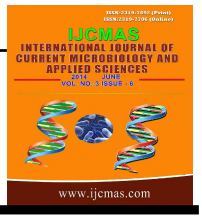
Int.J.Curr.Microbiol.App.Sci (2014) 3(6) 720-730

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 6 (2014) pp. 720-730**
http://www.ijcmas.com



Original Research Article

Evidence to prove why *Garcinia indica* Choisy leaves does not respond to hairy root induction by *Agrobacterium rhizogenes* mediated transformation along with positive antimicrobial activity

Geetika Kunder[1] and Varsha Parasharami[2]*

[1]Transient researcher, M.Tech (Integrated) Biotechnology, Padmashree Dr. D. Y Patil University, Department of biotechnology and bioinformatics, Cbd-Belapur, Navi Mumbai, India
[2]Senior Scientist, Plant Tissue Culture Division, CSIR-National Chemical Laboratory, Pune, India
**Corresponding author*

Keywords

Garcinia indica, *A.rhizogenes* transformation, Antimicrobial activity, Zone of inhibition

A B S T R A C T

Garcinia indica Choisy has many medicinal properties including anti-microbial, anti-cancer, anti-salmonella, anthelmintic, anti-ulcer, antacid, anti-ageing, anti-inflammatory, anti-oxidant and many others. Hairy root induction in any plant gives better chance of isolation of bioactive compound from that species. Hence, we have performed many *Agrobacterium rhizogenes* transformation experiment with the *in vitro* induced leaves of *Garcinia indica.* None of the leaves at any parameter showed the induction of the hairy roots. So the antimicrobial activity of the *in vitro* and *in vivo* leaves extract was checked by well diffusion method against three pathogenic bacterial species including activity against *A. rhizogenes* culture. All the microorganism including *A. rhizogenes* showed zone of inhibition which proves the antimicrobial activity of *G.indica*. The zone of inhibition obtained for both *in vitro* and *in vivo* leaf extracts were comparable against all microorganisms including *A. rhizogenes.* This proves the cause for nonresponsive nature of this plant for *A. rhizogenes* transformation.

Introduction

Agrobacterium rhizogenes mediated transformation system was found to be very useful in metabolic engineering (Hamill and Lidgett, 1997; Rao and Ravishankar, 2002), genetic manipulation of plants for the production of phytochemicals such as enzymatic pathways, key intermediates and critical regulation points and production of transgenic herbals (Fu *et al.*, 2006; Piatczak *et al.*, 2006; Shanks and Morgan, 1999), large scale secondary metabolite production (Choi *et al.*, 2000), monoclonal antibody production (Wongsamuth and Doran, 1997) and phytoremediation (Nedelkoska *et al.*, 2000). There are many reports that suggest the successful use of *A.rhizogenes* harbouring binary vectors with desired gene construct (Christey,

2001) for plant genetic transformation. The success of *A.rhizogenes* mediated transformation depends upon various parameters such as the species and the age of the plant tissue, density of the bacterial suspension (Park and Facchini, 2000).

Garcinia indica Choisy (Kokam) belonging to family Clusiaceae is an underexploited spice tree. It is a slender evergreen and polygamodioecious tree with dropping branches which attains pyramidal shape on maturity. In India, it is mostly found in Konkan region of Maharashtra, Goa, Karnataka, Kerala and Surat district of Gujarat on the West Coast of India and to some extent in the forests of Assam, Meghalaya, and West Bengal (Chandran, 1996).

Various parts of the plant are used in food preparations or for its medicinal properties. The bark is astringent and used for making vinegar (Watt, 1972). Young leaves are acidic in nature and are used in food preparations containing components similar to fruit (Rao, 1987). The leaves are also known to have anti-salmonella activity. The seeds of the tree contain fat and are used to make kokum butter which is proven to have sunprotective activity. The fruit rind is a rich source of phytochemicals making it medicinally important.

Kokam is traditionally been known in Ayurveda and used for treating various ailments such as allergic rashes, burns, chaffed skin and scalds; provide relief from sunstroke; tackle dysentery and mucus diarrhea; improve appetite and quench thirst; treat bleeding piles, tumors and heart problems and as a tonic for the heart and liver (Miguel et al, 2012).

