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Research Article

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Evaluation of Biological activities and Pharmacological analysis of *Ceropegia juncea* Roxb. (Asclepiadaceae) plant extract

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Abstract

In vitro conservation protocol for rare medicinal plant *Ceropegia juncea* was developed using nodal explants by culturing on Murashige and Skoog (MS) medium. The maximum number of greenish nodular callus was induced in MS + 6-benzylaminopurine (BAP) 1.5 mg/l + 2,4-dichlorophenoxy acetic acid (2,4-D) 1.5 mg/l and massive callus induction was in MS + Indole-3-Butyric Acid (IBA) 1.0 mg/l + BAP 1.0 mg/l + 2,4-D 0.5 mg/l. The greenish nodular calli were raised on the MS medium supplemented with BAP 3.0 mg/l + kinetine (Kin) 0.5 mg/l + Indole-3-Butyric Acid (IBA) 0.5 mg/l + Naphthalene Acetic Acid (NAA) 0.5 mg/l for shoot induction. Root induction was achieved in MS + Indole-3-Acetic Acid (IAA) 0.5 mg/l + NAA 0.5 mg/l + IBA 0.5 mg/l + BAP 1.0 mg/l. The plantlets were established, acclimatized and thrived in greenhouse and then in natural environmental condition. The *in vitro* conservation protocol developed in this study provides a basis for germplasm conservation of this medicinal plant. Aim of the present study is to investigate the phytochemical analysis and anti microbial activity of aqueous and methanol extracts of *Ceropegia juncea*. The enzymatic and non enzymatic (DPPH) method was employed to analyze the antioxidant property. Qualitative analysis of phytochemical screening reveals the presence of Alkaloids, Phenol, Saponins and Protein. Further presents of phytochemicals were detected by Thin Layer Chromatography (TLC), which is the standard technique for separating organic compounds. The extracts were purified using Column chromatography. The fraction 3 was subjected to GC-MS analysis to the compounds presents in the extract. Biologically synthesized silver nanoparticles are being widely used in the field of medicine. The present study is focused on extracellular biosynthesis of silver nanoparticles using stem extract of *Ceropegia juncea* (Asclepiadaceae) *ex situ* as well as *in vitro* plants.

Keywords: *Ceropegia juncea, in vitro,* Nanoparticles, Phytochemical, Expalnt.

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Introduction

India has a rich and varied heritage of biodiversity, encompassing a wide spectrum of habitats from rasin forests to alpine vegetation and from temperate forests to coastal wetlands. In India, about 1500 rare and threatened species of both flowering and non-flowering plant groups were identified during the last two decades. The threat to a majority of them could be attributed to anthropogenic factors like habitat destruction due to grazing, urbanization and other developmental activities and exploitation (Myers, 1988).

Ceropegia genus with 200 species are present throughout the world, also has distribution in tropical and subtropical Asian countries. In India 55 species have been reported, among them, 28 are endemic to peninsular India and 16 species of them are included in the Red Data Book (Nayar and Sastry, 1987). Documentation of 56 species, two subspecies and three varieties for India has been done (Karthikeyan *et al*. 2009), among them a few species are edible and few are domesticated as ornamental plants (Mabberley, 1997). These species of *Ceropegia* have many common names snake creeper, lantern flower, parasol flower, string of hearts, bushman's pipe, parachute flower, wine-glass vine, necklace vine and rosary vine. Several *Ceropegia* species are included under the categories of rare, endangered, vulnerable, extinct and threatened plants (Jagtap and Singh, 1999). Several workers attracted towards the *Ceropegia* genus in recent years due to its potent medicinal properties and rarity of its species. There are various reasons for threatened status of *Ceropegia* species such as severe anthropogenic pressures on the forest land, overexploitation of *Ceropegia* species for their tubers by humans and various animals and hence these species needs special attention and requires proper conservation strategies.

Botany and distribution

The genus *Ceropegia* L. belonging to the family Asclepiadaceae is a botanically curious genus with largest number of species native to Africa, Southern Asia and Australia. In India these species are mainly distributed in the Western Ghats (Jagtap and Singh, 1999). *Ceropegia* L. shows a variety of growth habits such as erect herbs, climbers; rarely sub shrubs, leafless succulents and non-succulent twiners. Check-list of 55 *Ceropegia* species, including status of taxa, phenology, and habit, importance of the conservational strategies, Indian and World distribution has been summarised (Ramamurthy *et al*. 2012). Most of the species are producing tuberous roots whereas few have fascicled roots (Surveswaran *et al*. 2009). Different *Ceropegia* species with their diagnostic features and distribution in India are shown in (Table 1).

