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RESEARCH ARTICLE

ESTABLISHED *CHONEMORPHA FRAGRANS* CALLUS EXHIBITED BIOLOGICAL ACTIVITIES AS THAT OF PLANT EXTRACT

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ABSTRACT

Chonemorpha fragrans is a medicinal plant used in Indian medicinal systems for the treatment of skeletal gynaecological disorder, stomach disorders, respiratory diseases, rheumatism, skin diseases, diabetes and jaundice. The plant extract shows antipyretic, antidiabetic, anti-parasitic, anticancer and anti-HIV activity. According to International Union for Conservation of Nature (IUCN), the plant is considered to be endangered medicinal plant in the Karnataka and vulnerable in Kerala state of India. The threats for the *C. fragrans* are due to exploitation of its natural habitat and overexploitation of plant for medicinal use. Conventional propagation by seeds has very low seed viability and the vegetative propagation through stem cutting has low frequency of rooting and low survival rate. The present investigation *Chonemorpha fragrans* was focused on standardizing the protocol for micropropagation, explore the biological potential such as antimicrobial and anticancer properties of the aqueous and methanolic extract of callus from leaves and leaves alone. The best explants for the direct regeneration were found to be shoot tip as well as single node followed by root and shoot induction. More importantly, the embryogenesis from callus was successfully obtained. Methanolic extract exhibited excellent broad-spectrum antimicrobial activity against human pathogens such as *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* than the aqueous extracts of leaves as well as callus. The anticancer activity revealed that methanolic extract of leaves exhibited inhibition of leukemic cells with the IC_{50} concentration of 0.3±0.02 and 0.29±0.02 µg ml^{-1} in HL-60 and K562 cells respectively than the 0.5±0.02 and 0.39±0.02 µg ml^{-1} methanolic extract of callus in HL-60 and K562 cells respectively. The aqueous extract of leaves showed IC_{50} concentration of 1.4±0.03 and 1.8±0.04 µg ml^{-1} in HL-60 and K562 cells respectively aqueous extract of callus showed 1.1±002 and 1.2±0.05 µg ml^{-1} in HL-60 and K562 cells respectively. These results concluded that the methanolic extract of both callus and leaves of the plant exhibited maximum anticancer activity than aqueous extract of both. The phytochemicals analysis revealed the maximum variety of compounds was present in the methanolic extract which corresponds with the considerable inhibition demonstrated on test microorganisms and cancer cells. These results revealed the optimum micropropagation of medicinal plant that can be used as a natural drug source for modern medicine.

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INTRODUCTION

Chonemorpha fragrans, a stout spreading laticiferous liana with soft greyish to rusty-brown bark which yields fibre of good quality; leaves simple, opposite, large, orbicular, fulvous tomentose beneath, prominently veined; flowers large, whitish to cream-yellow, fragrant, in terminal or pseudo-axillary cymose panicle; fruits long, straight, woody, parallel, follicular mericarps; seeds many, flat, shortly beaked with long white silky coma (Kulkarni *et al.,* 2011, 2010). The global distribution of plant is reported from India extending to Myanmar (earlier Burma) and SirLanka. In, India it is recorded in moist, semi-evergreen forest of Western Ghats of Karnataka, Kerala, Tamil Nadu, Maharastra, Goa and also Meghalaya, Assam, Arunachal Pradesh, Uttar Pradesh and Andaman and Nicobar Islands. This species is globally distributed in Indo-Malaysia. Within India, it occurs in dense moist forest throughout up to an altitude of 1500 m. *Chonemorpha fragrans* syn *C. grandiflora* or *C. macrophylla* is a medicinal plant used in Indian medicinal systems. It is used in different preparation sudarsansavam, kumaryasavam used in Kerala ayurvedic system and possess several medicinal properties such as antiamoebic (Chatterjee *et al*., 1987), antipyretic, antidiabetic (Chatterjee *et al*., 1987), anti-parasitic, anti-helmenthic (Yadav *et al*., 2011), anticancer (Pradnya *et al*., 2013, 2012), anti-HIV (Priel *et al*., 1991), skeletal muscle relaxant, gynaecological disorder**,** stomach disorder, chest