All the parts of this plant are known to possess medicinally important properties. The fruit and fruit rind is widely used and its extracts are reported to possess anti-microbial activity against *Escherichia coli*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Bacillus megaterium* and *Pseudomonas aeruginosa* (Varalakshmi et. al., 2010; Ghosh et. al., 2012). The stem bark crude, flavononlyflavone and proauthocyanin fraction extracts shows anti-microbial activity against *Staphylcoccus aureus*, *Escherichia coli* and *Salmonella typhi*.

The leaves of this plant contain constituents similar to fruits. Earlier report suggests the use of *in vitro* leaves of *G. indica* for plant regeneration through somatic embryogenesis and organogenesis (Thengane et. al., 2006; Devendra et. al., 2012). Chand et. al., in 2009 reported the medicinal use of the *in vivo* leaves of this plant as anti-salmonella activity against *Salmonella typhi*, *Salmonella paratyphi* A and *Salmonella typhyrium*.

The repeated attempts to induce hairy roots on *in vitro* leaves led to the present study to identify the cause for the non-responsive behaviour of the *in vitro* leaves for hairy root induction. Hence, *Garcinia indica in vivo* and *in vitro* leaf aqueous and methanol extract was evaluated for their anti-microbial activity.

Materials and Methods

Agrobacterium rhizogenes mediated transformation

Initial plant material

The transformation was performed using leaves obtained from *in vitro* raised shoot cultures. The mature shoots were maintained in culture for last four years with regular subculturing to fresh

multiplication medium containing WPM basal media with BAP 8.88 μM + Sucrose 3% w/v and solidified with Defco Agar 0.7% w/v. In total more than 500 *in vitro* leaves were used including for all the experiment of hairy root induction. For Antimicrobial activity, *in vivo* extracts were prepared from leaves which were freshly collected from Sarole village in Mulshi District 90 Km from Pune city (MS, India). For *in vitro* extracts leaves from the *in vitro* raised shoots from 2-3 culture vessels (bottles) were used.

Nutrient media:

The following nutrient media were used in the experiments:

1. **Yeast Mannitol Broth (YMB) media**

   For cultivation of *A. rhizogenes*, One litre of media contains 10 g/L Mannitol, 1 g/L Yeast extract, 0.5 g/L Potassium, 0.2 g/L Magnesium, 0.1 g/L Sodium, 1 g/l Calcium, pH 7.0 – 7.2, liquid medium was used.

2. **Woody Plant medium (WPM):**

   WPM (Lloyd and McCown 1980) enriched with 30 g/L sucrose and 7 g/L agar-agar for co-cultivation of the leaf explants without any hormone was used.

***Agrobacterium rhizogenes* Activation**

The *Agrobacterium rhizogenes* (ATCC 15834) cultures were revived by inoculating a single bacterial colony into 15-20 ml sterile YMB in tubes. These inoculated culture tubes were then kept on a shaker at 140 rpm overnight. Bacterial density was determined spectro-photometrically (UV-450 Shimadzu, Japan) by measuring the optical density at 600 nm wavelength.

Preparation of the explants and infection:

Minimum 100 *in vitro* grown leaves were used for each experiment. The *in vitro* leaves were obtained fresh on the day of the experiment. Almost 300 to 400 healthy fully elongated leaves could be collected from each culture bottle. With the help of a sterile blade tip, the leaves were injured by pricking along their midrib, petiole and lamina. The bacterial cultures of different optimum optical density (0.5-1.2) were poured over these prepared explants till they were submerged for time periods of 5h, 7h and overnight. *Nicotiana tabaccum* invitro leaves were used as control for which O.D. of 0.7 and infection time of 5 hr was chosen and prepared in a similar way. At least two parameters were done in one day and each experiment was repeated 3 times before making final conclusions.

Co-cultivation and Transformation:

After the completion of variable infection period at different Optical Density (Table 1), the leaves were shifted upon sterile filter papers to soak off the excess bacterial culture until the leaves were dry, and some leaves were desiccated. Then these leaves were shifted to WPM plain medium for a period of 24 h and 48 h for co-cultivation with the bacterium. After the completion of co-cultivation period, the leaves were shifted to WPM containing 500 mg/L of Cefotaxim to suppress excess bacterial growth. Acetosyringone (Sigma, USA) which is known to help in increasing transformation efficiency was also tried by addition into

the co-cultivation medium in the range of 50-150 µM. The acetosyringone was added in cocultivation medium for 24 hrs in all the experiments as shown in Table 1.

