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An improved protocol for inducing hairy roots in *Vitex negundo* using *Agrobacterium rhizogenes*

Bhaswatimayee Mahakur[1], Aryaputri Bedaprabha[1], Sandeep Kumar Mohanta[1], Sanjay Kumar Madkami[1], Debasis Mitra[2], Arpita Moharana[3], Swasti Swagatika Swain[4], Durga Prasad Barik[1]*

[1] Ravenshaw University, Department of Botany and Biotechnology, Cuttack, Odisha, India
[2] Graphic Era (Deemed to be University), Department of Microbiology, Uttarakhand, India
[3] Indian Council of Agricultural Research, National Rice Research Institute, Cuttack, Odisha, India
[4] Jatiya Kabi Bira Kishor Das Government College, Department of Botany, Cuttack, Odisha, India
*Corresponding author. E-mail: dpbarik@ravenshawuniversity.ac.in

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Abstract

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Vitex negundo L. contains numerous secondary metabolites in its roots. This experiment aimed to induce the growth of hairy roots that accumulate large quantities of secondary metabolites. The *Agrobacterium rhizogenes* (MTCC 532) strain was used to induce hairy roots in *Vitex negundo* explants. The transformation frequency was increased by adjusting the infection time, as well as the concentrations of the bacterial suspension and acetosyringone, and the co-cultivation period. *In vitro* leaf explants were collected from two-month-old mature shoot cultures, which exhibited the highest transformation frequency of 63.33%. Of the five infection durations and three acetosyringone concentrations tested, the highest transformation frequency was achieved with a 45-minute infection and 100 µM acetosyringone, respectively. A two-day co-cultivation period was found to be appropriate, and the explants were inoculated in hormone-free Murashige and Skoog medium to promote the spontaneous development of hairy roots.

Keywords: acetosyringone, *Agrobacterium rhizogenes*, secondary metabolites, *Vitex negundo*.

INTRODUCTION

India has a diverse range of medicinal plants that can be used as natural sources of products for maintaining human health. Many formulations containing these plants have been used to treat various diseases (Gade et al., 2023). *Vitex negundo* (Lamiaceae) is widely cultivated for commercial purposes due to its therapeutic properties. It is an aromatic, woody shrub that can grow into a small tree, and is most frequently found in tropical Africa, Sri Lanka, the Philippines, Burma, China, Pakistan, and India (Rana & Rana, 2014; Vasanthi et al., 2014). Its fruits, seeds, leaves, and roots have all been thoroughly studied and reported to have hepatoprotective, antioxidant, anti-inflammatory, and anticancer properties. *Vitex negundo* is used in homoeopathic and naturopathic pharmaceutical formulations, which are widely used to treat human diseases (Maurya & Rao, 2019; Nsenga Nkulu et al., 2022). *Vitex negundo* also influences

the behaviour and physiology of insects (Haridasan et al., 2017).

The roots of *Vitex negundo* contain a variety of significant secondary metabolites, including phytoene, vitamin A, acetyl oleanolic acid, sitosterol, vitexoside, agnuside, dibutyl malate, and vitexin. These metabolites exhibit various beneficial properties, including diuretic, antioxidant, anti-inflammatory, antifungal, anticancer and antimalarial activities (Kumar et al., 2022; Gade et al., 2023; Najafipour et al., 2023). Unlike the leaf extract, the methanolic root extract contains higher levels of flavonoids and glycosides (Gayathridevi et al., 2022).

The biotechnological synthesis of high-quality compounds is a suitable alternative to conventional cultivation. *In vitro* plant cultivation is often employed to enhance the production of bioactive compounds (Zhou et al., 2011). One such method involves developing hairy roots by infecting explants with *Agrobacterium rhizogenes* and subsequently transferring the Ti plasmid of T-DNA to the explants. By contrast, *in vitro* cultures of conventional roots grow slowly and require the addition of growth regulators. The interaction between the host plant and the gram-negative soil bacterium *Agrobacterium rhizogenes* leads to the development of hairy roots. "Hairy roots" then appear at the site of infection. These transformed hairy roots can grow indefinitely in a growth culture medium without the addition of growth hormones (Zhou et al., 2011). Hairy roots are characterised by their high growth rate in minimal medium, biochemical and genetic stability, high branching, lack of geotropism and ability to produce a considerable number of bioactive compounds, independent of seasonal changes. Using transformed roots also enables the successful cultivation of large-scale cultures in bioreactors (Grzegorczyk-Karolak et al., 2018; Rezazadehfar et al., 2024).