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disease, rheumatism, skin diseases, leprosy, scabies, dyspepsia, colic, constipation hyperacidity, cardiac debility, diabetes, jaundice, cough, bronchitis and intermittent fevers (Yadav *et al*., 2011, (Wall *et al*., 1966, Kedari *et al*., 2013). The plant has been assigned endangered in the Karnataka states and vulnerable in Kerala state and The International Union for conservation of Nature (IUCN) has included in the list of an endangered medicinal plant (Kolammal *et al*., 1978, Harun-ur-Rashid *et al*., 2013). The threats for the extinction of the *C. fragrans* are the exploitation of natural habitat and overexploitation of plant for medicinal use. Conventional propagation by seeds cannot keep the pace with the demand of this plant because of low seed viability and germination rate further curtail the propagation of *C. fragrans* through seeds. The plant can be vegitatively propagated through stem cutting but the frequency of rooting and survival is low. The research work carried out on micropropagation and suspension culture of *Chonemorpha fragrans* was inadequate, so more studies with detailed refinements of techniques were required (Sharmin *et al*., 2014). Hence, the present research on *Chonemorpha fragrans* carried out focus was on the influence of the various factors on the *in vitro* clonal propagation and in the establishment of suspension culture; to come up with a standard protocol for the micropropagation and cell suspension culture. The phytochemicals analysis was studied with antimicrobial and anticancer properties from propagated plant callus.

RESULTS AND DISCUSSION

Standardized protocol for efficient surface sterilization of explants

The explants shoot tip, single node, stem, petiole and leaves showed washed gently with running tap water and sterilized with tween (0.1%) few 5 min followed by 0.8% mercuric chloride ($HgCl_2$) for 2 min (Fig. 1A-D). Then, the explants was washed in sterilized distilled water and quickly dipped into 70% ethanol for 30 sec. Then, washed with sodium hypochlorite (4%) for 1 min and washed with sterilized with distilled water (Fig. 2A-D). This optimized protocol was used to reduce the contamination.

Tissue culture protocol for optimum micropropagation

Plant micropropagation is a biotechnological approach for the conservation of plant species and is successful for those plant species which have difficulties in propagation using conventional methods. It is an *in situ* conservation of plant species having special phenotypic characters by introduction of regenerated plantlets directly into its natural habitat (Juliani *et al*., 2010). The manipulation of plant growth regulator concentration is probably the most widely used technique for the induction of organogenesis and this methodology has the basis of the propagation of commercially important plant via tissue culture in recent years (George and Sherriagton, 1984). Optimum conditions for direct micropropagation of different explants by monitored by survival percentage of the explants with the increased incorporation of growth regulators. The optimum callus result was observed in the combination of $2mg^{-l}$ BAP in combination with 0.01 mg^{-l} NAA, 3 mg^{-l} BAP and 0.01 mg^{-l} NAA. Single node exhibited highest survival percentage (Fig. 2E-F). Shoot tip and stem explants survived with survival percentage of 83.3 ±1.2 and 82.14±1.3 % respectively followed by single node with the survival percentage of 70.58±1.4% where as leaves explants with the survival percentage of 68.4±1.4%. The petiole had lowest survival percentage of 20±0.3% and was not able to establis. Days taken for bud break were significantly affected by the growth regulators. Number of days taken for the bud break was drastically decreased in the growth regulator combination of 2mg l^{-1} BAP + 0.01 mg l^{-1} NAA. Though the stem explants was able to survive but no further development was observed, so the stem explants was not considered further. The shoot length in the direct regeneration of the established culture was measured after 30 days of inoculation. The height of shoot was more in the combination of 2mg l^{-1} BAP+0.01 mg l^{-1} NAA. The height of shoot decreased with the increase in BAP concentration after 2mg l^{-1}. The height of internode region was more in 2mg l^{-1} BAP while the internode region was reduced in the higher concentration of BAP and also in the combination of BAP and kinetin. The numbers of leaves was the factor of shoot height and the height of internode in per shoot in direct regeneration (Fig. 3A-D). Thought the numbers of leaves was greater in 2mg l^{-1} BAP+ 0.01 mg l^{-1} NAA, the numbers of leaves to height ratio was comparatively higher in the higher concentration of BAP and the combination of BAP and kinetin.

Rooting is carried out after the successful *in vitro* shoot regeneration (Fig. 3B); shoots were excised and transferred to root induction media for the generation of roots. The best result was obtained in the 3mg l^{-1} IBA+0.01mg l^{-1} BAP with the rooting percentage of 50%. Leaves explants exhibited the larger size callus followed by the shoot tip (Fig. 4A-C). The MS medium supplemented with 2mg l^{-1} 2,4-D+0.01mg l^{-1} BAP was found to best for the rapid proliferation of callus. Increase in photoperiod from 16h of light to 18h of light caused the browning of callus. Observation was similar for all explants and all growth regulators combinations. Some calli were kept in the higher temperature of 28±1^{0}C to observe the effect of temperature, the browning of callus was observed. So, the temperature was reduced to 20±1^{0}C. The reduction in temperature resulted in the halt in the browning process and callus development was proper.