Data scoring

Following incubation on antibiotic containing WPM, the leaves were regularly checked for bacterial growth and contamination. Regular shifting of the leaves was done and they were also observed for induction of hairy roots. No statistical analysis has been done as all the results were negative.

Antimicrobial Activity

Preparation of Plant Material

Fresh leaves collected as mentioned earlier were used. The collected leaves were washed, shade dried and converted into moderately coarse powder by mixer grinder. While the *in vitro* leaves were crushed into fine paste.

Preparation of Aqueous and Methanol Extracts

20 gm of the powdered *in vivo* leaf and *in vitro* leaf fine paste was extracted using 100 ml each of distilled water and methanol separately in 250 ml sterile conical flasks. These flasks were placed on shaker at 90 rpm for 72 hours. After 72 hours each of the extracts was filtered using a Whatman filter paper#1 (90 mm circles), the process was repeated 3 more times in next 72 hrs with both methanol and aqueous extracts. These filtrates were then concentrated in vacuum (Speed vac-Eppendorf Concentrator plus) at 45°C and stored at 4° C till further use.

Microorganisms Tested

The following microorganisms were used in the present studies which were procured from National Collection of Industrial Micro-organisms (NCIM), CSIR-NCL, PUNE excluding *Agrobacterium rhizogenes:*

1) *Staphylococcus aureus*: NCIM 2065
2) Escherichia coli : NCIM 2200
3) Pseudomonas aeruginosa: NCIM 5021
4) Agrobacteuim rhizogenes : ATCC 15834

Bacteria were grown on Nutrient Broth at 37°C for 24 hours before use.

Antimicrobial Activity

The modified agar well diffusion method (Perez *et al.*, 1990) was employed. Nutrient agar was used for bacteria. Once the agar was solidified, 50 µl of the different bacterial cultures were spread onto the plates using a sterile glass spreader. The plates were punched with six millimeter diameter wells and filled with 25 µl of the plant extracts and blanks (distilled water and methanol which were used as blank and served as the negative control). Simultaneously, Ampicillin (100µg/ml) was used as positive controls for bacteria. The tests were carried out in triplicates. The bacterial plates were incubated at 37°C for 24 hrs. The diameter of the zone of inhibition was measured in millimetres ruler scale after 24 hrs for all the bacteria.

Results and Discussion

Even after repeated attempts to induce hairy roots in *in vitro* leaves of *Garcinia indica* by *A. rhizogenes* mediated transformation, the *in vitro* leaf showed unresponsiveness.

Different optical density of *A. rhizogenes* cultures, soaking, desiccation, infection period, acetosyringone addition in the co-

cultivation medium and co-cultivation duration was also attempted as shown in Table.2 to exclude the possibility of any other reason.

The *in vitro* grown leaves *of Nicotiana tabaccum* which were used as control have shown the induction of hairy roots within 20 days after infection (Figure 1). But none of the parameters could induce hairy roots on *in vitro* leaves of *Garcinia indica* (Figure 1). The antimicrobial activity of the *in vivo* and *in vitro* leaf extracts (Aqueous and methanol) was screened against *A. rhizogenes*. It was found that *G. indica in vivo* and *in vitro* leaf possessed antimicrobial activity against *A. rhizogenes* (Figure 1. i). Since, it showed antimicrobial activity against *A. rhizogenes*, these extracts were tested against other pathogenic microorganism namely *E. coli, P. aeruginosa* and *S. aureus*. These are most common bacteria which have been reported to be involved in human infections. The results obtained were noteworthy and interesting. Both *in vivo* and *in vitro* leaf extract possessed antimicrobial activity against the three pathogenic microorganisms (Figure 2 ii; iii; iv).

The measured zone of inhibition as seen in Figure 2 and 3 against the microorganisms in the study after 24 hours for *in vivo* and *in vitro* aqueous and methanol leaf extract is presented in the Table 3 in mm. In order to compare the results between the *in vivo* and *in vitro* leaf extracts graphs were plotted as given below in Figure 4 and Figure 5.

