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Comparative Antibacterial Study Of *In Vitro* And *In Vivo* Plant Extracts Of *Coscinium Fenestratum* (GAERTN.) COLEBR., A Critically Endangered Medicinal Plant

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Abstract

This study investigates the, antibacterial, activity of methanolic extracts of *in vitro*-raised callus and different parts (roots, stems, leaves) of *in vivo*-grown plant extract of *Coscinium fenestratum* against a range of bacterial strains. Mature leaves were used as the explants for the initiation of callus and cultured into Murashige and Skoog (MS), medium with varying concentrations of Thidiazuron (TDZ). The highest frequency of callus formation reached 95% with a mean fresh weight of 2854.76 ± 0.30 mg for explants cultured on MS basal medium supplemented with 0.75 mg/l TDZ. Among the four different crude extracts tested for antibacterial activity, the most potent activity of (22 mm), 5 mg/disc was exhibited by root extract and stem extract exhibited highest activity (20 mm) at 5 mg/disc against *Klebsiella pneumoniae*. Methanolic extract of *in vitro* leaf derived callus of *C.fenestartum*, showed the highest inhibition (10 mm) at 5 mg/disc against *Enterococcus faecalis and Vibrio parahaemolyticus.* Methanolic leaf extract did not show any significant activity. The present study successfully developed an efficient protocol for *in-vitro* callus induction in *C.fenestartum* mature leaf explants and confirmed the antibacterial potential in both *in vitro*-raised callus extracts and *in vivo*-grown plant extract extracts which could be a rich source of antimicrobials for therapeutic usage.

KEYWORDS: *Coscinium fenestratum,* leaf explants, callus, antibacterial activity

INTRODUCTION

Plant-based therapeutics are sources of novel bioactive compounds. Medicinal plants have been used for centuries in traditional healing systems, and today they are gaining recognition for their accessibility, affordability, and pharmacological potential (Bencheikh et al., 2023). Increase prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection fighting agents (Edeoga et al.,2005).

Coscinium fenestratum (Gaertn.) Colebr., (Menispermaceae) is a critically endangered, dioecious medicinal liana (Ravikumar and Ved 2000). In India, it is restricted to the few habitats of Western Ghats, mostly in the high rainfall receiving wet evergreen forests, moist evergreen, semi semi-deciduous forests at an altitude of 500 to 750 m (Mohanan and Sivadasan 2002). 1997 IUCN Red list of threatened plants recorded the status of *Coscinium* as highly endangered in India, vulnerable in Vietnam, rare in Singapore and indeterminate in Srilanka (Walter and Gillet 1998). The stem and roots of this species is used in a variety of indigenous medicinal preparations in the treatment of skin infections, snake bite, diarrhea, tetanus, anorexia, chronic dyspepsia, anaemia and psiolosis (Kritikar and Basu 1935; Warrier *et al*., 1994) and used in over 62 ayurvedic preparations (Nambiar *et al*., 2000).

Safety duplicates of the living collections are established using alternative strategies of conservation and it is in this area that biotechnology contributed significantly by providing *in vitro* conservation options through tissue culture techniques (Engelmann and Engels, 2002). Plant tissue culture are the most frequently used biotechnological tools for applied purposes ranging from rescue of endangered plant species, commercial plant micropropagation, generation of transgenic plants and for the production of secondary metabolites of industrial and pharmaceutical interest. (Loyola and Ochoa, 2018).

The active principle of this plant was identified as berberine, $C_{20}H_{18}NO_4Cl$ an isoquinoline alkaloid having numerous biological activities (Birdsall and Kelly 1997). The other major components in wood and root of *C.fenestratum* include palmatine, tetrahydropalmatine, crebanine and jatrorhizine (Pinho et al. 1992). *Coscinium fenestratum* has been reported to possess immense pharmacological actions such as antidiabetic, antiplasmodial, anti inflammatory, hepatoprotective activities (Anjali et al. 2012).

