

ORIGINAL CONTRIBUTION

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Improved micropropagation of *Bacopa monnieri* (L.) Wettst. (Plantaginaceae) and antimicrobial activity of in vitro and ex vitro raised plants against multidrug-resistant clinical isolates of urinary tract infecting (UTI) and respiratory tract infecting (RTI) bacteria

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Abstract

Background: Nowadays the multidrug-resistant (MDR) bacterial pathogens are a major concern of the medical science. Medicinal plants may be considered as new sources for producing antibacterial agents. The present study aimed to standardize an improved method for micropropagation and in vitro biomass production of *Bacopa monnieri*. Second aim is to evaluate the antimicrobial potency of in vitro cultured and ex vitro field grown micropropagated plants against different MDR clinical isolates of human urinary tract infecting (UTI) and respiratory tract infecting (RTI) pathogens.

Methods: Micropropagation of *B. monnieri* were performed following standard tissue culture method. The role of 6-benzylaminopurine (BAP), kinetin and spermidine on multiple shoot induction were evaluated. Antimicrobial activity of ethanol, methanol and acetone extract of in vitro and ex vitro plants of *B. monnieri* were screened by agar cup method against five MDR-UTI bacteria, four MDR-RTI bacteria and three microbial type culture collection (MTCC) bacteria and two fungi. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were also determined.

Results: Synergistic effect of BAP and spermidine had improved shoot induction with a maximum of 123.8 shoot-buds per explant. Optimum micropropagation with 34.9 elongated shoots per explant was recorded in Murashige and Skoog medium containing 1.5 mg/L BAP and 2.0 mM spermidine. Methanolic extract of ex vitro plants showed maximum activity against MDR-UTI strain of *Escherichia coli* (sample-9) [ZI 18 ± 0.68 mm, MIC 2.5 $\mu\text{g/mL}$, MBC 5.0 $\mu\text{g/mL}$]. Acetone extract of ex vitro plant exhibited maximum inhibition against MDR-RTI strain of *Klebsiella pneumoniae* (sample-38) [ZI 14 ± 0.22 mm, MIC 5.0 $\mu\text{g/mL}$, MBC 7.5 $\mu\text{g/mL}$]. The extracts of *B. monnieri* were bactericidal rather than bacteriostatic against all UTI and RTI bacteria tested.

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(Continued from previous page)

Conclusions: The present manuscript demonstrated an efficient in vitro method for large scale biomass production of *B. monnieri*. Furthermore, the methanolic extract of *B. monnieri* have potential antimicrobial activity against clinical isolates of MDR-UTI and MDR-RTI bacterial strains. Hence this plant may further use to treat these infectious diseases. The comparative results show ex vitro grown plants have slightly better antimicrobial activities as compared to the in vitro plants.

Keywords: *Bacopa monnieri*, Clinical isolates, Micropropagation, Multidrug-resistant, RTI bacteria, Spermidine, UTI bacteria,

Background

In more than 80% of developed countries, plants have been traditionally used as remedy because they are the actual good source of different pharmacologically active compounds [1]. *Bacopa monnieri* (L.) Wettst. (family Plantaginaceae) is an important medicinal plant grown throughout the Indian subcontinent and traditionally been used in Ayurvedic system of medicine [2, 3]. This species is one of the best versatile ‘tonic herbs’ stimulates brain, heart, liver, and kidney health. It also used as a revitaliser of sensory organs, water retention and blood cleaning, relaxant, neuroprotective as well as in the treatment of allergic disorders of skin, snake bite, enlargement of the spleen, rheumatism, leprosy, epilepsy, asthma, hoarseness and anxiety. *Bacopa monnieri* extract has proven sedative, anti-inflammatory and antipyretic, anti-cancer, anti-oxidant, immuno-modulatory, antiulcerogenic, antistress, anti-leishmanial, antipyretic, analgesic and anti-aging properties [4–9]. Kishore [6] had designated *B. monnieri* as a “complete herbal medicine” due to multipurpose usage of

this wonder medicinal plant. The antimicrobial property of *B. monnieri* was previously screened by few scientists, but majority of this reports are against standard bacterial strains [10–12]. Although one report are available where clinical isolates of human pathogenic strains are tested, but these are not multidrug-resistant (MDR) strains [13]. Anti-bacterial resistance has become one of the grave public health concerns, globally, over the last two decades [14, 15]. In modern years, there has been a rising interest in investigating and evolving new antimicrobial agents from various sources to combat microbial resistance [16]. The MDR microbial pathogens imply a most important threat to human health. The worldwide surge in MDR bacteria and the proximity of urinary tract infectious and respiratory tract infectious disease demand alternative curative approaches to supplement the existing medications.