Traditional uses and preparations

Ceropegia species have sound and numerous ethnomedicinal values and hence is an important component in several indigenous drug preparations (Ayurveda). Bhilla tribal group in Northwest Maharashtra region are using *Ceropegia hirsuta* fresh root for treatment of stomach disorders (Kambale *et al*. 2008). In India, practise of taking a teaspoon of tuber paste daily once for 3 days is reported to be effective for treating the stomach related problems (Reddy *et al*. 2009). The whole plant extract used as an antidote for snack bite (Suresh and Paulsamy, 2010) and fleshy stem is used for treatments of stomach and gastric disorders in traditional and folk medicines. The combination of goat milk with crushed stems of *Ceropegia juncea* is taken orally for three days to cure decease ulcer by Paliyan/Paliyar tribe in Sirumalai hills of Southern India (Karuppuswamy, 2007). Starchy edible tubers with medicinal properties gained economic importance (Jagtap and Singh, 1999). Fresh tubers are boiled before consumption to remove bitterness. Tuberous roots are rich sources of starch, sugar, gum, albuminods and medicinally potent alkaloid 'cerpegin' (Anonymous, 1980) used as blood purifier and as nutritive tonic. Kani, the tribal group from Kerala-Tamil Nadu border used *Ceropegia spirallis* corm extract for purification of blood and in treating syphilis (Daset al. 2008). The alkaloid cerpegin

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extracted from the tubers of *Ceropegia* is a valuable constituent in the traditional Indian drug preparations against various ailments especially dysentery, diarrhoea and to cure sneezing, cold and eye diseases in Bihar region and also the seed paste has been used for treatment of Deafness (Kirtikar and Basu, 1935). *Ceropegia bulbosa* var. *bulbosa* Roxb. Tuber paste is applied on the inflammation of skin and decoction taken to get rid of urinary bladder stone. Aquaeous extract of *C. bulbosa* contains steroids, polyphenols, sugars and potassium (Khare, 2007). Tubers of *Ceropegia hirsuta* Weight Arm. are eaten raw or taken in the form of decoction for stomach ache, dysentery, diarrhoea and warm tuber paste is used for treatment of viral infections (Deshmukh and Rathod, 2013). Raw leaf and flower extracts of *C. pusilla* is used for the treatment of nervous weakness in India (Rajan *et al*. 2005). The whole plant extract of *C. ciliata* Wight is used for treatment of fever while *C. oculata* raw tubers are used for treatment of fever in children and stomach ache.

Chemical constituents

Tuberous roots of *Ceropegia* L. are sources of relatively rare alkaloid 'ceropegin' has been elucidated as1,1,5- trimethylfuro-[3,4-c]pyridine-3,4(1H,5H)-dione [1, 2] and identified and isolated from *Ceropegia juncea* as new pyridine type alkaloid that, possess a wide range of properties such as anti-inflammatory, analgesic, tranquilizer anti-ulcer, mast-cell stabilizing, hepatoprotective, antipyretic, local anaesthetic and antiulcer (Adibatti *et al*. 1991). *Cerpegia* is the only naturally occurred example of bicyclical furo [3,4-c] pyridine ring system, a convenient method was developed for pyridine alkaloid 'cerpegin' and its various new C-1 and N-5 derivatives in high yields (Hovhannisyan, 2014). Presence of different phytoconstituents such as steroids, terpenoids, anthocyanins, anthracene glycosides, coumarins, flavonoids, fatty acids, phenolic compounds, alkaloids, carotenoids have been reported in *Ceropegia juncea* (Karayil and Veeraiah, 2014; Kalimuthu and Prabakaran, 2013). The phenolic compounds such as gallic acid, vanillin, cathechol and ferulic acid, flavonoids and their antioxidant capacity were identified and reported from various extracts of three species of *Ceropegia* such as *Ceropegia spiralis*, *Ceropegiapanchganiensis* and *Ceropegia evansii* from the Western Ghats of India (Chavan *et al*. 2013). *Ceropegia pusilla* tubers were screened for secondary metabolites and confirmed the presence of glycosides, alkaloids, flavonoids, tannins, saponin, triterpenes phenol and steroids through phytochemical screening and GC-MS analysis (Kalimuthu and Prabakaran, 2013). In North Central Nigeria, *C. deightoniii* plant used for the treatment of various infectious diseases was screened for phytochemicals and revealed the presence of tannins, flavonoids, steroids, terpenes, saponins, anthraquinones, cardiac glycosides and triterpenes, glycosides are seems to be rare in *Ceropegia* (Awolowo, 2014). C. bulbosa tuberous roots consists of polyphenols, steroids, fats, albuminoids, sugars, potassium and the active constituent an alkaloid cerpegin (Monika *et al*. 2012). Tubers and leaves of different *Ceropegia* species are screened for ascorbic acid content and found that ascorbic acid content was highest in tubers of *C. hirsute* and lowest in *C. bulbosa* leaves, concluded that leaves contained higher ascorbic acid than the tubers. A novel coumarin, (E)-5-(4-methyl-2-oxo-2H- chromen-8-yl)-2-(4-methylbenzyl)-3-oxopent-4-enenitrile was isolated from *Ceropegia juncea* (Karayil *et al*. 2014). Coumarins have attracted the attention in recent years due to their diverse properties such as hepatoprotective, anti-inflammatory and antioxidant, antithrombotic, anti allergic and anti-carcinogenic activities (Karayil *et al*. 2014).