Combination of the rampant destruction of the forests along with over exploitation for the raw drug market and very slow rate of regeneration has seriously depleted its population in the wild, making conservational measures very urgent (Tushar and Udayan 2005). This paper aims to establish an efficient standard protocol for callogenesis from mature leaf explants using TDZ and to compare the antibacterial properties of *in vitro* and *in vivo* plant extracts of *C. fenestratum*.

Materials and Methods

Establishment of callus culture

The healthy mature plants were brought from the natural habitat in Thrissur, Kerala, India. The fully developed mature leaves were harvested and used as explants. The explants were washed with distilled water containing a few drops of detergent (Tween 20) for 5 min and rinsed 2–3 times with sterile distilled

water and then soaked in fungicide (Bavistin 1%) for 5 min followed by rinsing with sterile distilled water. Thereafter, the explants were surface disinfected with 70% ethanol for 30 s and rinsed 2–3 times with sterile distilled water, treated with 0.1% aqueous mercuric chloride for 3 min and thoroughly washed 4–5 times with sterile distilled water under aseptic condition.

Culture conditions:

The pH of the medium was adjusted to 5.8 before solidifying with 0.8% w/v Difco bacto agar. The chemicals used in this study were of analytical grade (Hi-media, Qualigens, SD fine chemicals, India). Molten medium (10 ml) was dispensed into 50 ml test tubes (Borosil) and plugged with non absorbent cotton plugs. The culture tubes containing the media were autoclaved at 121˚C for 15 min. All the cultures were maintained at 22 ± 2˚C under a 16 h photoperiod at a photosynthetic flux of 35-50 µmol/(m^{-2} s^{-1}), provided by cool daylight fluorescent lamps.

Callus induction:

Mature leaf explants were used for the callus induction. The explants were cut into small pieces and inoculated into MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and various concentrations of Thidiazuron (TDZ), a substituted phenylurea (N-phenyl-1,2,3-thidiazol-5-ylurea), at 0.0, 0.05, 0.1, 0.2, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l. The developed calluses were separated from the initial explants and healthy masses subsequently sub-cultured at regular intervals. The number of explants forming callus was scored to calculate callus formation frequency and their fresh and dry weight were recorded.

Preparation of plant and callus extracts

Healthy, disease free leaves, stems and roots of *Coscinium fenestratum,* were collected, washed thoroughly, shade dried in room temperature and grounded into powder. Similarly the leaf derived callus at the concentration (0.75 mg/l TDZ) grown on MS medium, was also shade dried and powdered. The powder was soaked in methanol. After 24 hrs the solution was filtered and the filterate was concentrated under reduced pressure using rotary vacuum evaporator. All the crude extracts were collected and stored at 4°C in air tight containers.

Antibacterial activity by disc diffusion method

The crude methanol extracts of *Coscinium fenestratum* are used to determine the antibacterial activity against a number of bacterial reference cultures, clinical isolates as well as drug resistance microbes cultures and clinical isolates by disc diffusion method (Murray et al.,1995). According to this method, 20 ml of sterile Mueller Hinton Agar (MHA) (Hi-media, Mumbai) was poured into sterile petri plates and solidified. The bacterial strains (100 µl of suspension containing 108 CFU/ml bacteria) were swabbed on top of the media and allowed to dry for 10 min. The tests were conducted at three different concentrations of the crude extract, i.e., 5, 2.5 and 1.25 mg/disc. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Streptomycin (10 µg/disc) was used as positive control. The plates were incubated for 24 h at 37°C. Zone of inhibition was recorded in millimeters and the experiment was repeated twice.