Organogenesis from callus

Number of shoot observed was restricted to one on both treatments; 2mg l^{-1} BAP+ 0.01 mg l^{-1} NAA and 3mg l^{-1} BAP+ 0.01 mg l^{-1} NAA. Thus, the micropropagation protocol was successfully optimized. The best explants for the direct regeneration were found to be shoot tip as well as single node. Both the explants was well proliferated in the MS medium supplemented with 2mg l^{-1} BAP and 0.01mg l^{-1} NAA attaining the highest shoot height. The root induction was best in the MS medium supplemented with 3mg l^{-1} IBA and 0.01mg l^{-1} NAA. The response for callus development of *C. fragrans* was paramount. Stem and leaves responded best and the MS medium with 2mg l^{-1} 2,4-D and 0.01mg l^{-1} BAP was found to be ideal for the development of callus for all explants. The organogenesis from callus was also successfully obtained (Fig. 4D). Transfer of callus in MS medium with 2mg l^{-1} BAP and 0.001 mg l^{-1} NAA favored the adventitious shoot regeneration from callus. The formation of somatic embryo was observed in the callus transferred in MS medium with 0.5mg l^{-1} 2,4-D. The optimum photoperiod was 16 h of day and 8 h of darkness, where as the optimal temperature were found to be 20^{o}C.

Fig. 1. Plant photo with flowers A Entire plant in natural habitat B Flower of plant C Fruit of plant D Plantlet obtained