Due to the significant role played by *Vitex negundo* roots in various therapeutic formulations, it is crucial to establish a method that can also be employed in the production of other bioactive compounds, such as sitosterol, vitexoside, agnuside and vitexin. Based on previous data, this study aimed to develop a hairy root induction technique to increase the production of key bioactive compounds and prevent the overharvesting of *Vitex negundo* in its natural habitats.

MATERIALS AND METHODS

In vitro shoot proliferation

The *Vitex negundo* plant was brought to the experimental garden at Ravenshaw University in Cuttack, Odisha, India, from the Athagarh village area in Cuttack. After being surface-sterilised with running tap water for 30 minutes, the *Vitex negundo* nodal explants were treated with 2% Teepol (Reckitt Benckiser Limited, India) for 10 minutes, followed by a further 10 minutes with 2% Bavistin (Antracol fungicide, Bayer, Crystal Crop Protection Ltd., India). In the final stage of surface sterilisation, the explants were immersed in a solution of 0.1% $HgCl_2$ (Merck, India) for seven minutes. The nodal explants were inoculated on four concentrations of Murashige & Skoog's (1962) medium (MS (100%), 1/2MS (50%), ¼ MS(25%), 1/8MS (12.5%)) either on their own or in combination with various concentrations (0.5–3.0 mg/L) of benzylaminopurine (Hi Media, India) to promote the growth of multiple shoots. For *Agrobacterium rhizogenes* infection, well-developed *in vitro* shoots were used as a source of explants (leaf and internode).

Maintenance of bacterial culture

A nutrient broth with a pH of 7.4 ± 0.2 (Hi Media, India) at a concentration of 1.3 g/100 mL was used to revive the *Agrobacterium rhizogenes* strain MTCC 532 (CSIR-IMTECH, MTCC, Chandigarh). The temperature was maintained at 26 ± 1 °C for 24 to 48 hours inside the incubator (Shaker and Incubator, N-Biotek, NB 205 QF) to promote bacterial growth. To induce hairy roots, the MTCC 532 bacterial culture was incubated for 24 to 48 hours, maintaining an optical density between 0.06 and 1.1. Once revived in nutrient broth, the culture was stored in a refrigerator at 4–5 °C on a nutrient agar medium (1.3 g/100 ml nutrient broth and 1.8 g/100 ml bacteriological-grade agar (Hi Media, India)) for later use. The collection, establishment, and *in vitro* shoot proliferation of *Vitex negundo* plants, along with the maintenance of *Agrobacterium rhizogenes* used in this study (Fig. 1B), have been previously described by Mahakur et al. (2024).

Transformation and induction of hairy roots in the internode and leaf

Well-developed *in vitro* shoots were used as a source of *in vitro* explants (leaves and internodes) for *Agrobacterium rhizogenes* infection for transformation, and hairy root induction experiments (Fig. 1A). *In vitro* shoots were collected from two-month-old shoot cultures and small internode portions (1.0–1.5 cm in length) were cut from them. Similarly, *in vitro* leaves were cut into small pieces of approximately 1 cm long and 0.5 cm wide. Both internode and leaf explants were then pierced using a sterile needle. The pierced leaf and internode explants were then dipped in a mixed solution of Murashige and Skoog liquid medium, MTCC 532-grown suspension culture and acetosyringone (Hi-Media, India). Acetosyringone was used at three different concentrations: 50 µM, 100 µM and 150 µM. The explants were dipped for various periods of time (15, 30, 45, 60, and 75 minutes). After the infection period, the explants were dried with sterile tissue paper and then inoculated onto 0.6% agar-gelled Murashige and Skoog (0) medium (Hi-Media, India; pH 5.8 ± 0.01) (Fig. 1, D and E). The co-cultivation period was maintained for two days at 26 °C inside an incubator. After co-cultivation, cefotaxime (100–200 mg/L) was used to remove any excess bacterial growth. The explants were then transferred to new flasks containing 30 ml of Murashige and Skoog (0) medium and stored in a dark environment at 25 ± 1 °C.

