and b. The density of the crystals was also considered along with its size were presented in Fig. 2c and d, respectively. No variation was observed between plant and callus derived calcite crystals surface analysis. Comparative study was made among the callus and plant derived calcite crystals to bring forth the calcite formation *in vitro*. Size of the crystal was found to be 2 mm in diameter in wild plant extract and was circular in shape. Crystal from callus was less than 1 mm and irregular in morphology. Individual crystals were rod shaped and hexagonal in transectional view (Fig. 2c). Dense nano rod showed 50-60 μm in length. Difference in size and arrangement of the crystals might be because of changes in the composition of elements. In crystallization method calcium is crystallized as Magnesium calcite. According to the shape of crystals, Calcite crystals are trigonalrhombohedral whereas magnesium calcite is rod shaped. These crystals may be replaced instead of hydroxyapatite in bone fracture healing and other applications (Pekov *et al.*, 2003). The property of the plant in bone healing has to be ascertained by further experiments on animal models.

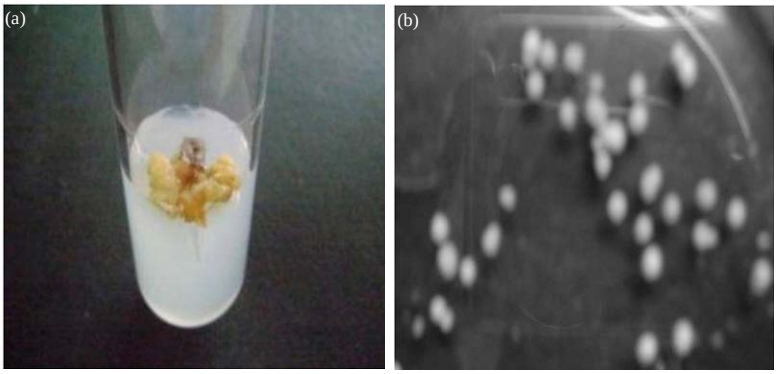

Fig. 1(a-b): (a) Callus obtained from *Ormocarpum cochinchinense* and (b) Calcite crystals obtained from the callus extract of *Ormocarpum cochinchinens*