Bacopa monnieri was positioned second in a priority list of the most significant medicinal plants, assessed on the basis of their medical reputation, commercial value, and potential for advance research and development by the

Table 1 Antimicrobial activities of nine clinical isolates of the human UTI and RTI bacteria against 15 well known drugs

Antibiotics	UTI Pathogens					RTI Pathogens			
	Sample 5 (<i>Escherichia coli</i>)	Sample 9 (<i>Escherichia coli</i>)	Sample 11 (<i>Escherichia coli</i>)	Sample 19 (<i>Klebsiella pneumoniae</i>)	Sample 26 (<i>Klebsiella pneumoniae</i>)	Sample 31 (<i>Klebsiella pneumoniae</i>)	Sample 35 (<i>Klebsiella pneumoniae</i>)	Sample 36 (<i>Klebsiella pneumoniae</i>)	Sample 38 (<i>Klebsiella pneumoniae</i>)
Amoxiline	R	S	S	R	R	R	R	R	R
Amikacin	S	S	S	R	R	S	S	R	R
Aztreonam	R	R	R	R	R	NT	R	NT	NT
Ceftriaxone	R	R	S	R	R	R	R	R	S
Cefuroxime	R	R	S	R	I	R	R	R	R
Ciprofloxacin	R	NT	I	I	I	R	R	R	R
Piperacillin	S	S	S	R	S	S	NT	R	NT
Cefoperazone	R	NT	S	R	R	S	S	R	R
Ceftazidime	R	R	S	R	R	NT	NT	R	R
Ofloxacin	R	R	R	I	S	NT	NT	NT	NT
Sulbactam	S	R	S	R	S	R	R	S	NT
Nitrofurantoin	S	R	S	NT	S	R	R	S	S
Meropenem	S	NT	NT	I	R	S	R	R	R
Doxycycline	S	R	S	S	S	NT	S	S	R
Norfloxacin	R	S	S	I	S	R	R	R	R

R resistant, S sensitive, I intermediate, NT not tested

National Medicinal Plants Board of India (2004) [17]. Due to illegal over-collection of the pharmaceutical companies, it has now been depleted from its natural habitat and recognized as an endangered plant of India [3, 18]. In this circumstances, the demand for good quality planting material of this wonder curative herb is increasing day-after-day, which necessitate the improvement of the existing micropropagation methods for rapid and constant supply of planting materials round the year. On other hand, land is not available for medicinal plant cultivation as compare to crop plants [19], so in vitro biomass production is alternative option to produce huge amounts of pharmaceutically active compounds round the year without any cultivation land. Although micropropagation of *B. monnieri* has been studied by several group of researchers for more than four decades [2, 3], yet based on their

wonder medicinal properties, the additional research is much needed for further improvement of the micropropagation protocol. The present study was thus aimed to the—(1) development of high frequency micropropagation method, (2) screening of the antimicrobial activities of different solvent-extracts of *B. monnieri* against various pathogenic strains of MDR bacteria and standard laboratory strains (MTCC) of bacteria and fungi.

Methods

Surface sterilization and initial culture establishment

Shoot tips (8–10 mm) of *Bacopa monnieri* (L.) Wettst. (family Plantaginaceae) were collected from the healthy plants grown in an experimental garden of our college and were washed with 2.5% (w/v) systematic fungicide (Bavistin) for 15 min followed by 2.5% liquid detergent