The results from the present study revealed the possible cause for the unresponsiveness of *G. indica in vitro* leaves to *A. rhizogenes* transformation is its antimicrobial activity against the organism. This conculsion was drawn after repeated attempts of *A. rhizogenes* mediated transformation. All the parameters of the experiments were varied such as optical density, infection period, acetosyringone addition in medium and co-cultivation duration. Still there was no induction of the hairy roots. It also revealed that the *in vivo* and *in vitro* leaf extracts possess antimicrobial activity against three pathogenic microorganism namely *E. coli, P. aeruginosa* and *S. aureus.*

The antimicrobial activity was screened by well diffusion method and compared to standard antibiotic drug Ampicillin (100 µg/ml). Potent antimicrobial activities were recorded with all the four bacterial species. The Aqueous *in vivo* and *in vitro* leaf extracts showed 16.3 mm and 12.1 mm zone of inhibition against *A. rhizogenes*, 17 mm and 15.1 mm against *E. coli*, 18.7 mm and 14 mm against *P. aeruginosa* and 17.3 mm and 17 mm aginst *S. aureus* respectively. Similarly, methanol *in vivo* and *in vitro* leaf extracts showed 20.6 mm and 18.1 mm zone of inhibition against *A. rhizogenes*, 18 mm and 16.3 mm against *E. coli*, 22 mm and 20.2 mm against *P. aeruginosa* and 20.6 mm and 18 mm aginst *S. aureus* respectively. These results are comparable showing that *in vitro* leaves posses antimicrobial activity similary to the *in vivo* leaves as shown in figure-4 and figure-5.

Earlier reports on *G. indica* show proof of the antimicrobial activity of fruits and fruit rinds against various pathogenic microorganisms. Aqueous extract of the fruit rind shows antimicrobial activity against *Escherichia coli, Bacillus subtilis* and *Enterobacter aerogenes* and *Staphylococcus aureus* with minimum inhibitory concentrations of 0.5 mg/ml, 5

mg/ml, 5 mg/ml and 50 mg/l respectively (Varalakshmi et al, 2010**).** Similarly, in another report Ghosh *et. al.* suggested that aqueous extract of the fruit rind exhibits a zone of inhibition of 7 mm against *Micrococcus aureus*, 9 mm zone of inhibition against *Bacillus megaterium* and 13 mm zone of inhibition against *Pseudomonas aeruginosa*. Also, ethanol and methanol extract of the fruit rind shows a zone of 10 mm against *Micrococcus aureus*, 8 mm and 10 mm zone against *Bacillus megaterium* and 11 mm and 16 mm zone of inhibition against *Pseudomonas aeruginosa* (Ghosh *et al.*, 2012). Not only fruits extract but also stem bark crude, flavononlyflavone and proauthocyanin fraction extracts have antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* (Lakshmi *et al.*, 2011). Also, the leaves of the plant report to possess anti-salmonella activity against three Salmonella species i.e. *Salmonella typhi, Salmonella paratyphi* A, and *Salmonella typhimurium.* The aqueous extract of *Garcinia indica* shows activity against all the three Salmonella species with zone of inhibition of 25 mm. While the methanolic extract shows zone of inhibition of 22 mm against *Salmonella typhi* and *Salmonella paratyphi A* and a zone of inhibition of 20 mm against *Salmonella typhimurium* (Chand *et al.*, 2009). These results for the anti-salmonella activity and anti-microbial activity of the fruits are comparable to the results obtained during present study.

The result of the present study provides proof that *G. indica in vivo* and *in vitro* leaves possess potent antimicrobial activity. This is the cause leading to hindrance in hairy root induction on the leaves of this specific plant. Even though the cause is identified the active molecule responsible for such an activity requires further identification and isolation. Also the exact mechanism by which hairy root induction is inhibited needs to be identified.