Growth parameters measurement

The induction of hairy roots was measured by calculating the transformation frequency, which was determined by dividing the number of explants that induced hairy roots by the total number of explants infected by *Agrobacterium rhizogenes*. The induced hairy roots from the explants were cut and transferred to a 100 ml flask containing 50 ml of Murashige and Skoog (0) liquid medium. The flask was then placed in a dark location inside the culture room and kept at 25 ± 1 °C to allow the roots to grow.

Statistical analysis

The experimental data are represented as the mean ± standard error (SE) of three separate experiments and were analysed using the Kruskal-Wallis test at a significance level of $p < 0.05$.

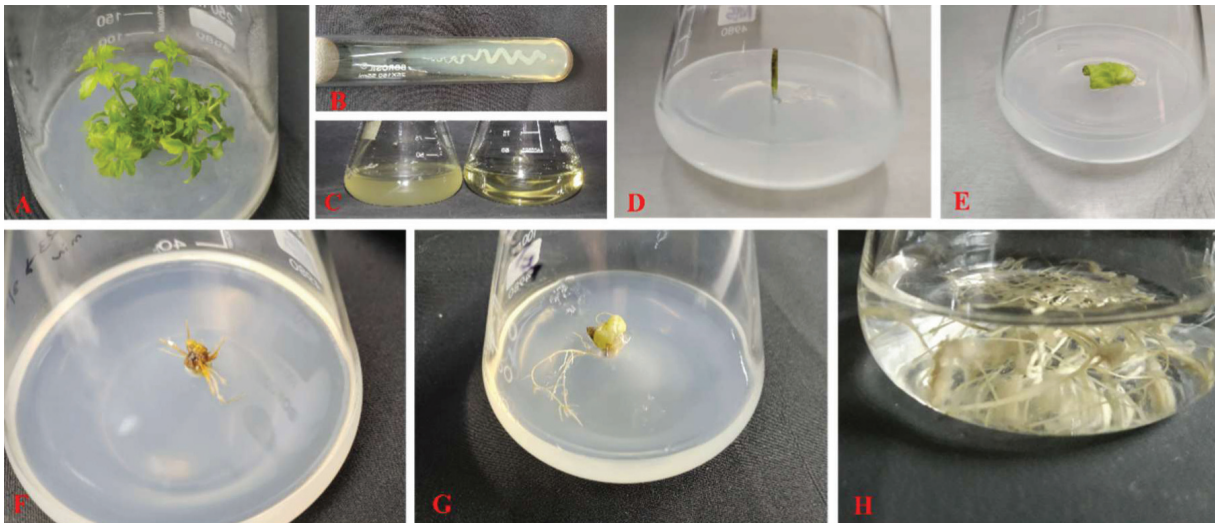

Fig. 1. Hairy root culture of *Vitex negundo*. A – *Vitex negundo in vitro* culture; B – *Agrobacterium rhizogenes* pure culture; C – MTCC532 working culture; D – *Agrobacterium* infected *in vitro* internode culture inoculated in Murashige and Skoog solid medium; E – *Agrobacterium* infected *in vitro* leaf culture inoculated in Murashige and Skoog solid medium; F – hairy root induction from *in vitro* internode culture; G – hairy root induction from *in vitro* leaf culture; H – multiplication of hairy root in Murashige and Skoog liquid medium.

RESULTS

The *Agrobacterium rhizogenes* strain MTCC 532 responded positively to the induction of hairy roots. Hairy root induction was examined using five distinct infection durations (15, 30, 45, 60, and 75 minutes) at varying optical density (0.064, 0.149, 0.279, 0.348, 0.503, and 1.102) (Fig. 2), a two-day co-cultivation period, and three distinct acetosyringone concentrations (50 µM, 100 µM, and 150 µM) (Fig. 3). After 20 days of infection, hairy roots started emerging from the midrib and side cut of the infected *in vitro* internodes and leaves. The optimal transformation frequency, or 56.66% with an average of 3.30 hairy roots per explant in the internode (Fig. 4; Fig. 1F) and 63.33% with an average of 3.30 hairy roots per explant in the leaves (Fig. 1G), was demonstrated by the 48-hour growth of MTCC 532 at optical density 600 nm = 0.348 (i.e. 0.3 × 107 cells/mL), 100 µM of acetosyringone, 45-minute infection time, and two-day co-cultivation period. In a 100 mL flask, induced hairy root multiplication was observed in liquid MS media at 25 ± 1°C under dark culture (Fig. 1H).