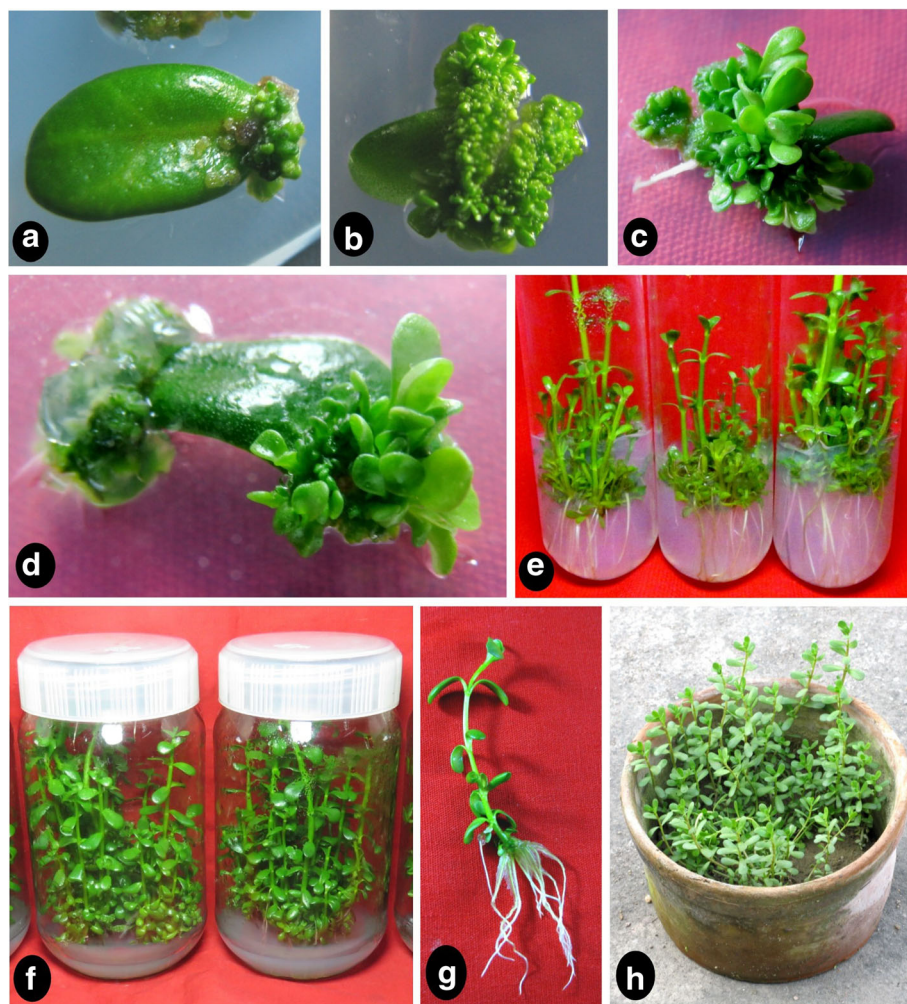


Fig. 1 Different stages of micropropagation of *Bacopa monnieri*. **a** Induction of shoot buds from the cut edges of leaf after 8 days of inoculation in medium containing 0.5 mg/L KIN. **b** Shoot buds induced in medium containing 1.5 mg/L BAP after 12 days of inoculation. **c** & **d** Shoot buds start to elongate in medium containing 1.5 mg/L BAP and 2.0 mM spermidine after 21 days of inoculation. **e** Elongated shoots after 42 days of inoculation. **f** Plants grown in half strength MS medium without any PGR. **g** A complete plantlets with well developed root system ready for field transfer. **h** Tissue culture derived ex vitro field grown micropropagated plants

(Tween-20) solution for 2 min. Finally, the explants were surface-disinfected with mercuric chloride (HgCl_2) solution (0.1%, w/v) for 8 min and rinsed 3 times with sterile distilled water. Then all the explants were implanted on Murashige and Skoog (MS) [20] basal medium without any plant growth regulator (PGR) for the establishment of an initial sterile culture.

Micropropagation

The mature leaves (12–15 mm) were cut from the in vitro established infection-free plants and were used as initial explants for micropropagation purpose. Two different cytokinins viz. 6-Benzylaminopurine (BAP) and Kinetin (KIN) are tested in six different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L). The effect of Spermidine (1.0, 1.5, 2.0, 2.5 mM) in combination with optimum cytokinin was also evaluated for better results. After 3 weeks, all the explants were subcultured to their respective fresh medium for the elongation of the adventitious shoots. After another 3 weeks, all cultures were transferred to half-strength MS medium without any PGR for further elongation and in vitro rooting. Ultimately, the micropropagated plants with well developed root systems, were acclimatized and transferred to the field conditions following the methods previously standardized by us [21].