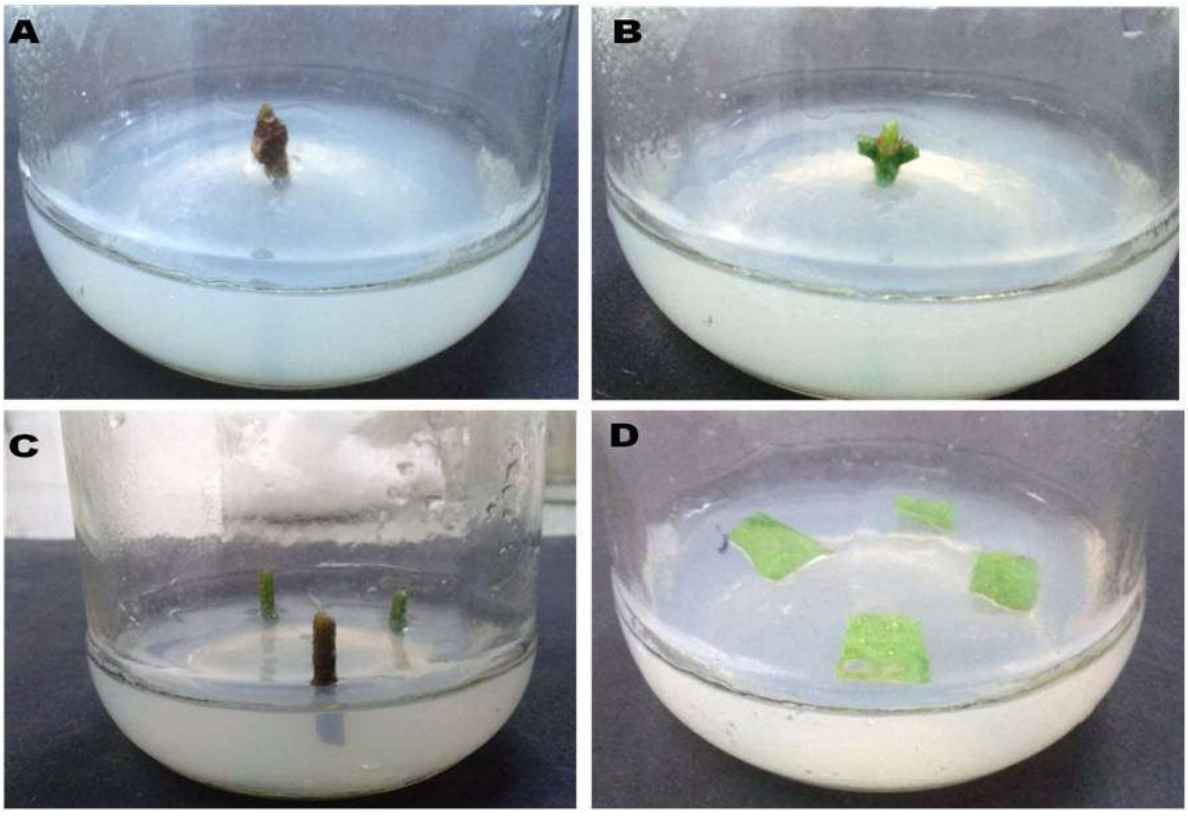

Fig. 2. Explants inoculated to medium A Shoot tip B Shoot node C Stem D Leaves

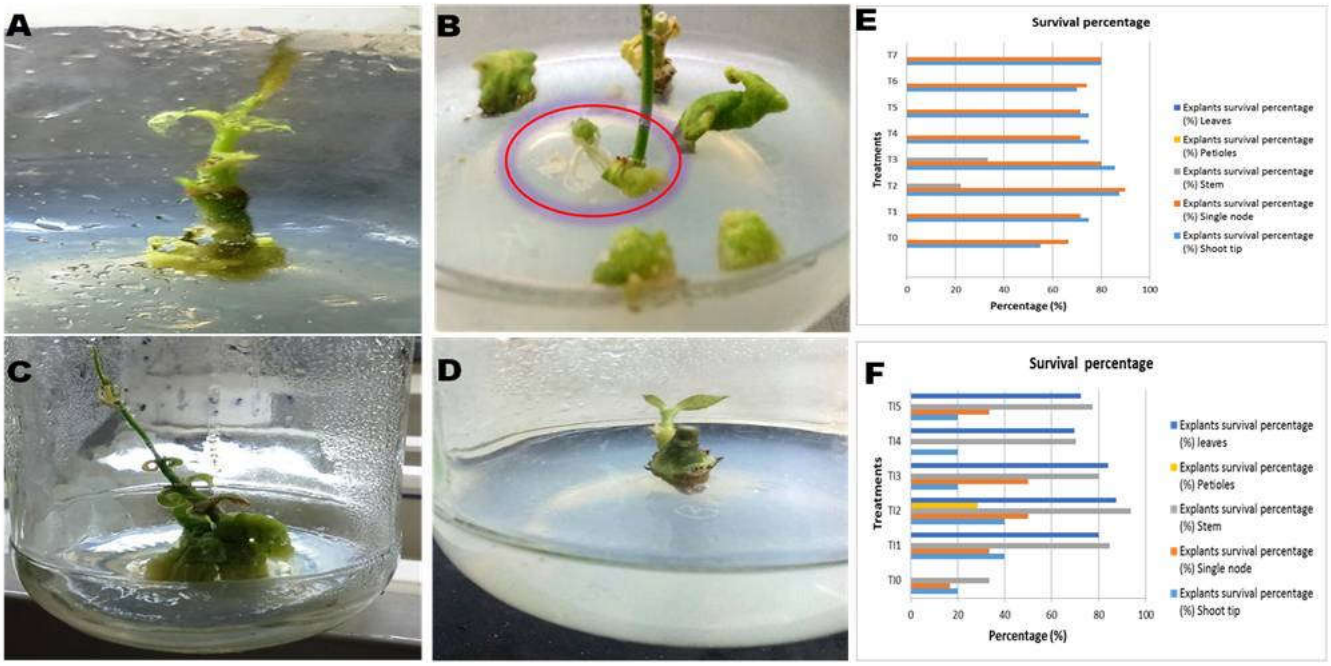

Fig. 3. Root and Shoot regeneration A and B photographs showed root induction C and D Shoot regeneration E Optimum growth regulators for direct regeneration from different explants F Effect of growth regulators on the survival percentage of explants for direct regeneration