Results and discussion

The present study was carried out in *Coscinium fenestartum* plant and callus extracts. There is a substantial growing global demand for this plant because of its uniqueness. *In vitro* culture method offers new ways to conserve and multiply rapidly valuable, rare and endangered medicinal plants. An established protocol including plant regeneration would be helpful for preventing extinction. A review of available literature revealed no previous report on callus induction from mature leaf explants. In this study we successfully induced callus from mature leaf of *Coscinium fenestartum* using TDZ. These callus cells were used to test the antibacterial activity. This study would help us to find the presence of various phytochemical compounds in plant and callus extract, which will help us in the future, development of medicines in pharmaceutical industries.

***In-vitro* callus induction**

In vitro regeneration analyses were conducted to evaluate the potential for developing a faster and more reliable method for callus induction in *Coscinium fenestartum*. Murashige and Skoog (MS), was tested to determine their suitability for *in vitro* cultures of *Coscinium fenestratum*. Medium formulation displayed a strong effect on the fresh and dry weight of the callus. The highest percentage of callus formation from mature leaf explants reached 95% with a highest callus growth in terms of mean fresh weight (2854.76 ± 0.30 mg) (Table 1) was observed in MS medium fortified with 0.75 mg/l TDZ. This effect on MS medium seems to be in relation to the calcium and nitrogen concentrations which have eight and four fold higher calcium and nitrogen levels than the B5 and woody plant medium (Zouzou et al. 2000). Inorganic nitrogen has a determining action on callogenesis (Grimes and Hodges 1990) and this probably explains the maximum response of callus fresh weight on MS medium in *Coscinium fenestratum*.

Leaf explants grown on MS medium supplemented with various concentrations of TDZ formed calli from the cut ends within 2 weeks of culture (Fig. 1A), and at the end of fifth week, the entire surface of explants was covered with callus (Fig. 1B). The calli were slow growing, creamish yellow and compact in texture. During incubation on medium containing TDZ, expansion, swelling and thickening of leaf explants were observed. This could probably due to intense cell division as in the work reported on *Pelargonium capitatum* (Arshad et al. 2011). The first stage in the morphogenic process is the development of an undifferentiated cell mass commonly known as callus (Murthy et al. 1998) and the process of callus formation is called callogenesis, which is the primary step in the stimulation of shoot reproduction via indirect mode and adventitious organs regeneration. Thidiazuron is a potent cytokinin for promoting callus formation from woody explants, especially when used at ≥0.1 µM (Huetteman and Preece 1993). N-phenyl-N'-1,2,3-thidiazol-5-ylurea or thidiazuron is a substituted phenylurea which was first developed as a cotton defoliant and now is used as a potent growth regulator in diverse plant species for eliciting a wide spectrum of *in vitro* responses (Murthy et al. 1998) However, the utilization of TDZ for callus induction in *Coscinium fenestratum* had never been employed before.

There have been several reports of significant TDZ effects on callus formation in other species (karami et al. 2009; Jones et al. 2007). The growth of callus initiated on 0.75 mg/l TDZ could not be sustained during subsequent subcultures on the same medium, but remained prolific only at 0.2 mg/l TDZ. Whereas repeated subcultures on 0.5 to 1.5 mg/l TDZ concentrations triggered a shift to a static state and ceased repetitive growth within 2-3 wk. Callus induced in 0.2 mg/l TDZ were morphologically similar to those raised in 0.75 and 1.0 mg/l TDZ supplemented medium. Since TDZ at 0.2 mg/l was optimum for maintenance and proliferation of callus, it was subsequently used for regular maintenance (Fig. 1 D,E,F)