Extract preparation for antimicrobial screening

Fresh leaves of both in vitro cultured and ex vitro, field grown micropropagated plants were collected and air

dried in closed and dark environment at 40 °C for 7 days. Dry leaves were powdered in mixture grinder and kept at 4 °C for further use. The powdered plant material was extracted in three different solvent (methanol, ethanol, acetone) for 12 h with the help of Soxlet apparatus. Then the extracts were air dried for 24 h and finally dissolved in dimethyl sulfoxide (DMSO) to prepare dilution for further study.

Collection and maintenance of test organisms

A total of 14 microorganisms (9 pathogenic and 5 standard laboratory microorganisms) were used in this experiment. The pathogenic organisms are MDR clinical isolates of human UTI and RTI bacteria (Table 1). The microbial type culture collection (MTCC) strains of three bacteria and two fungi were used as standard test organisms. Different pathogenic and standard bacterial strains of gram negative namely *Klebsiella pneumoniae*, *Escherichia coli*, gram positive *Bacillus subtilis*, *Staphylococcus aureus* and two standard fungal stains of *Aspergillus fumigatus*, *Candida albicans* were tested.

Antimicrobial screening by agar cup method

The antimicrobial activity was screened by agar well diffusion method. Saborouds dextrose agar was used as the culture media for fungi and Muller Hinton agar medium was used for bacteria. The inoculums were prepared in fresh nutrient broth from well-maintained slant culture. The inoculums were standardized following McFarland

Table 2 Effect of two different cytokinins supplemented with MS basal medium and culture duration (i.e., regeneration cycles) on adventitious shoot induction from leaf explant of *Bacopa monnieri*

Concentration of plant growth regulators			Response (%)	Number of shoot buds induced per leaf explant (after 12 d of implantation)	No. of elongated (≥ 1 cm) shoot buds per leaf explant (after 42 d of implantation)
BAP (mg/L)	KIN (mg/L)	Spermidine (mM)			
–	–	–	0	0 ^a	0 ^a
0.5	–	–	96.7	53.5 \pm 1.7 ^{cd}	11.7 \pm 0.3 ^c
1.0	–	–	100	76.7 \pm 1.2 ^{de}	16.1 \pm 0.2 ^d
1.5	–	–	100	92.4 \pm 3.7 ^{ef}	22.5 \pm 0.4 ^{ef}
2.0	–	–	100	84.1 \pm 3.2 ^e	19.2 \pm 0.5 ^e
2.5	–	–	100	78.2 \pm 2.4 ^e	13.8 \pm 0.2 ^{cd}
–	0.5	–	90.0	45.0 \pm 1.3 ^{bcd}	9.5 \pm 0.6 ^c
–	1.0	–	93.3	62.8 \pm 1.9 ^d	13.6 \pm 0.7 ^{cd}
–	1.5	–	100	73.5 \pm 2.2 ^{de}	16.0 \pm 0.3 ^d
–	2.0	–	100	81.3 \pm 2.5 ^e	18.2 \pm 1.0 ^{de}
–	2.5	–	100	75.4 \pm 1.9 ^{def}	15.4 \pm 0.8 ^d
1.5	–	1.0	100	106.0 \pm 3.2 ^f	27.3 \pm 0.7 ^{fg}
1.5	–	1.5	100	114.2 \pm 2.6 ^{fg}	30.7 \pm 0.9 ^g
1.5	–	2.0	100	123.8 \pm 3.4 ^g	34.9 \pm 1.2 ^h
1.5	–	2.5	100	117.6 \pm 3.1 ^{fg}	31.4 \pm 1.1 ^{gh}

Each value represents the mean \pm standard error, $n = 30$ (3 set, 10 samples in each set). Mean followed by the same letters in each column are not significantly different at $P < 0.05$ according to Tukey's multiple range test. BAP 6-Benzylaminopurine, KIN Kinetin

Table 3 Antimicrobial activities of the three different solvent extracts of ex vitro *Bacopa monnieri* plants