Pharmacological activities

Ceropegia species has been subjected to various pharmacological investigations by various researchers. The pharmacological importance of the genus *Ceropegia* L. is mainly due to the presence of 'cerpegin', a pyridine alkaloid**.**

Antimicrobial activity

Studies on antibacterial activity of three *Ceropegia* species such as *C. spiralis*, *C. juncea*

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and *C. candelabrum* var. *candelabrum* on human pathogens *Klebsiella pnemoniae, Staphylococcus aureus, Escherichia coli* and *Pseudomonas* has been carried out, findings showed that ethanol extract from the *In-vitro* plant showed a higher antibacterial activity as compared to chloroform and aqueous extracts. Antimicrobial activity was assayed with whole plant extract of *Ceropegia pusilla* against five species of bacteria (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei, Bacillus* sp.) and four species of fungi (*Candida albicans, Aspergillus* sp., *Penicillium* sp., *Mucor* sp.) by agar well diffusion method. The crude extract of *C. deightonii* showed antimicrobial activity against pathogens *Staphylococcus aureus; Streptococcus feacalis; Escherichia coli; Shigella dysenteriae* and *Candida albicans*[5]. Leaf extract of *C. thwaitesii* used for the plant mediated synthesis of AgNPs (silver nanoparticles) showed antimicrobial activity against *S. typhia* and *B. subtilis*.

Materials and Methods

Plant material and explants preparation

The plants of *C. juncea* were collected from a naturalized population in Nartharmalai (Pudukottai District), Tamil Nadu, India (Figure 1, a,b,c). For periodic harvest of explants, stocks were maintained in the herbal garden at Department of Microbiology, AVS College of Arts and Science, Salem, Tamil Nadu. Nodal portions of succulent stem were tested for culture initiation. The young succulent stems were excised from the plants and kept under tap water with Tween 20 (4-5 drops) for 20 min. Nodal segments (1-2 cm long) was cut from these stems and was treated with fungicide Bavistin (0.5- 1.0% w/v) and antibiotic streptomycin (0.05-0.1% w/v) for 10 min each. Each time the nodal segments were rinsed thrice in distilled water and then they were surface sterilized with aqueous solution of $HgCl_2$ (0.1% w/v) for 2-3 min and washed thrice with sterilized distilled water. The explants were cut gently with a sterilized blade and explants were inoculated in culture tubes with modified MS media under aseptic conditions.

Culture medium and conditions

Basal Murashige and Skoog medium (Murashige and Skoog, 1962). (MS medium) was prepared using Hi media and Sigma chemicals. Sucrose (30% w/v) was added to medium and pH of medium was adjusted to 5.8 before adding agar-agar (8% w/v). Different growth regulators (BAP, 2, 4-D, IBA, Kin, NAA and IAA) at different concentrations and combinations were added to the medium. In the present study, the media were autoclaved at 121° C and 15 lbs pressure for 20 min. All the *in vitro* cultures were maintained at 24± 2° C and illuminated for 16 h with fluorescent light (18-24 µ mol/m/sec) followed by 8 h dark period and the relative humidity was about 60-80% within the 25×150 mm culture tubes covered with cotton plug.