Six distinct optical densities (0.064, 0.149, 0.279, 0.348, 0.503, and 1.102) based on the MTCC 532 growing period (12–72 h) were employed to examine the leaf and internode transformation frequency. At an optical density of 0.348, the leaf explant with the best transformation efficiency was MTCC 532 after 48 hours of growth, compared to the internode.

The optimal transformation frequency was

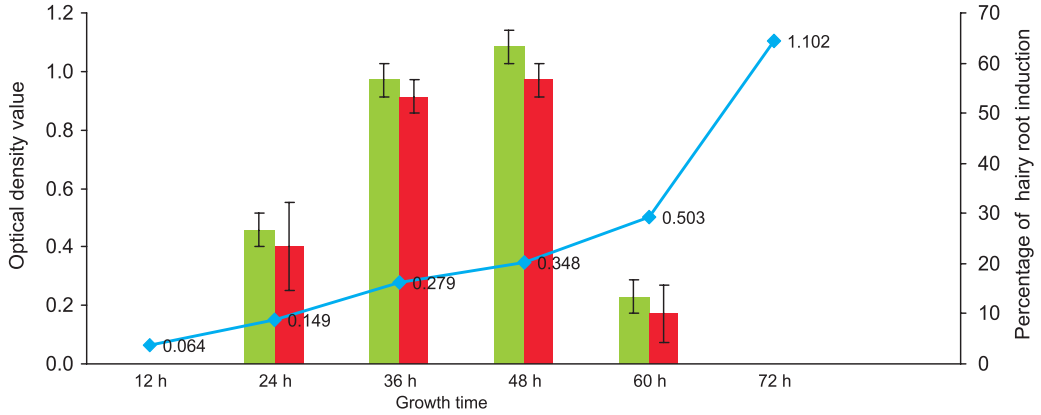

Fig. 2. Percentage of hairy roots induced in leaves and internodes affected by optical density value. The information is the average ± standard error of three independent experiments. Mean values shown by different lowercase letters in the same column indicate significant differences ($p < 0.05$). Legend: blue curve – optical density value; green bar – percentage of hairy root induction in leaf; red bar – percentage of hairy root induction in internode.

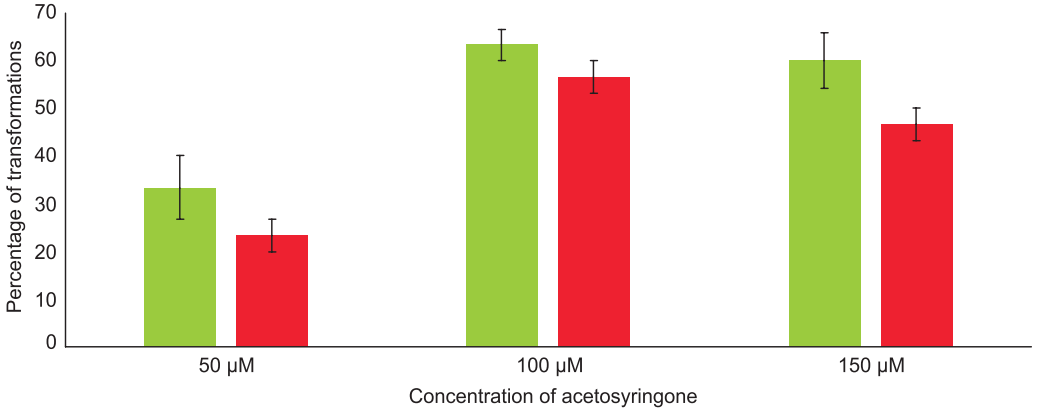

Fig. 3. Transformation frequency in leaves and internodes affected by acetosyringone. The data signify the mean ± SE of three independent experiments. Mean values shown by different lowercase letters in the same column indicate significant differences ($p < 0.05$). Legend: green bar – percentage of transformations in leaf; red bar – percentage of transformations in internode.