Solvent	Extract conc. (mg/cup)	Zone of Inhibition (mm)																					
		MDR-UTI bacteria									MDR-RTI bacteria									MTCC bacteria		MTCC fungus	
		Sample 5	Sample 9	Sample 11	Sample 19	Sample 26	Sample 31	Sample 35	Sample 36	Sample 38	Sample 38	Sample 38	Sample 38	Sample 38	Sample 38	Sample 38	Sample 38	Sample 38	Sample 38	MTCC 870	MTCC 1637		
Acetone	0.75	-	12 ± 0.11	-	R	12 ± 0.22	-	9 ± 0.28	-	-	-	-	-	-	-	-	-	-	-	8 ± 0.08	-		
	1.5	11 ± 0.11	13 ± 0.26	-	R	14 ± 0.32	-	11 ± 0.58	10 ± 0.56	-	-	-	-	-	-	-	-	-	-	8 ± 0.36	10 ± 0.38	12 ± 0.22	
	3.0	13 ± 0.24	15 ± 0.66	-	R	15 ± 0.36	9 ± 0.22	12 ± 0.11	11 ± 0.32	8 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	12 ± 0.11	11 ± 0.32	11 ± 0.28	14 ± 0.18	
	4.5	15 ± 0.36	16 ± 0.33	11 ± 0.11	R	16 ± 0.14	11 ± 0.11	12 ± 0.56	13 ± 0.88	9 ± 0.28	14 ± 0.26	14 ± 0.26	14 ± 0.26	14 ± 0.26	14 ± 0.26	14 ± 0.26	14 ± 0.26	14 ± 0.26	14 ± 0.26	13 ± 0.44	12 ± 0.56	16 ± 0.38	
	6.0	15 ± 0.22	16 ± 0.48	13 ± 0.24	R	16 ± 0.18	11 ± 0.58	12 ± 0.22	13 ± 0.44	9 ± 0.22	14 ± 0.44	14 ± 0.44	14 ± 0.44	14 ± 0.44	14 ± 0.44	14 ± 0.44	14 ± 0.44	14 ± 0.44	14 ± 0.44	15 ± 0.48	12 ± 0.88	19 ± 0.12	
Ethanol	0.75	-	14 ± 0.18	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	1.5	-	16 ± 0.38	-	R	-	8 ± 0.28	9 ± 0.48	-	-	-	-	-	-	-	-	-	-	-	-	-	10 ± 0.26	
	3.0	-	17 ± 0.22	12 ± 0.32	R	-	9 ± 0.44	10 ± 0.22	-	10 ± 0.21	11 ± 0.32	11 ± 0.32	11 ± 0.32	11 ± 0.32	11 ± 0.32	11 ± 0.32	11 ± 0.32	11 ± 0.32	8 ± 0.38	10 ± 0.22	10 ± 0.21	14 ± 0.46	
	4.5	11 ± 0.18	17 ± 0.18	14 ± 0.11	R	-	11 ± 0.22	11 ± 0.48	8 ± 0.32	11 ± 0.44	13 ± 0.22	13 ± 0.22	13 ± 0.22	13 ± 0.22	13 ± 0.22	13 ± 0.22	13 ± 0.22	13 ± 0.22	10 ± 0.22	10 ± 0.22	11 ± 0.26	18 ± 0.55	
Methanol	0.75	13 ± 0.22	17 ± 0.66	14 ± 0.62	R	10 ± 0.11	11 ± 0.22	12 ± 0.56	10 ± 0.44	11 ± 0.22	15 ± 0.48	15 ± 0.48	15 ± 0.48	15 ± 0.48	15 ± 0.48	15 ± 0.48	15 ± 0.48	15 ± 0.48	12 ± 0.22	16 ± 0.48	11 ± 0.18	21 ± 0.38	
	1.5	11 ± 0.26	13 ± 0.78	-	R	8 ± 0.33	7 ± 0.56	-	-	10 ± 0.28	11 ± 0.48	11 ± 0.48	11 ± 0.48	11 ± 0.48	11 ± 0.48	11 ± 0.48	11 ± 0.48	11 ± 0.48	-	10 ± 0.22	12 ± 0.18	12 ± 0.18	
	3.0	12 ± 0.88	16 ± 0.18	-	R	10 ± 0.24	9 ± 0.88	-	9 ± 0.88	12 ± 0.32	13 ± 0.66	13 ± 0.66	13 ± 0.66	13 ± 0.66	13 ± 0.66	13 ± 0.66	13 ± 0.66	13 ± 0.66	-	12 ± 0.48	14 ± 0.11	16 ± 0.77	
	4.5	13 ± 0.48	18 ± 0.11	-	R	11 ± 0.18	11 ± 0.22	8 ± 0.11	11 ± 0.88	13 ± 0.12	13 ± 0.78	13 ± 0.78	13 ± 0.78	13 ± 0.78	13 ± 0.78	13 ± 0.78	13 ± 0.78	13 ± 0.78	9 ± 0.48	14 ± 0.55	16 ± 0.22	18 ± 0.34	
	6.0	14 ± 0.54	18 ± 0.66	10 ± 0.24	R	12 ± 0.38	12 ± 0.38	8 ± 0.88	12 ± 0.48	13 ± 0.21	13 ± 0.48	13 ± 0.48	13 ± 0.48	13 ± 0.48	13 ± 0.48	13 ± 0.48	13 ± 0.48	13 ± 0.48	10 ± 0.52	14 ± 0.33	16 ± 0.38	18 ± 0.72	
6.0	14 ± 0.22	18 ± 0.68	13 ± 0.34	R	13 ± 0.62	13 ± 0.62	9 ± 0.32	12 ± 0.48	14 ± 0.22	13 ± 0.28	13 ± 0.28	13 ± 0.28	13 ± 0.28	13 ± 0.28	13 ± 0.28	13 ± 0.28	13 ± 0.28	12 ± 0.66	14 ± 0.68	16 ± 0.18	18 ± 0.38		