Callus, shoot and root induction and acclimatization

The healthy and normal nodal segment explants were inoculated in MS medium with different combinations of growth regulators. When the growth regulators failed to induce a specific response in callus, direct organogenesis and multiple shoot production at the end of the first cycle, it was marked as inappropriate combination. Twenty cultures were raised for each treatment and all experiments were repeated thrice. BAP, 2, 4-D, IAA and IBA combinations were tested to estimate the callus production. BAP, Kin, IAA, IBA and NAA combinations were used for shoot induction. The regenerated shoots were inoculated for rooting medium supplemented with IAA, IBA, NAA and BAP combinations. The rooted shoots were washed with sterile distilled water to remove the traces of medium. The *in vitro* rooted plantlets were transferred to cups containing autoclaved sand. Those cups were covered initially with polythene bags to maintain humidity and placed in mist chamber. After every alternative day, quarter strength MS medium salt solution was supplied to the plantlets. After two weeks of growth, the complete plants were established, acclimatized and thrived in green house condition. The acclimatized plants after two weeks were then transferred to natural environment and established into healthy plants (Figure 1, l)

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Collection and Processing of Plant:

Matured stem of *C. juncea* were collected from a naturalized population in Nartharmalai (Pudukottai District), Tamil Nadu, India (Figure 1, a,b,c). For periodic harvest of explants, stocks weremaintained in the herbal garden at Department of Microbiology, AVS College of Arts and Science, Salem, Tamil Nadu. Nodal portions of succulent stem were tested for culture initiation. That plant leaves were washed with tap water to remove soil and unwanted dust particles. Then the leaves were shaded, dried, and then powdered by using mechanical blender and stored in air tight bottles.

Extract Preparation: The powdered plant stem and leaves were soaked with (10g/100ml) in different solvent (aqueous and methanol), for overnight in rotator shaker.

Qualitative Analysis of Phytochemicals

Test for Alkaloids: To 1 ml of extract added 1 ml of Mayers reagent and few drop of Iodine solution. Formation of yellow colour precipitate indicates the presence of Alkaliods.

Test for Terpenoids: To 1 ml of crude extract add 1 ml of concentrated H_2SO4 and heated for 2 minutes. A grayish colour indicates the presence of Terpenoids.

Test for Phenol and Tannins: To 1 ml of crude extract added 1 ml of $FeCl_3$. A blue green or black colour indicates presence of Tannins.

Test for reducing Sugar: To 1 ml of extract added 1 ml of Fehling's A solution and 1 ml of Fehling's B solution. Formation of red colour indicates the presence of sugar.

Test for Saponins: To 1 ml of extract added 2 ml of distilled water, shaken well and formation of 1 cm layer of foam indicates presence of Saponins.

Test for Flavonoids: To 1 ml of extract added few fragments of magnesium ribbon and added few drops of concentrated Hcl drop wise. Appearance of pink scarlet colour confirmed the presence of flavonoids. Test for Quinines: To 1 ml of extract added 1 ml of 1% NaOH and mixed well. Appearance of blue green or red indicates presence of Quinines.

Test for Protein: To 1 ml of extract added few drop of mercuric chloride. Formation of yellow colour indicates the presence of protein.

Test for Steroids: 1 ml of extract mixed with 1 ml of chloroform and concentrated H_2SO4 sidewise. A red colour presence at the lower chloroform layer indicates presence of steroids.

Antibacterial Assay:

The agar well diffusion method was used for antibacterial assay. Petri plates were prepared by pouring 20 ml of Nutrient Agar medium and allowed to solidify. Plates were solidified and 20 µl of bacterial culture *E. coli, S. aureus, P. aeruginosa* and *B. subtilis* was poured and uniformly spread. The excess inoculum was drained away and the inoculum was allowed to dry for 5 minutes. Agar well of 5 mm in diameter were prepared with the help of a sterilized stainless cork borer. The wells were labelled appropriately and to each well were loaded with 10 µl, 20 µl and 30 µl plant extract, along with disc and plant extract using a micro-pipette. Standard reference antibiotic Amikacin(methanol) and Neomycin(water) (25 mcg/disc) was used as controls for the tested bacteria. The plates were incubated at 37° C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of zones of inhibition against the tested bacteria.