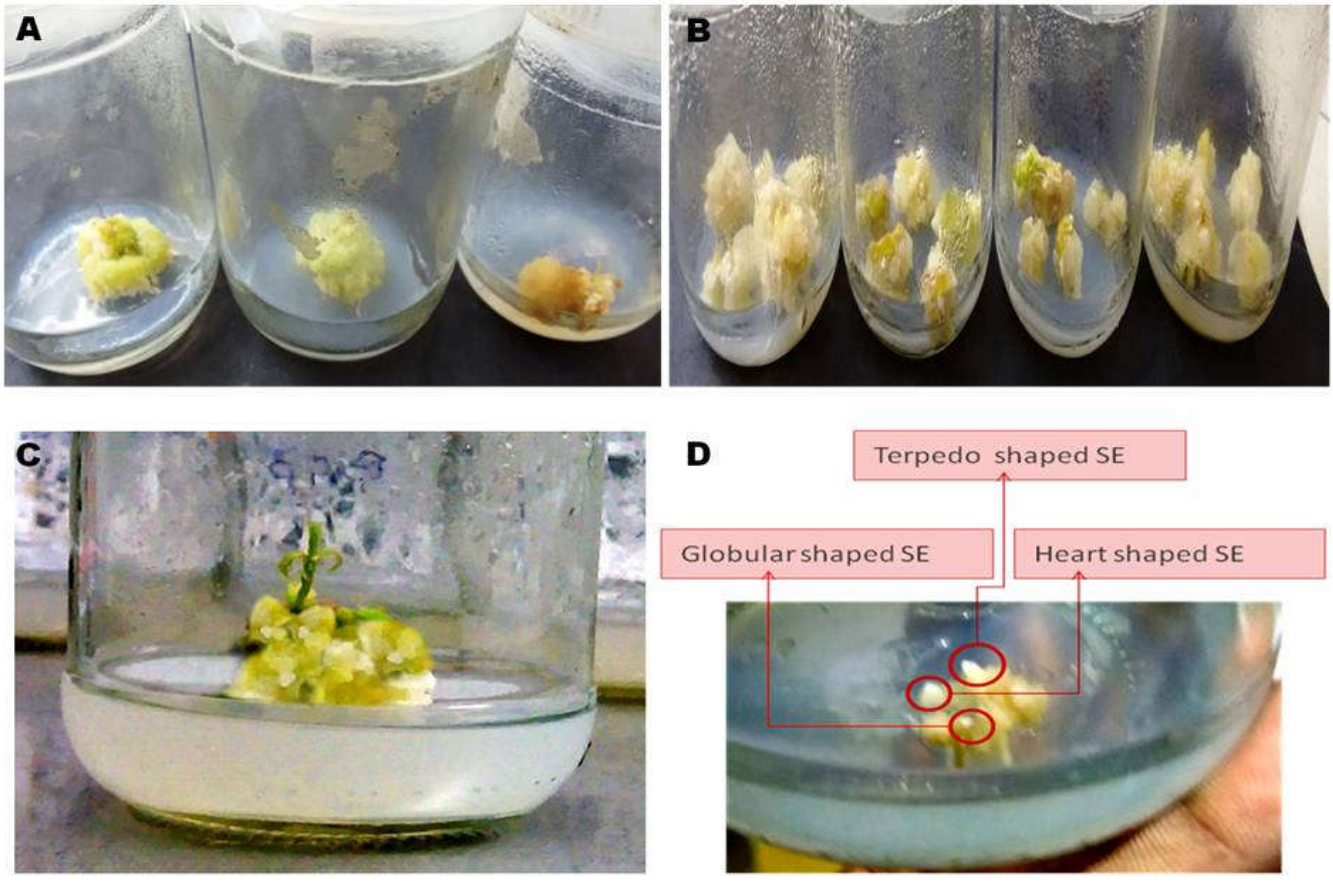

Fig. 4. Indirect regeneration of plant A Callus from stem explants on various treatments B Callus from leaves explants in various treatments C Shoot regeneration on various treatment D Developmental stages in somatic embryogenesis from callus