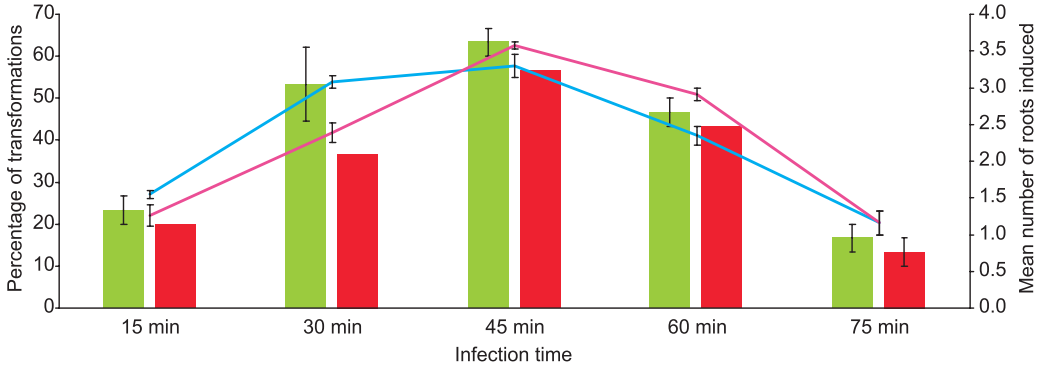

Fig. 4. Effect of infection time on the percentage of transformation and numbers of hairy roots in leaves and internodes. The data signify the mean ± SE of three independent experiments. Mean values shown by different lowercase letters in the same column indicate significant differences ($p < 0.05$). Legend: blue curve – number of roots induced in leaf; pink curve – number of roots induced in internode; green bar – percentage of transformations in leaf; red bar – percentage of transformations in internode.

achieved with acetosyringone at 100 µM, when using three different acetosyringone concentrations (50 µM, 100 µM, and 150 µM). From two distinct explants (leaf and internode), the best outcome was obtained at a concentration of 100 µM acetosyringone in the leaf.

Based on five distinct infection times (15, 30, 45, 60, and 75 minutes) and two explants (leaf and internode), the leaf explant at 45 minutes of infection time exhibited the highest transformation frequency, or 63.33% with an average of 3.30 hairy roots per explant, while the internode showed 56.66% with an average of 3.58 hairy roots per explant.

DISCUSSION

Agrobacterium rhizogenes MTCC 532 showed a higher transformation frequency in leaves compared to the internode of *in vitro*-grown shoots of *Vitex negundo* in our experiment. Karwasara & Dixit (2009) have also identified that *in vitro* leaf explants of *Abrus precatorius* L. show the maximum transformation frequency with MTCC 532. Similar results with MTCC 532 have also been reported in *Withania somnifera* L. (Thilip et al., 2015), *Berberis aristata* DC. (Brijwal & Tamta, 2015), *Nothapodytes foetida* (Grah.) Mabb. (Malik & Laura, 2019). Gill & Siwach (2022) have reported that the transformation frequency is highest in leaf explants than in the nodal and apical explants. In *Solanum trilobatum* L. the leaf explant also shows better transformation efficiency than the internode explant (Shilpha et al., 2015).

Infection time varies depending on the explant and plant species. Identifying the ideal infection time is crucial for improving the transformation efficiency of that specific plant. In our study, an infection time of 45 minutes gave the best result among the different infection times (15, 30, 45, 60, and 75 minutes). Bhagat et al. (2019) have also attempted 15 to 45 minutes at 15-minute intervals in *Rauwolfia serpentina* L. In *Gymnema sylvestre* (Retz.) Schult, Nagella et al. (2013) have reported that a 30-minute infection time shows the best transformation frequency. According to Zolfaghari et al. (2020), the optimal time for inducing hairy roots in *Trigonella foenum-graecum* L. is 20 minutes. By increasing the infection time from 15 to 45 minutes, the transformation frequency increases in *Abrus precatorius* (Singh & Dixit, 2009). Contrary to our result in *Raphanus sativus,* 10 minutes of infection time shows the maximum percentage of hairy root induction (Balasubramanian et al., 2018). The highest transformation frequency has been obtained from the explants of *Chlorophytum borivilianum* Santapau & R.R.Fern. cultured in bacterial suspension for 20 minutes (Bathoju et al., 2017).