Anti-Oxidant Activity:

DPPH radical scavenging activity:

This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. 0.1 ml, 0.2 ml and 0.3 ml of methanol leaf extracts were mixed with 1 ml of 0.1 mM DPPH. To all the tubes added 0.4 ml of 50 mM Tris-HCl. Incubate the reaction mixture at room temperature for 30 minutes. The absorbance of the reaction

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mixture was read at 517 nm. The percentage of free radical scavenging was calculated as formula mentioned below. Estimation of total flavonoids: 1 ml of methanol and aqueous extract was mixed with 0.1 ml of 10% AlCl3, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled H2O. The mixture was incubated in room temperature for 30 minutes. Then the absorbance was measured at 415 nm in spectrophotometer. Estimation of total phenol: Total phenol content was determined by the Folin- ciocalteau reagent method. 1 ml of methanol and aqueous extract was mixed with 1 ml of folin's phenol reagent and 1 ml of 20% sodium carbonate. The mixture was allowed it for incubation at 45 C for 45 minutes and the absorbance was measured at 765 nm in spectrophotometer.

Thin layer Chromatography:

The aqueous and methanol extracts were added as spot using capillary tubes on the one end of the thin layer plate at above 1 cm. Plate was allowed it for air dry, then it was placed in a beaker containing solvent Ethyl acetate: Methanol in the ratio of 6: 4. The samples were allowed to run towards the other end of the plate. The sheet was removed and allowed it to air dry and 2% of ninhydrin was sprayed and again allowed to air dry for 10 minutes. The plate was then visualized under the UV light and violet colour spot was absorbed on the plate. Column chromatography 1.0 gm of Silica gel 100- 200 Mesh was added to 50 ml sterile distilled water and kept for overnight soaking. To this 10 ml of methanol leaf extract was added carefully to the top of the gel and allowed to pass into the gel by running the column. After 30 minutes the samples were eluted and the fractions were collected at 15 minutes interval each in 5 eppendorf tubes and stored at -20º C.

GC-MS analysis

GC-MS analysis were performed using a Perkin-Elmer GC clauses 500 system and Gas Chromatograph interfaced to a mass spectrometer (GC-MS) equipped with a Elite-1, fused silica capillary column (30 m 0.25 mm ID ´ 1 m df, composed of 100% Dimethyl poly siloxane). For GC/MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min and an injection volumeof 2 ml was employed (Split ratio of 10:1) injector temperature 250°C; ion-source temperature280°C. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C/min to 2000°C, then 5°C/min to 280°C, ending with a 9 min isothermal at280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 45 to450 Da. Total GC running time was 36 minutes. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass.

Nanoparticles synthesis

Synthesis of Silver nanoparticles was carried out by the procedure formulated by Ponarulselvam *et al .*, (2012).

Plant material:

The *Ceropegia juncea* stem material is dried in shade, powdered and stored for further use.

Preparation of silver nanoparticles:

1 gram of dried powder material was taken and 1mM silver nitrate solution was added.

It is then kept in a water bath with temperature 50º-70º C for 20- 30 minutes.

Change of colour (dark brown) was noticed. It was then observed in UV-Vis spectroscopy at 280-580 nm w/l.

The bio-reduction of silver ions in solution was noticed at the absorption peak at 420 nm w/l.

The spectra were recorded at 2 min, 10 min, 20 min intervals for the confirmation of silver nanoparticles.

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The solution was then centrifuged at 12000 rpm for 15 min, the supernatant was discarded, the pellet was collected, it was then dried in hot air oven and the dried sample was used for SEM-EDX pictures.

SEM-EDX picture

The SEM picture of silver nanoparticle was taken from 500 nm magnifications the nanoparticles were spherical in shape and well defined.

The EDX data provided the elements present in silver nanoparticles of *C.juncea* plant.

Results and Discussion

Plant Tissue Culture

The combination of BAP, IBA and 2,4-D induced an excellent amount of massive callus from the nodal segments of *Ceropegia juncea* and the morphology of the callus was friable, yellowish green and greenish in colour and nodular in its nature (Table 1).