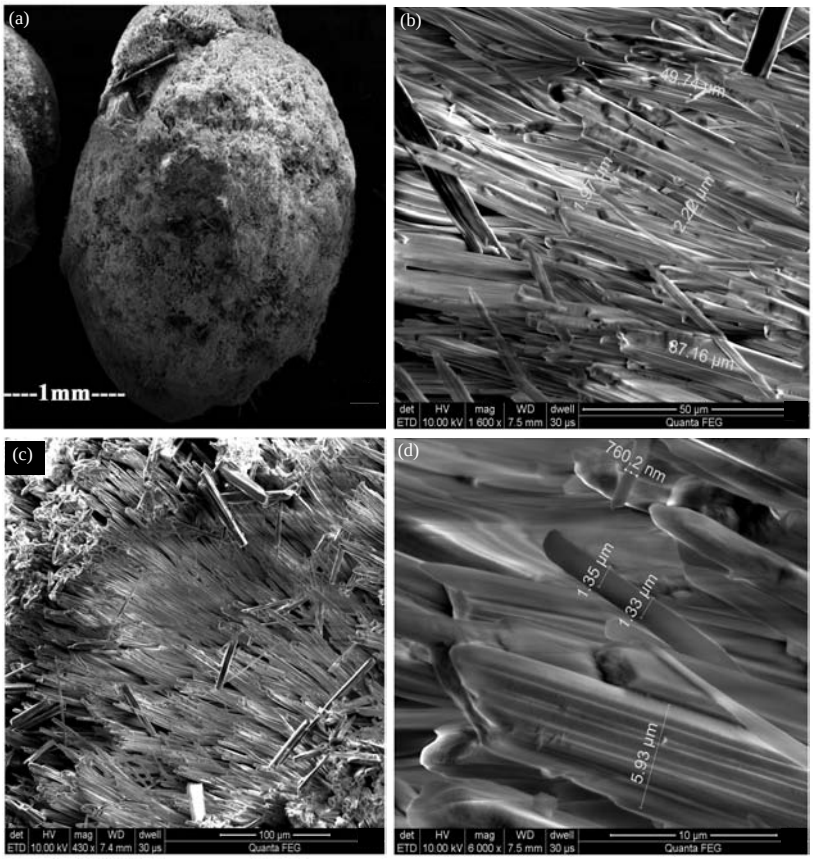

Fig. 2(a-d): Scanning electron microscope image of calcite crystals from *Ormocarpum cochinense in vitro*, (a) Syrface if tge crtstaks ivtaubed frin cakkys, (b) Magnified view of crystals at 50 µm, (c) Dense nano rod of 50-60 µm and (d) Magnified view of crystals at 10 µm

The level of minerals of plant and callus were examined and presented in Fig. 3. Calcium level of callus was less (79 mg L^{-1}) when compared to the Ca level in plant extract (103 mg L^{-1}). Same trend was observed with Mg (35, 29 mg L^{-1}) and phosphorus (11, 3 mg L^{-1}) levels of plant and callus but the levels of decrease were not varying high. The plant extract serves as a rich source of calcium ions which when reacted with magnesium sulphate and ammonium carbonate, led to the formation of calcite crystals of highly irregular morphology indicating that bioorganic molecules present in the extract modulate the crystal morphology. Calcium which is very abundant in the natural environment also required an element for plant growth and development. Calcium served as structural component of cell wall, a signal in various physiological and developmental pathways and an osmoticum. The cell wall and the vacuole provide major sinks for calcium in plants (Webb, 1999).

Calcium oxalate crystals (CaOx) are found in most organs and tissues of many plant species. *Conyza canadensis* (L.) Cronq. and *Conyza bonariensis* (L.) Cronq were studied by (Meric, 2008) for the Calcium oxalate crystals (Meric, 2008). Different plant extracts of *Ammodaucus luecotrichus*, *Erica multiflora* and *Stipa tenacissima* were studied to compare the calcium oxalate

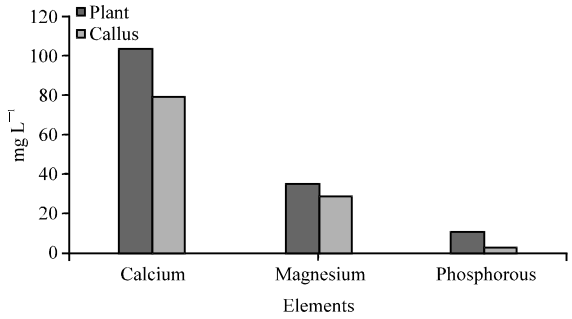

Fig. 3: Comparative study between wild plant and callus for the availability of elements

crystal growth and analyzed at different concentration and time. The crystal of calcium oxalate monohydrate is monitored by polarized light microscopy (Beghalia *et al*., 2008).

There are results stating the antiplasmodial effect of the genus but, the medicinal property of *O. cochinchinense* was not available (Dhooghe *et al*., 2010). Role of plants on bone healing such as *Cissus quadrangularis* by Sanyal *et al*. (2005), *R. curculiginis* and *R. drynariae* extracts by Wong *et al*. (2007) and the biomeneralizations were studied extensively. But the use of *O. cochinchinense* is not yet revealed for its calcite growth. Chen *et al*. (2010) used *Scindapsus aureum* petioles for the mineralization of calcium but here we used callus tissue for calcium mineralization.

CONCLUSION

Biological synthesis of calcite crystals using callus obtained from *O. cochinchinense* was revealed and presented. No variation in terms of SEM was observed between plant and callus derived calcite crystals. The minerals of the crystals were compared by Indulgence coupled plasma optical emission spectroscopy. Results revealed that the variation in terms of mineral content was present between callus and plant tissue. Even though the variation was noticed, it can be an alternative for biomenaralization of calcium.

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