Each value represents the mean ± standard error. R Resistant, Sample 5, 9, 11 – *Escherichia coli*, Sample 19, 26, 31, 35, 36, 38 – *Klebsiella pneumoniae*, MTCC 109 – *Klebsiella pneumoniae*, MTCC 441 – *Bacillus subtilis*, MTCC 3160 – *Staphylococcus aureus*, MTCC 870 – *Aspergillus fumigatus*, MTCC 1637 – *Candida albicans*

standards by regulating the turbidity of the broth culture by adding sterile broth. Petri dishes were spread with the inoculums and holes (8 mm) were punctured using sterile cork-borer. The plant extracts of different solvents were dissolved in DMSO and were added to the respective holes. DMSO being the negative control exhibited no zone of inhibition against any of the tested microorganisms. Experimental cultures were incubated for 24 h at 37 °C (for bacteria) and 72 h at 26 °C (for fungi). After incubation, the zones of inhibition (ZI) were measured and photographed with Canon 'PowerShot SX700 HS' camera.

Minimum inhibitory concentration (MIC)

The MIC was determined by two fold broth dilution method [22] for showing antimicrobial activity of the plant extract against all the test organisms. The dried plant extracts were resuspended in DMSO to make 100 mg/mL concentration, then added to broth media using serial dilution. Thereafter 100 µl inoculum was added to every tube and incubated at 26 °C for 72 h (for fungi) or 37 °C for 24 h (for bacteria). The MIC value of both fungi and bacteria was taken as the lowermost concentration of the plant extracts in the tube that exhibited no turbidity following incubation.

Minimum bactericidal and fungicidal concentration (MBC and MFC)

The minimum bactericidal and fungicidal concentration (MBC and MFC) was determined by the viable cell count method [23] by subculturing 50 µl of each dilution used in MIC experiment. Minimum concentration of plant extract, showing no visible growth on subculturing, was taken as MBC or MFC.

Statistical analysis

In present experimental design, each treatment of micropropagation was repeated thrice with 10 explants per treatment. All data were subjected to one-way analysis of variance (ANOVA) with the help of SPSS software (IBM® SPSS, version 21.0, Chicago, IL). After conducting an ANOVA, the means were further separated using Tukey's test at $P \leq 0.05$ [24]. All types of antimicrobial tests are also repeated three times.

Results

Micropropagation

The effect of different concentrations (ranging from 0.5 mg/L to 2.5 mg/L) of BAP and KIN alone on in vitro adventitious shoot multiplication from leaf explants, was investigated (Fig. 1 a–d). The adventitious shoot-buds