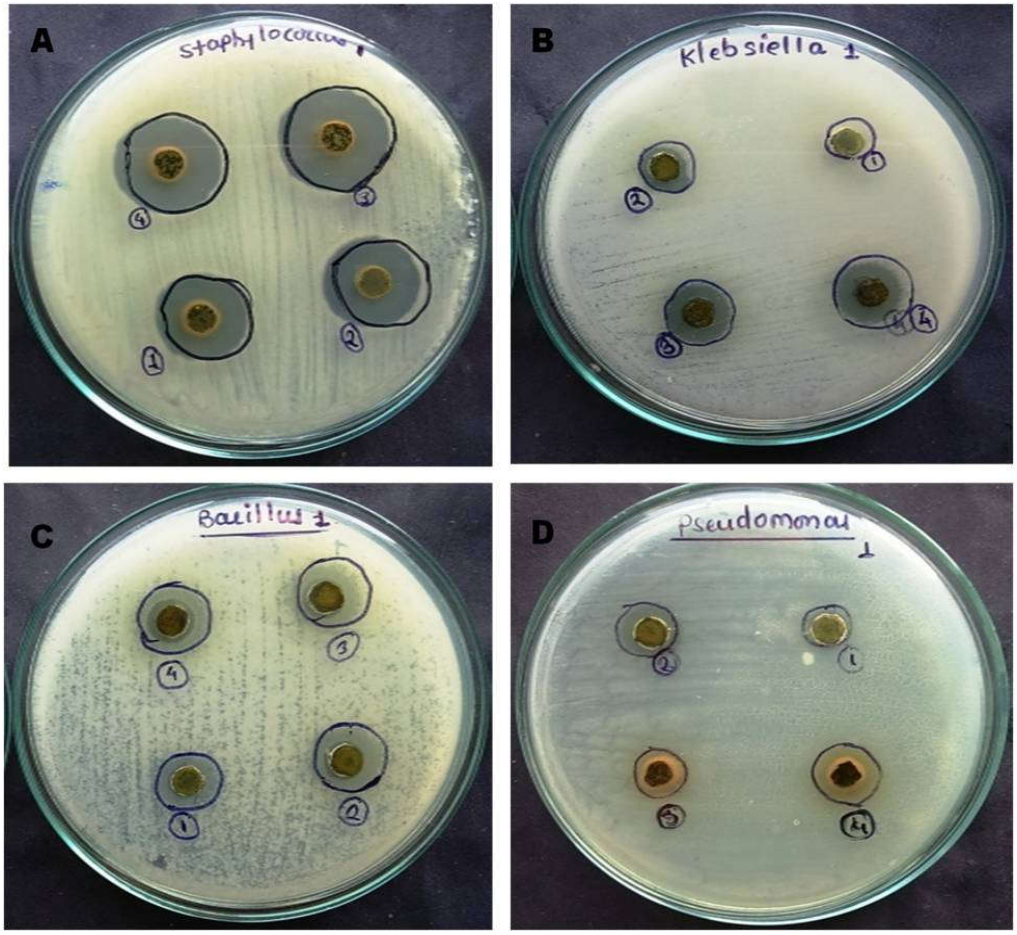

Fig. 5. Antimicrobial activity of aqueous and methanolic extract of leaves and callus obtained from the leaves Representative photographs showing the antimicrobial activity in methanolic extract A *Staphylococcus aureus*, B *Klebsiella pneumonia*, C *Pseudomonas aeruginosa* and D *Bacillus subtilis* (1-0.1; 2-0.2; 3-0.5 and 4-1µg ml^{-1})

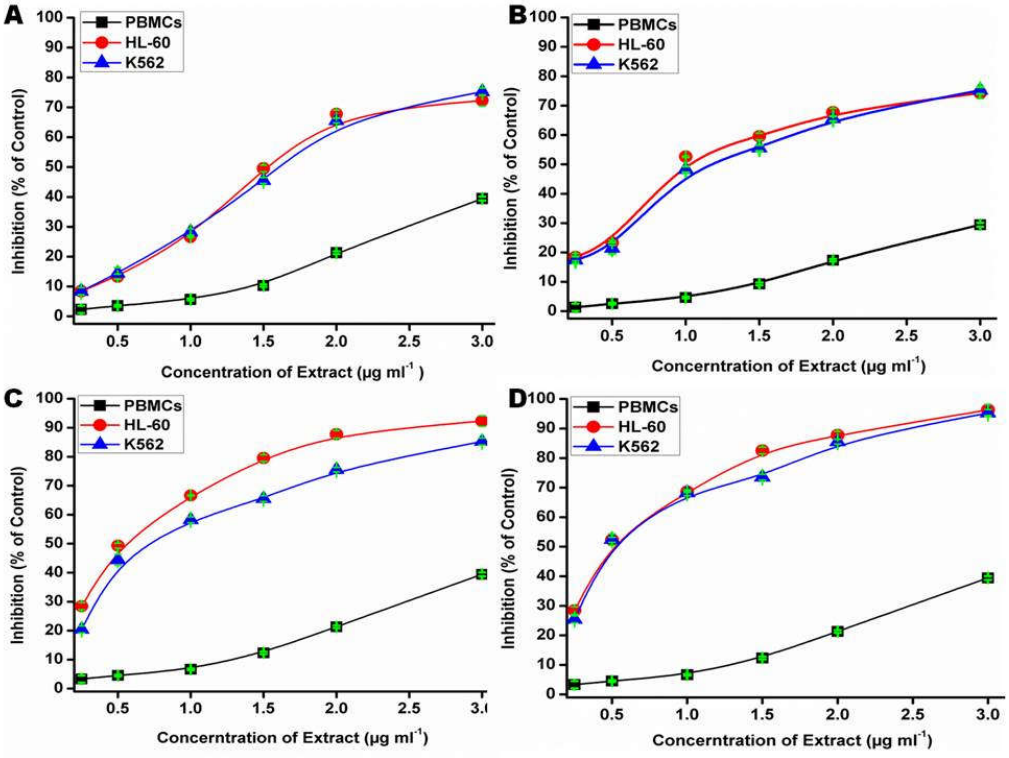

Fig. 6. Anticancer activity of different solvent extracts of leaves and callus obtained from the leaves A Dose-response study of aqueous extract of leaves B Dose-response study of aqueous extract of callus obtained from the leaves C Dose-response study of methanolic extract obtained from leaves and D Dose-response study of methanolic extract obtained from callus