Progressive browning of the callus as well as culture medium was the major constraint encountered in establishing calli cultures of *Coscinium fenestartum*, which is probably due to oxidation and production of phenolic compounds. Browning of excised explants and the resultant discolouration of culture media is a major challenge in plant tissue culture systems (Huang et al. 2002). (Nair et al. 1992) proposed that in media with high PGR concentrations, the production and release of berberine into the culture media could result in browning. However, (Figueiredo et al. 2000), who observed a similar phenomenon in cell suspension cultures of *Rollinia mucosa* with 2,4-D, suggested that the PGR itself could be involved in the formation of phenols. In the present study, frequent subculture of the calli to the fresh medium for every five days was found to be effective in reducing the browning and maintenance of the callus. Similar results was reported by (prakash et al. 1999). This practice was used to control the blackening of the cultures in a considerable number of species such as *Euphorbia lathyris* (Ripley and Preece 1986) and *Pisonia alba* (Jagadish et al. 1999). The use of natural products for medicinal purposes is increasing, and at present, researchers aim to produce substances with anti-tumor, anti-viral, hypoglycemic, antiinflammatory, anti-parasite, antimicrobial tranquilizer and immunomodulating activities through tissue culture technology (Nalawade et al., 2003). Advances in the area of cell cultures for the production of medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids (Suman Kumar et al.,2010) and, (Rajashekar M. Bhande et al 2006),

Antibacterial activity of plant extract

The methanolic extracts of leaf, stem, root and leaf derived callus of *Coscinium fenestartum* were screened against various bacterial reference cultures and clinical isolates listed in (Table 2 & 3). All the bacterial cultures were obtained from IMTECH, Chandigarh, India and the clinical isolates were obtained from Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India.

Zones of inhibition of the crude extracts were noted. Among the four different crude extracts tested for antibacterial activity, the most potent activity of (22 mm), 5 mg/disc was exhibited by root extract and stem extract exhibited highest activity (20 mm) at 5 mg/disc against *Klebsiella pneumoniae.* In the case of *in vitro* leaf derived callus of *C.fenestartum*, methanolic extract showed the highest inhibition (10 mm) at 5 mg/disc against *Enterococcus faecalis and Vibrio parahaemolyticus.* Moderate activity was observed in methanolic leaf extract. The methanolic extract of stem and root showed the maximum activity against bacterial pathogen compared to *in vitro* and *in vivo* leaf extract. The antibacterial activity of *Coscinium fenestratum* is mainly due to the presence of berberine (Nair et al., 2005). Ethanolic extract of *Coscinium fenestratum* stem had displayed a strong inhibitory effects against *Propionibacterium acnes* and

Staphylococcus epidermidis. (Kumar et al., 2007). Previous chemical analysis of the acetone and methanol extracts, in *C.fenestratum* indicated the presence of alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, and sterols showing antimicrobial activity as reported by (*Mala et al., 2022)*. Previous study (Goveas, S.W. and Abraham, A. 2013). revealed that the methanol extract inhibited the growth of *E. coli* moderately at the concentration of 150 mg/ml with the zone inhibition of 17 mm. This finding confirms that the extracts may have the capability to block the growth of all tested microbes at a higher concentration of more than 1,000 ppm. In conclusion, we described here a standard and reproducible callus induction method for *C.fenestratum* that would be helpful for large scale isolation of components from this endangered species. Evidence of strong antimicrobial components in the stem, root and leaf derived callus extracts would be useful in developing antimicrobial substances in pharmaceuticals industries.

Conclusion

Coscinium fenestratum has been reported to possess immense pharmacological actions with antibiological activities. The above results confirms that the methanolic stem extracts of *Coscinium fenestratum* showed potent activity against a wide range of drug-resistant bacteria compared to root and callus extracts. The present study also successfully developed an efficient protocol for *in-vitro* callus induction in *C.fenestartum* mature leaf explants and confirmed the antibacterial potential in both *in vitro*- and *in vivo* plant extracts.

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Table 1: Effect of different concentrations of TDZ on callus induction using mature leaf explants of *Coscinium fenestratum* grown on MS medium after 60 days of incubation.