Acetosyringone is a monocyclic phenolic compound that is involved in T–DNA transfer and *vir* gene activation (Ridgway et al., 2004). Acetosyringone plays a significant role in the transformation of hairy roots. Among the different concentrations (50, 100, and 150 µM), acetosyringone 100 µM shows the best transformation frequency. Other researchers have also tested various concentrations of acetosyringone for hairy root induction, such as 100 µM (Thilip et al.,

2015); 50–150 µM (Bhagat et al., 2019); 100, 200, 300 µM (Ji et al., 2023); and 100 µM (Kumar et al., 2023). In *Ficus religiosa* L., among three different concentrations of acetosyringone, i.e. 50 µM, 100 µM, 150 µM, the highest transformation frequency has resulted at 150 µM (Gill & Siwach, 2022). The maximum efficiency for inducing hairy roots has been observed at 100 µM of acetosyringone, among the various concentrations tested (25–125 µM) (Balasubramanian et al., 2018). According to Singh & Dixit (2009), the concentration of acetosyringone at 100 µM has a keen effect on hairy root transformation efficiency. However, Shilpha et al. (2015) have found that 200 µM of acetosyringone is best suited for hairy root induction in *Solanum trilobatum.* Following Cervera et al. (1998) findings, acetosyringone at 50 to 100 µM/ L is required for the development of hairy roots.

The co-cultivation period was maintained in hormone-free MS medium for two days and showed the best results in our experiment. Similar to our study, Sujatha et al. (2013) in *Artemisia vulgaris*, Sharifi et al. (2014) in *Tribulus terrestris* L., Thilip et al. (2015) in *Withania somnifera*, Bhagat et al. (2019) in *Rauwolfia serpentine*, Kumar et al. (2023) in *Plumbago zeylanica* L. have also reported that a two-day co-cultivation period shows the best result. A 48-hour co-cultivation period is also effective for *Podophyllum hexandrum* Royle., *Glycyrrhiza glabra* L. and *Linum mucronatum* Bertol. (Giri et al., 2001; Mehrotra et al., 2008; Samadi et al., 2012). Co-cultivation is typically restricted to two or three days and has been demonstrated to be sufficient for transformation, as prolonged co-cultivation beyond three days can increase bacterial cell densities in the selection medium (Singh & Dixit, 2009). Similar to our findings, Balasubramanian et al. (2018) have also reported that a 2-day co-cultivation period is most suitable for inducing hairy roots. Contrary to our result, Shilpha et al. (2015) have found that a 3-day co-cultivation is optimally efficient for transformation. Bacterial overgrowth triggers tissue damage when the co-cultivation time exceeds 48 hours (Amali et al., 2014; Bathoju et al., 2017).

After the co-cultivation period, cefotaxime (100–200 mg/l) was used to remove excess growth of *Agrobacterium. rhizogenes* from the explants. According to Sharifi et al. (2014), Thilip et al. (2015) and Sajjalaguddam & Paladugu (2016) in *Tribulus terrestris*, *Withania somnifera*, and *Abrus precatorius* (100 to 350 mg/l, 200 mg/l, and 100, 200, 300 mg/L, respectively. Contrary to our report, other researchers have used 400 mg/L and 500 mg/L cefotaxime to remove excess bacteria (Nagella et al., 2013; Sujatha et al., 2013; Kumar et al., 2023; Ji et al., 2023). In *Chlorophytum borivilianum*, the optimum concentration of cefotaxime is 200 µg/ml, which resulted in a 98% transformation frequency for bacterial growth control (Sharafi et al., 2013; Bathoju et al., 2017).

This investigation effectively developed a method for inducing hairy root induction in *Vitex negundo*. The establishment of a bioreactor for hairy root upscaling, improvement of hairy root biomass, confirmation of transformed roots, and parameter optimisation for the generation and improvement of secondary metabolites of particular significance should all be part of future research. The synthesis of the essential secondary metabolites found in *Vitex negundo* roots on a large scale may benefit from the use of hairy root cultures.

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Author contributions. Conceptualisation and literature analysis were performed by B.M., A.B., S.K.Ma. and S.K.Mo. The first draft of the manuscript was prepared by B.M. S.S.S., D.M. and A.M. critically analysed and gave suggestions to finalise the manuscript. Supervision was conducted by D.P.B. All authors read and approved the final manuscript.

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BM: https://orcid.org/ 0000-0002-6404-4593
AB: https://orcid.org/0009-0005-1678-2531
SKMo: https://orcid.org/0009-0006-5919-0031
SKMa: https://orcid.org/ 0009-0001-7688-2893
DM: https://orcid.org/0000-0003-0525-8812
AM: https://orcid.org/ 0000-0001-5050-3288
DPB: https://orcid.org/ 0000-0002-0319-968X