Biological activity of leaves and callus extracts

The biological activity such as antimicrobial spectrum and anticancer properties of aqueous and methanolic extract of leaves and callus obtained from leaves was performed. Methanolic extract exhibited excellent broad-spectrum antimicrobial activity against human pathogens such as *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* than the aqueous extracts of leaves as well as callus. The maximum inhibition (average zone of inhibition was 21±2 mm) was observed against *Staphylococcus aureus*, *Bacillus subtilis,* followed by *Klebsiella pneumonia* and *Pseudomonas aeruginosa* in methanolic extract of callus than the aqueous extract (Table No. 1). Anticancer properties of extracts were observed against the leukemic cells such as HL-60 and K562 cells and normal peripheral blood mononuclear cells (PBMCs) (Fig.5). The methanolic extracts showed dose-dependent inhibition of leukemic cells than normal PBMCs. The anticancer activity revealed that methanolic extract of leaves exhibited inhibition of leukemic cells with the IC_{50} concentration of 0.3±0.02 and 0.29±0.02 µg ml^{-1} in HL-60 and K562 cells respectively than the 0.5±0.02 and 0.39±0.02 µg ml^{-1} methanolic extract of callus in HL-60 and K562 cells respectively at 48 h (Table No. 2). The aqueous extract of leaves showed IC_{50} concentration of 1.4±0.03 and 1.8±0.04 µg ml^{-1} in HL-60 and K562 cells respectively aqueous extract of callus showed 1.1±002 and 1.2±0.05 µg ml^{-1} in HL-60 and K562 cells respectively. These results concluded that the methanolic extract of both callus and leaves of the plant exhibited maximum anticancer activity than aqueous extract of both (Table 2).

Phytochemical analysis of extracts

All seven phytochemical analyzed such as alkaloids, triterpenoid and carbohydrates were present methanol extract and only carbohydrates and trace of alkaloids were present in the aqueous extract of both leaves and callus from leaves (Table 3). The phytochemicals analysis reveals the maximum variety of compounds in methanolic extract which corresponds with the considerable inhibition demonstrated on test microorganisms and cancer cells. The inhibition of as *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* against human pathogens by methanol extract of callus of *C. fragrans* reveals its potent use of callus than the whole plant in the modern medicine to reduce the endangered species.

Concluding Remarks

Plant micropropagation is a biotechnological approach for the conservation of plant species and is successful for those plant species which have difficulties in propagation using conventional methods. The plant cell suspension though does not directly help in the conservation of plants; it contributes to prevent the over-exploitation for the fulfillment of demand of phytochemicals they produce. As, the plant cell suspension culture provides an attractive alternative source that could overcome the limitations of extracting useful metabolites from limited natural resources. And it is now widely being employed as a model system to investigate the production of specific secondary metabolites because it offers experimental advantages both to basics and applied research and to the development of models with scale up potential. The best explants of *C. fragrans* for the direct regeneration were found to be shoot tip as well as single node. The response for callus development of *C. fragrans* was paramount. Stem and leaves responded best and was found to be ideal for the development of callus. The organogenesis from callus was also successfully obtained and favored the adventitious shoot regeneration from callus. The formation of somatic embryo was observed in the callus transferred in MS medium with 2,4-D (0.5mg $^{-l}$). The phytochemical analysis of the plants is very important commercially and has great interest in pharmaceutical companies for the production of the new drugs for curing of various diseases. Starting from the ancient time, medicinal plants have been used to prevent and treat various health problems. The root powder of *C. fragrans* contains phytochemicals like alkaloids, carbohydrates, flavonoids, saponins, triterpines, phytosterol, tannins, glycosides, protein, amino-acids and phenolic compound. The major alkaloids present in *C. fragrans* are chonemorphine, funtumafrine and camptothecine. Chonemorphine gives the characteristic fragrance present in this species and makes it a potential raw material of perfume industries. Camptothecin, an anticancer compound was isolated and characterized for the first time form a Chinese tree *Camptotheca accuminata*. In our work the phytochemicals analysis reveals the maximum variety of compounds to present in the methanolic extracts showed considerable inhibition of test microorganisms and cancer cells. These results confirmed the presence of bioactive phytochemicals in the methanol extract of leaf and callus obtained from callus might be primary responsible for the antimicrobial and anticancer potential, which has to identified in future aspects. In our present investigations, we have successful in micropropogation of *C. fragrans* with optimized conditions and phytochemical present in the *C. fragrans* exhibited excellent antimicrobial and anticancer properties. Further, studies are focused on the identification of active phytochemicals responsible for antimicrobial and anticancer activities.