TDZ conc. (mg//l)	% of callus formation	Fresh weight of callus (mg) mean ± SE	Dry weight of callus (mg) mean ± SE	Callus colour
0.00	-	-	-	-
0.05	70	410.05 ± 0.45^{g}	17.29 ± 0.29^{g}	Creamy yellow
0.1	65	726.85 ± 0.20^{e}	35.00 ± 0.17^{e}	Creamy yellow
0.2	90	1510.59 ± 0.18^{c}	75.00 ± 0.11^{c}	Creamy yellow
0.5	80	2329.21 ± 0.32^{b}	116.23 ± 0.11^{b}	Yellow green
0.75	95	2854.76 ± 0.30^{a}	142.85 ± 0.15^{a}	Yellow green
1.0	60	1375.67 ± 0.30^{cd}	68.00 ± 0.20^{cd}	Creamy yellow
1.5	55	966.55 ± 0.27^{d}	46.85 ± 0.20^{d}	Light yellow
2.0	50	512.37 ± 0.21^{f}	24.77 ± 0.13^{f}	Yellow brown
2.5	45	369.74 ± 0.28^{h}	17.37 ± 0.11^{h}	Brownish
3.0	40	245.25 ± 0.55^{i}	11.33 ± 0.10^{i}	Brownish black

Table 2: Antibacterial activity of methanolic extract of leaf, stem, root and callus from *Coscinium fenestratum*

Name of the Microbe (Reference Cultures)	Zone of Inhibition (mm)												S10
	Methanolic extract												
	(LEAF) mg/disc			(STEM) mg/disc			(ROOT) mg/disc			(CALLUS) mg/disc			
	1.25	2.5	5	1.25	2.5	5	1.25	2.5	5	1.25	2.5	5	
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	9 ± 0.12^{d}	11 ± 0.24^{d}	7 ± 0.15^{e}	10 ± 0.18^{c}	12 ± 0.13^{d}	-	-	-	16 ± 0.06^{d}
Klebsiella pneumoniae ATCC 15380	-	-	-	15 ± 0.13^{a}	17 ± 0.28^{a}	22 ± 0.23^{a}	16 ± 0.22^{a}	18 ± 0.25^{a}	20 ± 0.12^{a}	7 ± 0.12^{a}	8 ± 0.18^{a}	9 ± 0.28^{b}	18 ± 0.04^{b}
Enterococcus faecalis ATCC 29212	-	-	-	9 ± 0.12^{b}	11 ± 0.26^{b}	15 ± 0.18^{b}	10 ± 0.25^{b}	12 ± 0.16^{b}	16 ± 0.18^{b}	7 ± 0.13^{a}	8 ± 0.21^{a}	10 ± 0.23^{a}	20 ± 0.03^{a}
Yersinia enterocolitica MTCC 840	-	-	-	8 ± 0.18^{c}	10 ± 0.22^{c}	12 ± 0.17^{c}	7 ± 0.09^{e}	9 ± 0.19^{d}	10 ± 0.25^{f}	7 ± 0.21^{a}	-	9 ± 0.12^{b}	18 ± 0.05^{b}
Erwinia sp. MTCC 2760	-	-	-	7 ± 0.21^{d}	9 ± 0.21^{d}	10 ± 0.25^{e}	7 ± 0.19^{e}	9 ± 0.12^{d}	10 ± 0.21^{f}	-	-	-	16 ± 0.04^{d}
Vibrio parahaemolyticus MTCC 451	-	-	-	7 ± 0.25^{d}	9 ± 0.13^{d}	11 ± 0.12^{d}	7 ± 0.23^{e}	8 ± 0.18^{e}	10 ± 0.12^{f}	-	8 ± 0.13^{a}	10 ± 0.18^{a}	-
Enterobacter aerogenes MTCC 111	-	-	-	7 ± 0.19^{d}	9 ± 0.12^{d}	11 ± 0.19^{d}	9 ± 0.18^{c}	10 ± 0.13^{c}	12 ± 0.18^{d}	-	7 ± 0.13^{b}	8 ± 0.13^{c}	17 ± 0.05^{c}
Bacillus subtilis MTCC 441	-	-	-	9 ± 0.13^{b}	10 ± 0.26^{c}	11 ± 0.13^{d}	8 ± 0.25^{d}	10 ± 0.16^{c}	12 ± 0.26^{d}	7 ± 0.21^{a}	8 ± 0.16^{a}	9 ± 0.13^{b}	16 ± 0.06^{d}
Escherichia coli ATCC 25922	-	-	-	7 ± 0.18^{d}	10 ± 0.21^{c}	12 ± 0.18^{c}	8 ± 0.16^{d}	10 ± 0.22^{c}	14 ± 0.28^{c}	-	7 ± 0.12^{b}	9 ± 0.16^{b}	15 ± 0.04^{e}
Proteus vulgaris MTCC 1771	-	-	-	7 ± 0.21^{d}	9 ± 0.22^{d}	11 ± 0.21^{d}	7 ± 0.22^{e}	9 ± 0.17^{d}	11 ± 0.13^{e}	-	-	-	15 ± 0.03^{e}
Eubacterium lentum MTCC 43055	-	-	-	7 ± 0.12^{d}	8 ± 0.13^{e}	10 ± 0.18^{e}	7 ± 0.25^{e}	9 ± 0.25^{d}	12 ± 0.17^{d}	-	8 ± 0.26^{a}	9 ± 0.22^{b}	14 ± 0.05^{f}
Candida albicans MTCC 227	-	-	-	8 ± 0.13^{c}	9 ± 0.26^{d}	12 ± 0.17^{c}	8 ± 0.15^{d}	10 ± 0.09^{c}	12 ± 0.21^{d}	7 ± 0.12^{a}	8 ± 0.21^{a}	9 ± 0.17^{b}	-