Callus initiation was observed in MS+ 2,4-D 1.5 mg / l or MS + BAP 1.5 mg /l, produced in MS +2,4-D + BAP 1.5mg / l+ 1.5 mg/ l combination. (Figure 1, d,e). MS + IBA 1.0 mg/l + BAP 1.0 mg/l + 2,4-D 0.5 mg/l produced the maximum number of massive calli induction (Figure 1, f) followed by MS + IBA 0.5 mg/l + 2,4-D 0.5 mg/l., MS+ IAA 1.0mg/l + BAP 1.0 mg/l., whereas MS + 2,4-D, MS +2,4-D + IAA, MS + IAA or MS + IBA, failed to induce a specific response. The maximum greenish nodular calli was induced in MS + BAP 1.5 mg/l + 2, 4-D 1.5 mg/l followed by MS + BAP 2.0 mg/l + 2, 4-D 2.0 mg/l combinations (Fig. 1, g). Callus induction in *Ceropegia* spp. has been studied by many workers *viz. C. jainii* and *C. bulbosa* (Patil, 1998), C. *candelabrum* (Beena et al., 2003), *C. pusilla* (Kondamudi et al., 2010) and *C. hirsute* (Nikam et al., 2008). In the present study the callus induction in *C. juncea* showed variations with the findings of those workers.

MS medium supplemented with BAP, Kin, IBA, IAA and NAA responded variable effects on shoot induction in nodal explants (Figure 1, h and Table 2). The maximum number of shoots was recorded on MS + BAP 3.0 mg/l + Kin 0.5 mg/l +.

Table 1. Callus induction from nodal explants of *C. juncea* cultured on MS medium supplemented with various concentrations of growth regulators

Concentrations of Plant Growth Regulators	No. of explants inoculated	No. of explants forming callus	Morphology of callus
MS + BAP 1.5 mg/l +2,4-D 1.5 mg/l	20	15±0.49	G, N, F
MS + BAP 2.0 mg/l +2,4-D 2.0 mg/l	20	10±0.99	G, N, F
MS + IAA 1.0 mg/l + BAP 1.0 mg/l	20	5±1.48	YG, M, F
MS + IBA 1.0 mg/l + BAP 1.0 mg/l	20	17±0.49	YG, M, F
+ 2,4-D 0.5 mg/l 2017±0.49YC, M, F			
MS + IBA 0.5 mg/l + 2,4-D 0.5 mg/l	20	10± 1.48	YG, M, F

F = Friable, G = Greenish, M = Massive, N = Nodular, YG = Yellowish Green Value represents means ± SD from 20 replicates.

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Table 2. Shoot induction from nodal explants of *C. juncea* cultured on MS medium supplemented with various concentrations of growth regulators

Concentrations of Plant Growth Regulators	No.of explants inoculated	No.of explants produced shoots	No. of shoots developed
MS + BAP 3.0 mg/l + Kin 0.5 mg/l + IAA 0.5 mg/l	20	4±0.49	1
MS + BAP 3.0 mg/l + Kin 0.5 mg/l + IBA 0.5 mg/l +NAA 0.5 mg/l	20	18±0.49	2
MS + BAP 3.0 mg/l + Kin 0.5 mg/l + IBA 0.5 mg/l	20	10± 1.48	2

Value represents means ± SD from 20 replicates

Table 3. Root induction from nodal stem explants of *C. juncea* cultured on MS medium supplemented with various concentrations of growth regulators

Concentrations of Plant GrowthRegulator	No. of shoot andclones inoculate	No. of clones produced roots	No. of roots Developed
MS + IAA 0.5 mg/l + NAA 0.5 mg/l + IBA 0.5 mg/l +BAP 1.0 mg/l	20	12 ± 0.99	2
MS + IBA 0.5 mg/l + NAA 0.5 mg/l + BAP 1.0 mg/l	20	6 ± 0.99	2

Value represents means ± SD from 20 replicates

IBA 0.5 mg/l + NAA 0.5 mg/l (Figure 1, i , j) followed by MS + BAP 3.0 mg/l + Kin 0.5 mg/l + IBA 0.5 mg/ l and MS + BAP 3.0 mg/l + Kin 0.5 mg/l + IAA 0.5 mg/l. The other combinations MS+ IAA 3.0mg/l+ Kin 0.5 mg/l and MS+ IBA 3.0 mg/l+ Kin 0.5 mg/l+ NAA 0.5 mg/l are not significant for shoot regeneration.

Similar observations were noticed in *Ceropegia hirsute* (Nikam et al., 2008) in which BAP concentrations showed vital role in shoot induction. The present results are in agreement with previous reports on *C. bulbosa* and *C. jainii*(Nikam et al., 2008) and reveal that the BAP alone can induce axillary shoot multiplication from nodal segments(Patil, 1998). On the other hand, a synergistic effect of a range of growth regulators in combinations with BAP for shoot regeneration was well documented for members of Asclepiadaceae *viz., C.* candelabrum(Beena et al., 2003), *Holostemma ada-kodien* (Martin, 2008), *Hemidesmus indica* (Sreekumar et al., 2000) and *Leptadenia reticulate* (Arya et al., 2003).