Figure.1 Absence of hairy root induction on *In vitro* infected leaves of *Garcinia indica* and positive control *Nicotiana tabaccum* with hairy roots

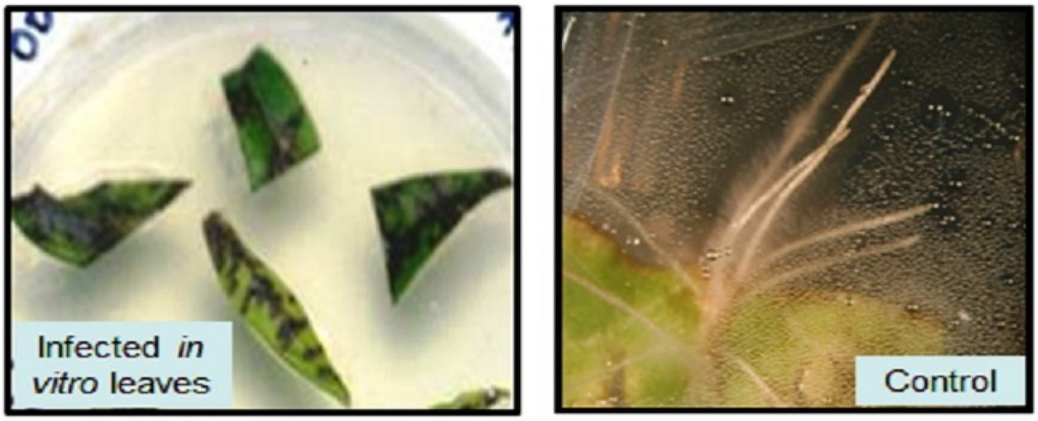


Figure.2 (i) Antimicrobial activity against *A. rhizogenes*; (ii) Antimicrobial activity against *E.coli*; (iii) Antimicrobial activity against *P. aeroginosa* and (iv) Antimicrobial activity against *S. aureus* for the *in vivo* and *in vitro* aqueous leaf extract in A and B respectively

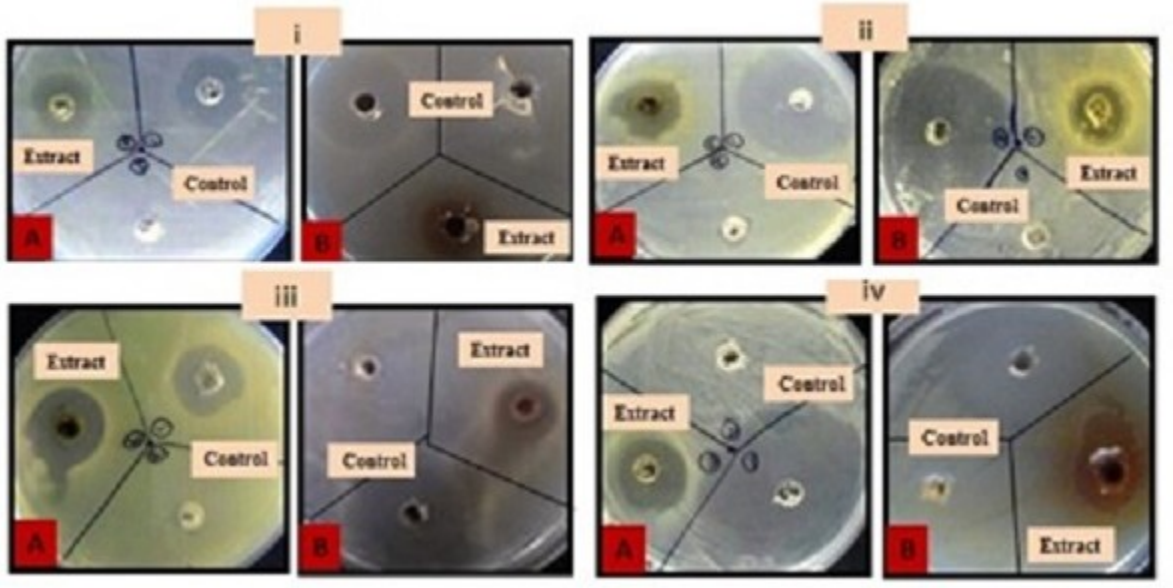


Figure.3 (i) Antimicrobial activity against *A. rhizogenes*; (ii) Antimicrobial activity against *E. coli*;(iii) Antimicrobial activity against *P. aeroginosa* and (iv) Antimicrobial activity against *S. aureus* for the *in vivo* and *in vitro* methanol leaf extract