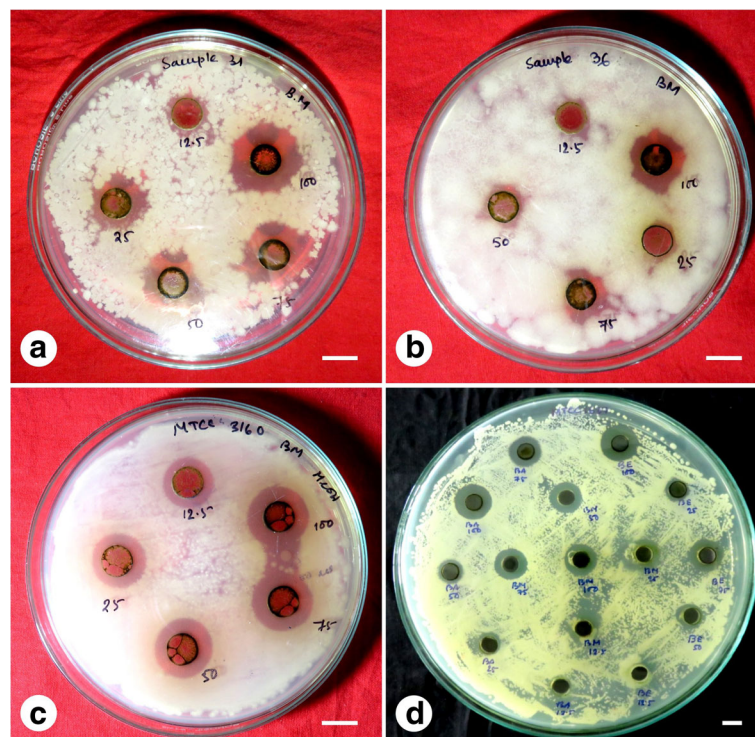


Fig. 2 Antimicrobial activities of *Bacopa monnieri* [bar = 10 mm]. **a** Activities of methanol extract against MDR-RTI clinical strain *Klebsiella pneumoniae* [Sample 31], **b** Activities of methanol extract against MDR-RTI clinical strain *Klebsiella pneumoniae* [Sample 36], **c** Activities of methanol extract against standard bacterial strain *Staphylococcus aureus* [MTCC 3160], **d** Activities of ethanol, methanol, and acetone extracts against standard fungal strain *Candida albicans* [MTCC 1637]

were induced from the cut edges within 6 to 7 days of implantation, followed by swelling at the cut edges. A total of 92.4 shoot buds were induced after 12 days of culture in MS basal medium containing 1.5 mg/L BAP, whereas maximum of 81.3 shoot buds were induced in the presence of KIN (2.0 mg/L) after same duration (Table 2). Greater concentration of cytokinin is not only abridged the quantity of shoot buds, but also suppressed the growth of the multiplied shoots. Spermidine in combination with the optimum concentration of BAP (1.5 mg/L) significantly increased the rate of shoot induction. MS basal medium supplemented with 1.5 mg/L BAP and 2.0 mM spermidine was found to be the best combination of growth regulators with a maximum of 123.8 shoot buds per leaf explant, after 12 days of culture.

Although the huge numbers of shoot buds were induced, the elongation of these newly induced buds was a real problem and few of them had elongated at a time in presence of cytokinin only. Spermidine had played an affirmative role on shoot bud elongation in *B. monnieri*. Only 22.5 shoot buds are elongated in the presence of 1.5 mg/L BAP alone, but this numbers increased up to 34.9 when supplemented with 2.0 mM spermidine along with 1.5 mg/L BAP (Fig. 1e). Further elongation and in vitro rooting was achieved in half strength of MS medium without any PGR (Fig. 1f). A maximum of 13.2 roots per shoot with a length of 4.5 cm was noted within 18 day of implantation (Fig. 1g). In vitro derived plantlets were successfully acclimatized with a survival rate of 96.7%. All the plants were grown healthily with a morphology alike to that of the source plants (Fig. 1h).

Table 4 MIC and MBC/MFC (mg/mL) of the three different solvents of ex vitro plants of *Bacopa monnieri*