The different concentration and combination of IAA, NAA and BAP induced rooting. One week old regenerated shoots were transferred to the different combinations of rootingmedium MS + IAA 0.5 mg/l + NAA 0.5 mg/l + IBA 0.5 mg/l + BAP 1.0 mg/l was found to be best suited concentration and combination of growth regulators for root induction (Figure 1, k and Table 3) this is followed by MS + IBA 0.5 mg/l + NAA 0.5 mg/l + BAP 1.0 mg/l. The other combinations MS+ IAA 1.0mg/l., MS+ IBA 1.0mg/l and MS+ IBA1.0 mg/l+ NAA 1.0 mg/l did not respond for rooting.

The combination of BAP + IBA inducedrooting in *C. pusilla* (Kondamudi *et al.,* 2010). Highest rooting percentage was reported with higher

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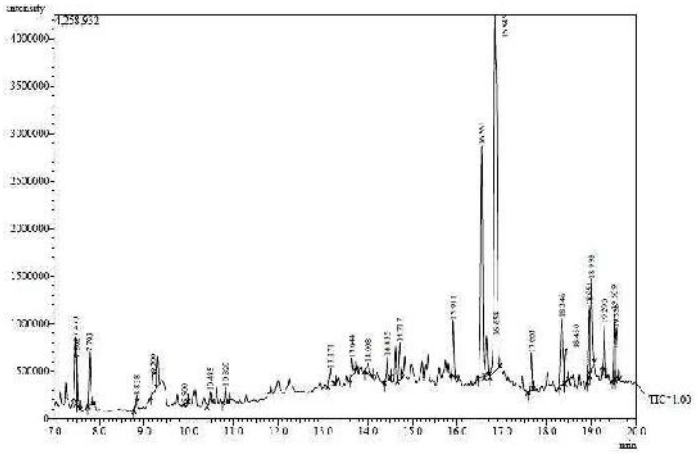
concentration of IAA 2.85-11.42 µm/l than NAA (2.69-10.74 µm/l) in *C. bulbosa* (Divya Goyal and Seema Bhadauria, 2003). However, the role of BAP + IBA in root induction is more significant from the present study as reported by earlier study (Kondamudi et al., 2010).

Thin layer chromatography & GC-MS

Compound identification was done using silica gel coated thin layer chromatography in water and methanol extracts. Light violet colour at visible light mode was present in the tracks of paper identified as compound in the sample**.**



Peak Report TIC

Peak#	R Time	I Time	F Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	7.470	7.425	7.492	1595075	1.60	680206	2.24	2.34		1,3-CYCLOHEXADIENE, 2-(
2	7.508	7.492	7.575	948575	0.95	490180	1.62	1.94	V	Sulfuric acid, diethyl ester
3	7.729	7.750	7.858	1294531	1.30	560866	1.85	2.31		BENZENE, METHYL(1-MET
4	8.838	8.775	8.858	2018619	0.20	94615	0.31	2.01	MJ	BENZENE, 1-METHYL-4-(1
5	9.220	9.158	9.283	1234953	1.24	209237	0.69	5.90		Levoglucosenone
6	9.900	9.892	9.983	-153412	-0.15	-2137	-0.01	0.00	MJ	Guaiol
7	10.482	10.408	10.525	325193	0.33	132226	0.44	2.44	MJ	ETHYL 2-ACETYLOCTANO
8	10.820	10.758	10.900	353898	0.36	167158	0.55	2.12	MJ	dienaldehyde, 2,4 camethyl
9	13.171	13.108	13.283	662666	0.66	182953	0.60	3.62	MJ	1 DODECANOL
10	13.644	13.600	13.742	507581	0.51	183854	0.61	2.68	MJ	TRICYCLO[4.4.0.0(2,7)]DE(
11	14.008	13.942	14.117	425830	0.43	112301	0.37	3.83	MJ	1E-CYCLOPROP[E]AZULEI
12	14.493	14.375	14.517	618064	0.62	160573	0.86	3.96	MJ	2-Isopropenyl-4a 8-dimethyl-
13	14.717	14.658	14.767	1139966	1.14	391630	1.29	2.91	V	ALPHA.-SELINENE
14	15.911	15.850	15.967	1367992	1.37	585609	1.93	2.34		1,1,4,7-TETRAMETHYLDEU
15	16.553	16.475	16.608	6899366	6.92	2537187	8.04	2.72		2-Naphthalenemethanol, 1,2,3
16	16.658	16.608	16.708	1108127	1.20	425277	1.40	2.82	V	NAPHTHALENE, 1,2,3,4,4a,
17	16.845	16.725	16.950	16012785	16.06	3756432	12.39	4.26		2-Naphthalenemethanol, decal
18	17.665	17.583	17.700	931504	0.93	392251	1.29	2.42	MJ	Benzenepropanamide, N-(2-b
19	18.346	18.233	18.400	2870087	2.88	697939	2.30	4.17		Benzene, 1-(1,1-dimethylethy
20	18.450	18.400	18.492	1703150	1.71	548329	1.81	3.61	V	2-Naphthalenemethanol, 1,2,4
21	18.951	18.908	18.967	1656655	1.66	750677	2.48	2.21		ROSIFOLIOL