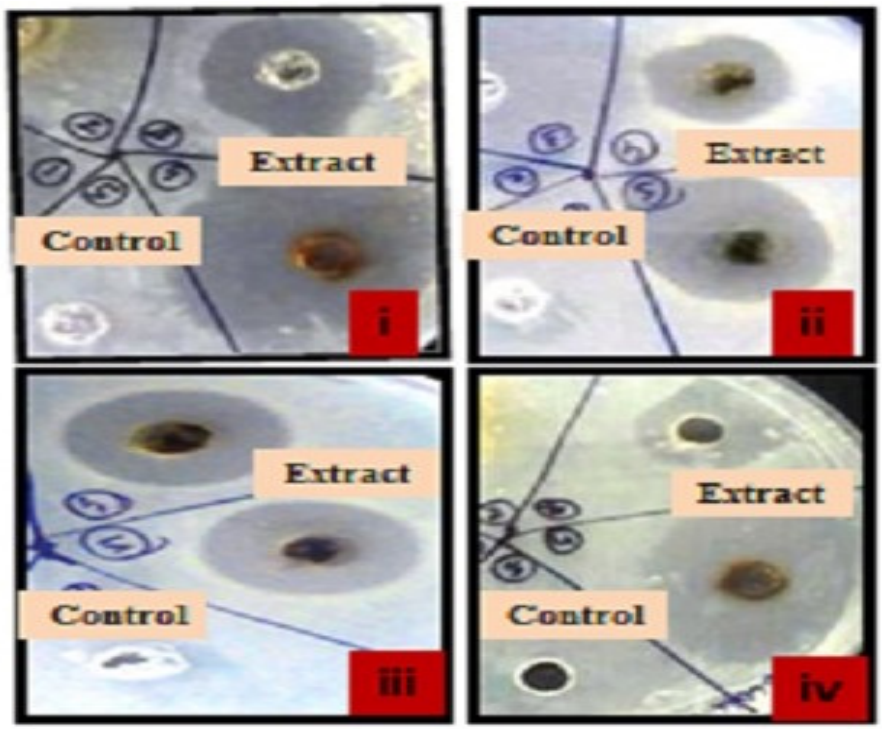
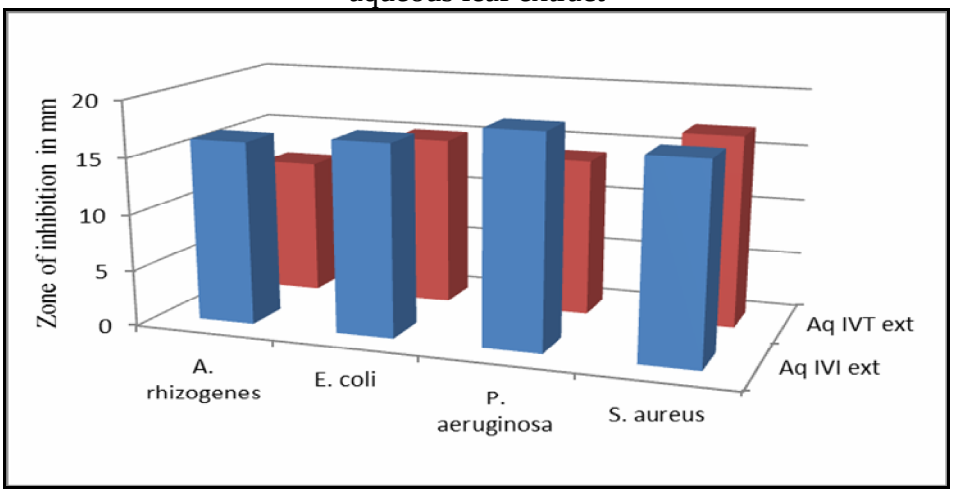
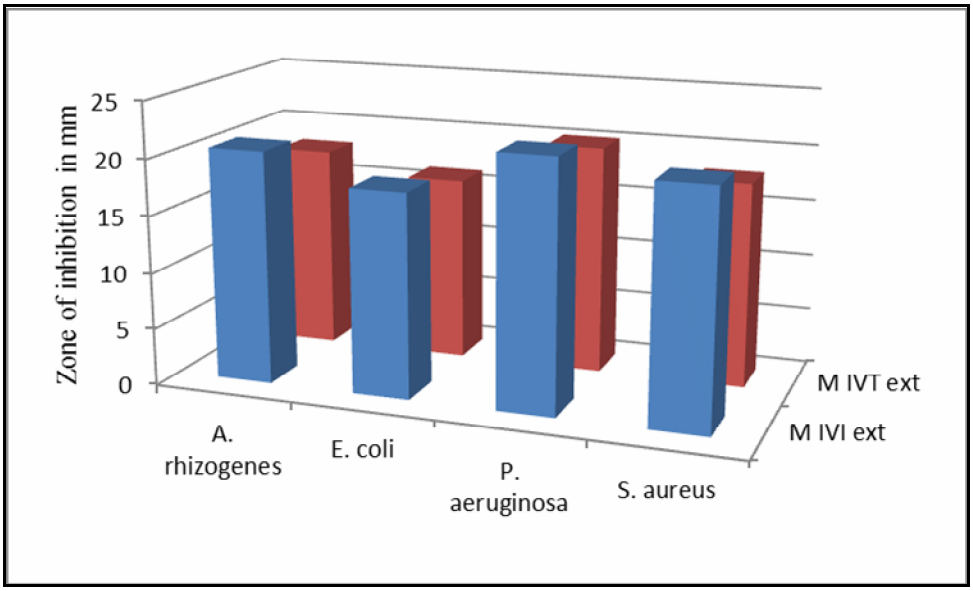