Among the four different crude extracts tested, the highest activity (22 mm), at 5 mg/disc was observed in methanolic stem extract against *Klebsiella pneumoniae* followed by root extract. In the case of leaf derived callus methanolic extract showed the highest inhibition (10 mm) at 5 mg /disc against *Enterococcus faecalis* and *Vibrio parahaemolyticus*.

Table 3: Antibacterial activity of methanolic extract of leaf, stem, root and callus from *Coscinium fenestratum*

Name of the Microbe (Clinical isolates)	Zone of Inhibition (mm)												S10
	Methanolic extract												
	(LEAF) mg/disc			(STEM) mg/disc			(ROOT) mg/disc			(CALLUS) mg/disc			
	1.25	2.5	5	1.25	2.5	5	1.25	2.5	5	1.25	2.5	5	
ESBL Klebsiella (ICMR-6) 17	-	-	-	-	7± 0.12^{c}	8± 0.24^{e}	7± 0.13^{b}	8± 0.16^{d}	10± 0.12^{c}	-	7± 0.13^{b}	8± 0.16^{c}	12± 0.04^{c}
Proteus vulgaris 41	-	8± 0.12^{a}	9± 0.21^{a}	-	7± 0.13^{c}	9± 0.23^{d}	-	7± 0.18^{e}	9± 0.18^{d}	-	8± 0.18^{a}	10± 0.18^{a}	12± 0.06^{c}
Salmonella paratyphi-B	-	-	-	7± 0.21^{c}	8± 0.17^{b}	10± .18^{c}	7±0.16^{b}	9± 0.21^{c}	12±0 .13^{a}	7± 0.12^{a}	8± 0.16^{a}	10± 0.12^{a}	15± 0.05^{b}
Staphylococcus aureus (Methiciline Sensitive S.aureus)21	-	-	-	-	7± 0.15^{c}	8± 0.17^{e}	-	7± 0.25^{e}	9± 0.21^{d}	-	-	-	16± 0.03^{a}
MRSA (Methiciline resistant S.aureus) ICMR-5 15	-	-	-	8± 0.25^{b}	10± 0.21^{a}	12±0.25^{b}	8± 0.12^{a}	12± 0.23^{a}	11± 0.17^{b}	-	-	-	12± 0.04^{c}
Pseudomanas (clinical isolates)	-	-	-	9± 0.19^{a}	10± 0.12^{a}	12±0.12^{b}	7± 0.17^{b}	8± 0.17^{d}	10± 0.24^{c}	-	-	-	15± 0.05^{b}
MRSA clinical pathogens	-	-	-	8± 0.13^{b}	10± 0.18^{a}	14±0.18^{a}	8± 0.18^{a}	10± 0.16^{b}	12± 0.21^{a}	-	7± 0.21^{b}	9± 0.18^{b}	16± 0.04^{a}
ATCC- 29213 (MSSA) 7	-	-	-	-	8± 0.13^{b}	10±0.13^{c}	7± 0.12	9± 0.12	10± 0.12^{c}	-	7± 0.25^{b}	8± 0.21^{c}	16± 0.04^{a}