Experimental

Tissue culture protocol for optimum micropropagation

The plantlets of *Chonemorpha fragrans* were procured from Institute of Ayurveda and Integrative Medicine (IAIM), Yelahanka, Bangalore, India. And the plantlets were maintained in the green house of REVA University Educational Park and the explants were excised in the time of need from the healthy plant by cutting the plant with a sharp scalpel. Single node, shoot tip, stem, petiole and leaves were used as the explants. The plant material was collected from IAIM and sterilized as described (Badoni *et al* 2010, Adel *et al* 2012). The explants such as shoot tip, single node, stem, petioles and leaves were obtained, gently washed in running tap water for few minutes. The explants were surface sterilized by Mercuric Chloride ($HgCl_2$) and ethanol (70%) for required time and washed with sterilized distilled water. After washing, the explants were placed onto MS medium without plant growth regulators (Murashige and Skoog, 1962). Cultures were maintained in air conditioned chamber at 24°C for 20 days under 16 h light period regimes with irradiance of 80 µm m^{-2} s^{-1} PAR. The cultures were checked macroscopically every 7-days.

Selection of the best propagation medium and conditions

The MS medium was used with different growth regulator combination (George *et al*., 1984). For direct shoot

regeneration basal media was supplemented with varying concentration of BAP (1-5 mg^{-l}), NAA (0.01 mg^{-l}), kinetin (1-2 mg^{-l}) and inoculated with shoot tip (approximately 2 cm), single node (approximately 2 cm), stem (approximately 2 cm), petiole (approximately 1 cm) and leaves (approximately 1 cm) was used as explants. For callus induction, the basal media supplemented with varying concentration of 2,4-D (1-3 mg^{-l}), BAP (0.01 mg^{-l}) and kinetin (0.01 mg^{-l}) using same explants. For organogenesis and somatic embryogenesis using callus, different growth regulator combination of 2,4-D (1-0.5 mg^{-l}), BAP (1-3 mg^{-l}) and kinetin (0.01 mg^{-l}) was used.

Extraction and antimicrobial potential of plant extracts

The callus of *C. fragrans* was also shade dried in room temperature and powdered by using grinder. Around 3 g of the dried powder was soaked in 30 ml of solvent (methanol and water) for 24 h on shaker at 150 rpm and extracted using soxhet apparatus. The extracts were concentrated by evaporating in the rotary evaporator and stock concentration was prepared in methanol (1 mg ml^{-1}). The stock solution was filtered, further diluted and used for the evaluation of MIC. The microorganism used in the study was *Klebsellia, Bacillus, Styphylococcus, E. coli* and *Pseudomonas.* Agar well diffusion method according to the protocol and 50µg ml^{-1} pure streptomycin was used as positive control (Kulkarni *et al*. 2011). The solvent itself was used as negative control at 37^0C for 24 h. The inhibition zone was observed and diameter was measured in millimeters using scale. Aqueous, chloroform and methanol extracts of *in vivo* and *in vitro* from the callus were subjected to standard phytochemical analysis to determine the presence of alkaloids, flavonoids, triterpenoids, tannins, sterols, saponins and carbohydrates (Chandra *et al*., 2011).

Anticancer potential of extracts

The anticancer activity of extract (1mg ml^{-1}) was dissolved in DMSO, diluted in medium and stored in 4°C until use. The cytotoxicity of extract was performed using leukemic cells (HL-60 and K562) and peripheral blood mononuclear cells (PBMCs) was used as normal cells as described (Veena *et al*., 2016). Briefly, the leukemic and PBMCs was seeded at the density of $1X10^6$ cells per ml and treated with different concentrations of extract (0.25 to 50 µg ml^{-1}) and incubated for 48 h. After treatment, MTT reagent (5 mg ml^{-1}) was added and incubated for 4 h under dark at 37°C. After 4h, the cells were centrifuged at 5000 rpm for 5 min, the supernatant was removed and the formazan formed inside the cells was dissolved in DMSO (100 µl) and incubated for 10 min. The absorbance was read at 595 nm in ELISA reader and the IC_{50} concentrations was obtained from dose-response curve using graph pad prism software.

Statistical analysis

The statistical analysis was performed by ANNOVA test with level of significance $P<0.05$ of three independent experiment.

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