Figure.4 Comparison between Antimicrobial activity of *Garcinia indica in vivo* and *in vitro* aqueous leaf extract



(Aq IVT ext- Aqeous *in vivo* extract; Aq IVI ext- Aqueous *in vitro* leaf extract)

Figure.5 Comparison between Antimicrobial activity of *Garcinia indica in vivo* and *in vitro* aqueous leaf extract



(M IVT ext- methanol *in vivo* extract; M IVI ext- Methanol *in vitro* leaf extract)

Table.1 *A. rhizogenes* Transformation parameters for *in vitro* leaves of *G. indica* Choisy

Parameters	**Values**				
Optical Density (OD)	0.5	0.7	0.9	1	1.2
Infection period (hours)	5		7	Overnight (15h to 17h)	
Co-cultivation period (hours)	24+ Acetosyringone		48		

Table.2 *Agrobacterium rhizogenes* mediated transformation parameter details

Sr. No.	**Optical Density**	**Co-cultivation in hours**	**No of explants**	**Infection period (hours)**	**Observation**
1.	0.5	24 + Acetosyringone	18	5	-
			14	7	-
			18	Overnight	-
2.	0.5	48	16	5	-
			14	7	-
			20	Overnight	-
3.	0.7	24 + Acetosyringone	15	5	-
			18	7	-
			15	Overnight	-
4.	0.7	48	20	5	-
			10	7	-
			15	Overnight	-
5.	0.9	24 + Acteosyringone	19	5	-
			15	7	-
			20	overnight	-
6	0.9	48	18	5	-
			19	7	-
			15	overnight	-
7	1	24 + Acetosyringone	17	5	-
			22	7	-
			13	overnight	-
8	1	48	12	5	-
			21	7	-
			19	overnight	-
9	1.2	24 + Acetoosyringone	16	5	-
			21	7	-
			17	overnight	-
10	1.2	48	16	5	-
			20	7	-
			14	overnight	-

Table.3 Antimicrobial activity of *in vivo* and *in vitro* leaf extracts of *Garcinia indica*

Zone of Inhibition in mm					
Microorganisms	*In vivo* leaf extract		*In vitro* leaf extract		Control Ampicillin (100µg/ml)
	Aqueous	Methanol	Aqueous	Methanol	
A. rhizogenes	16.3	20.6	12.1	18.1	30.2
E. coli	17	18	15.1	16.3	27
P. aeruginosa	18.7	22	14	20.2	26
S. aureus	17.3	20.6	17	18	32.7

Acknowledgement

Authors would like to thank CSIR-NCL in house funding committee for funding. The Director CSIR-NCL, Pune and Head of the Department, Padmashree Dr. D. Y Patil University, Department of Biotechnology and Bioinformatics, Cbd-Belapur, Navi Mumbai, for permission to work in NCL for first author.