The extracts were also tested against clinical isolates, which have shown moderate activity. Among them, drug resistant pathogens were also inhibited by the methanolic stem and root extracts

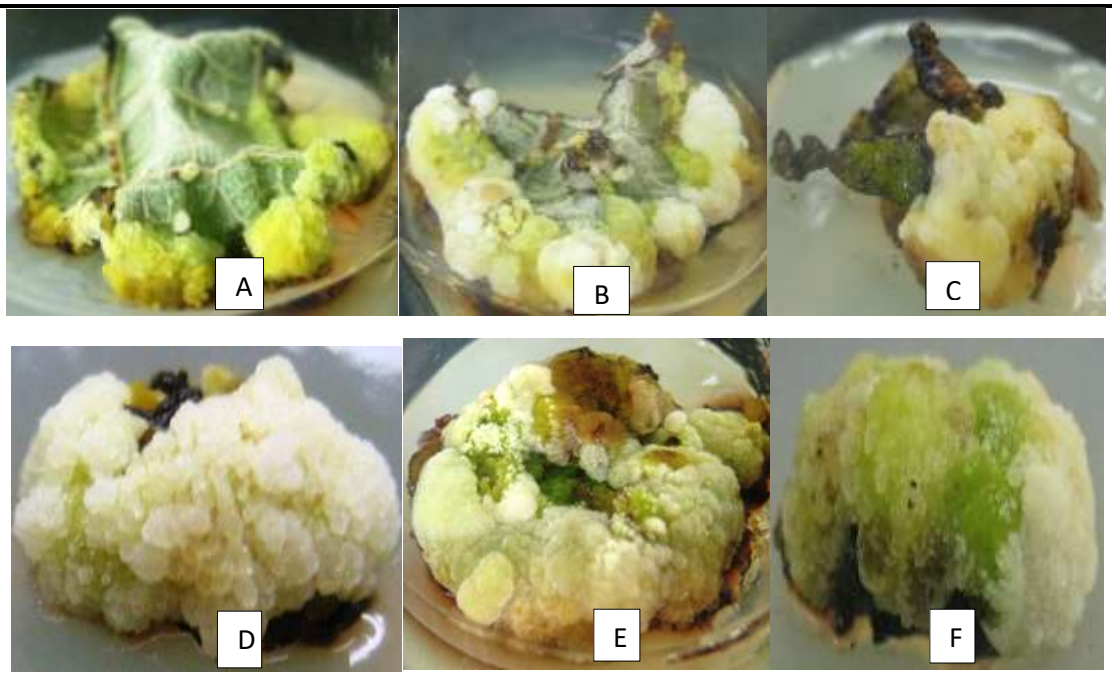

Fig 1: Callogenesis of *Coscinium fenestratum* (Gaertn.) Colebr., from mature leaf explants: A, B&C) Initiation of callus from leaf explants after 12 days of culture in MS medium supplemented with 0.75mg/l TDZ. (Bar 10 mm). D,E&F) Yellowish green coloured callus subcultured in the MS medium supplemented with 0.2 mg/l TDZ.