GC-MS Spectroscopy

Antibacterial activity of *C.juncea* plant silver nanoparticle on Gram (+) bacteria in *ex situ and in vitro* condition

The antibacterial activity of silver nanoparticle on Gram (+) bacteria is presented in (Table-4) the antibiogram have shown the maximum inhibitory effect in *Ex situ* plant extract. In case of *ex situ* the maximum zone of inhibition 21.0 mm was exhibited by *Bacillus subtilis* and *Clostridium butyricum* followed by 19.0 mm for *Staphylococcus aureus.* In *in vitro* condition the antibacterial effect of silver nanoparticle was 14 mm for *C.butyricum* and 12.0 mm for *B.sutilis and S. aureus.*

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Table-4 Antibacterial activity of *C.juncea* plant silver nanoparticle on Gram (+) bacteria in *ex situ* condition

Bacterial strain (+)	C	C1	S (mm)	C.J	C.J Ag (N)
Bacillus subtilis	-	-	27.0	16.0	21.0
Clostridium butyricum	-	8.5	27.0	16.0	21.0
Staphylococcus aureus	-	8.5	27.0	16.0	19.0
Gram (+) bacteria in *in vitro* condition					
Bacillus subtilis	-	-	27.0	9.0	12.0
Clostridium butyricum	-	8.5	27.0	14.0	14.0
Staphylococcus aureus	-	8.5	27.0	12.0	12.0

Antibacterial activity of *C.juncea* plant silver nanoparticle on Gram (-) bacteria in *ex situ and in vitro* condition

The antibacterial effect of silver nanoparticle on Gram (-) bacteria is presented in (Table-5) the antibiogram have shown the maximum inhibitory effect in *ex situ* plant extract. In case of *ex situ* the maximum zone of inhibition 24.0 mm was exhibited by *Salmonella typhi* followed by 22.0 mm for *E.coli and E.aerogens In vitro* condition the antibacterial effect of silver nanoparticles was 16 mm for *E.coli and E.aerogens* and 15 mm for *S.typhi.*

Table-5 Antibacterial activity of *C.juncea* plant silver nanoparticle on Gram (-) bacteria in ex situ condition

.Bacterial strain (-)	C	C1	S (mm)	*C.J*	*C.J Ag (N)*
Escherichia coli	-	8.5	21.0	16.0	22.0
Entrobacter aerogens	-	10.0	24.0	16.0	22.0
Salmonella typhi	-	-	24.0	20,0	**24.0**
Gram (-) bacteria in *in vitro* condition					
Escherichia coli	-	8.5	21.0	9.0	16.0
Entrobacter aerogens	-	10.0	24.0	12.0	16.0
Salmonella typhi	-	-	24.0	15.0	**15.0**

In conclusion the present study reported successful callus, shoot and root induction protocol that can be employed in the *in vitro* propagation of rare medicinal plant *Ceropegia juncea* and helps in conservation and domestication, thereby minimizing the pressure on wild populations of the valuable medicinal plants of natural ecosystem offorest.

In conclusion, *C. juncea* stem extract can serve as a natural plant antioxidant to the food industry. In addition it can also be a potential antibacterial source and the bioactive compounds will find a place in the formulation of herbal medicine to treat bacterial mediated infections.

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