References

Chand, P., S. Sayeed, Md. A. Sadath and Ziaullah, Md. K. 2009. Antisalmonella Activity of Selected Medicinal Plants. Turk J Biol. 33: 59-64.

Chandran, M. D. S., 1996. Nature watch. The Kokum tree. Resonance. 1: 86–9.

Christey, M. C, 2001. Use of Ri-mediated transformation for production of transgenic plants. *In Vitro* Cellular and Development Biology-Plant. 37: 687-700.

Devendra, C. T., A. K. Dass, A. Lima, and Malik, S. K. 2012. Direct organogenesis from leaf explants of *Garcinia indica* Choisy: An important medicinal plant. Indian Journal of Biotechnology. 11: 215-219.

Fu, D.Q., B. Z. Zhu, H. L. Zhu, W. B. Jiang and Luo, Y. B. 2006. Virus-induced gene silencing in tomato fruits. Plant J. 31: 299–308.

Lakshmi, C., K. K. Akshaya , T. J. Dennis, and Sanath, K. T. S. S. P. N. S. 2011. Antibacterial Activity of Polyphenols of *Garcinia indica*. Indian J. Pharm.Sci. 73: 470-473.

Miguel B., A. Shirodkar, D. B Jayarama and Krishnan, S. 2012. Resource book on Kokum (*Garcinia indica* Choisy). Pp: 7-8.

Nedelkoska, V. Tatjana, and Doran, P. M. 2000. Hyperaccumulation of cadmium by hairy roots of *Thlapsi caerulescens*. Biotechnology and Bioengineering. 67: 607-615.

Park, S.U. and Facchini, P.J. 2000. High - efficiency somatic embryogenesis and plant regeneration in California poppy, *Eschscholzia californica* Cham. Plant Cell Reports. 19: 421–426.

Perez, C., M. Pauli and Bazevque, P. 1990. An antibiotic assay by the agar well diffusion method. Acta Biologiae et Medicine Experimentalis. 15: 113-115.

Piatczak, E., A. Krolicka and Wysokinska, H. 2006. Genetic transformation of *Centaurium erythraea* Rafn by

Agrobacterium rhizogenes and the production of secoiridoids. Plant Cell Rep. 25: 1308–1315.

Rao, R. M., 1987. Flowering Plants of Travancore. India International Book Distributors.

Rao, S. R., and Ravishankar, G. A. 2002. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances. 20: 101-153.

Shailendra, R., D. Shailesh, R. Shruti, K. Rashi, B. Deepti, D. Ajit, K. Jitendra, R. Pratap, K. Manasi, and Komal, B. 2013. Identification of antibacterial component from extracts of *Garcinia indica* fruit rinds using LC/MS/MS. Shimadzu 1: 1-5.

Shanks, J.V., and Morgan, J. 1999. Plant hairy root culture. Curr. Opin. Biotechnol. 10: 151-155.

Sutar, R. L., S. P. Mane and Ghosh, J.S. 2012. Antimicrobial activity of extracts of dried kokum (*Garcinia indica* C). International Food Research Journal. 19: 1207-1210.

Thengane, S. R., S. R. Deodhar, S. V. Bhosale, and Rawal, S. K. 2006. Repetitive somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss. In vitro Cellular & Developmental Biology-Plant. 42:256-261.

Varalakshmi, K. N., C. G. Sangeetha, A. N. Shabeena, S. R. Sunitha and Vapika, J. 2010. Antimicrobial and Cytotoxic effects of *Garcinia indica* Fruit Rind Extract. American-Eurasian J. Agric. & Environ. Sci. 7: 652-656.

Vinod, K., S. Ashwani, C. N. P. Bellur, B. G. Harishchandra and Gokare, A. R. 2006. *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone Treatment. Electronic journal of biotechnology. 9: 349-357.

Watt, G., 1972. Dictionary of the Economic Products of India. Delhi, Periodical Expert.

Wongsamuth, R., and Doran, P. M. 1997. Production of monoclonal antibodies by tobacco hairy roots. Biotechnology and Bioengineering. 54: 401–415.