Microorganisms			Inhibition type	Inhibiting/cidal concentrations (mg/mL) of different solvent extracts			
Type	Bacteria / fungi	Strain		Acetone	Ethanol	Methanol	
Pathogenic	MDR-UTI bacterial strains	Sample 5	MIC	5.0	7.5	5.0	
			MBC	10.0	12.5	12.5	
		Sample 9	MIC	5.0	2.5	2.5	
			MBC	10.0	5.0	5.0	
		Sample 11	MIC	5.0	5.0	7.5	
			MBC	12.5	10.0	12.5	
		Sample 19	MIC	R	R	R	
			MBC	R	R	R	
		Sample 26	MIC	2.5	25.0	10.0	
			MBC	5.0	30.5	17.5	
		MDR-RTI bacterial strains	Sample 31	MIC	7.5	7.5	10.0
				MBC	10.0	12.5	15.0
			Sample 35	MIC	7.5	5.0	15.0
				MBC	12.5	7.5	17.5
			Sample 36	MIC	5.0	10.0	7.5
				MBC	10.0	15.0	15.0
			Sample 38	MIC	15.0	12.5	5.0
				MBC	17.5	17.5	7.5
Standard	MTCC bacterial strains		MTCC 109	MIC	5.0	5.0	7.5
				MBC	12.5	12.5	12.5
			MTCC 441	MIC	7.5	12.5	10.0
				MBC	10.0	17.5	15.0
		MTCC 3160	MIC	10.0	15.0	2.5	
			MBC	12.5	22.5	7.5	
	MTCC fungal strains	MTCC 870	MIC	2.5	10.0	2.5	
			MFC	7.5	12.5	5.0	
		MTCC 1637	MIC	7.5	7.5	1.25	
			MFC	12.5	12.5	5.0	

R Resistant, Sample 5, 9, 11 – *Escherichia coli*, Sample 19, 26, 31, 35, 36, 38 – *Klebsiella pneumoniae*, MTCC 109 – *Klebsiella pneumoniae*, MTCC 441 – *Bacillus subtilis*, MTCC 3160 – *Staphylococcus aureus*, MTCC 870 – *Aspergillus fumigatus*, MTCC 1637 – *Candida albicans*

Antimicrobial activity

In the present study, all UTI and RTI strains, collected from the patients, were routinely categorized in our laboratory, and examined individually for the resistance pattern against multidrug (Table 1). Result in the present study revealed that all the extracts (ethanol, methanol and acetone) of *B. monnieri* were more or less effective against all the test organisms except one (Sample 19) (Table 3). Overall, methanolic extract proved more effective than other two extracts with very clear ZI (Fig. 2a–c). In case of MDR-UTI strain of *E. coli* (Sample 9) methanolic extract exhibited maximum inhibition (ZI 18 mm), whereas another strain of *E. coli* (Sample 5) and *K. pneumoniae* (Sample 26) showed maximum inhibition (ZI 15 mm and 16 mm respectively) with acetone extract. For MDR-RTI bacteria, methanol extract showed maximum inhibition (ZI 14 mm) against *K. pneumoniae* (Sample 38). In case of all three MTCC bacteria, the maximum inhibition was noted with acetone extract. The fungal strain of *C. albicans* (MTCC 1637) showed maximum inhibition (ZI 21 mm) with ethanolic extract (Fig. 2d).

All the three types of extracts of *B. monnieri* were found to show potential anti-UTI and anti-RTI activity with MIC values ranging between 2.5–25.0 mg/mL. The MIC range was noted to be comparatively lower (1.25–10.0 mg/mL) against two fungal strains. In case of UTI and RTI bacteria, all the extracts exhibited MBC/MIC ratio less than “32”, which indicated that the test extracts of *B. monnieri* were bactericidal rather than bacteriostatic. Methanol was found to be the most potent (MIC 2.5–15.0 mg/mL), while acetone and ethanol showed more or less similar inhibitory activity (MIC 2.5–25.0 mg/mL and 2.5–30.0 mg/mL respectively) against all the microorganisms tested including MDR-UTI and MDR-RTI pathogens. Therefore, among all the test extracts, methanol was most potent against both bacteria and fungi (Table 4).

The antibacterial and antifungal activity of ex vitro plants is slightly better compared to the in vitro plants; however, the potency of the in vitro plants is not also negligible (Fig. 3).

Discussion

The cytokinin BAP or KIN alone was sufficient for induction of adventitious shoot buds from the leaf explants of *B. monnieri*. Higher concentration of cytokinin had reduced the number of shoots, a finding similar to that noted in *Tylophora indica* [19]. BAP was found to be more effective than KIN for adventitious shoot induction in *B. monnieri*. Similar results were reported in *Capsicum frutescens* where BAP proved to be the best among four of the cytokinins tested [25]. Micropropagation methods of *B. monnieri* have been studied by many groups for more than four decades

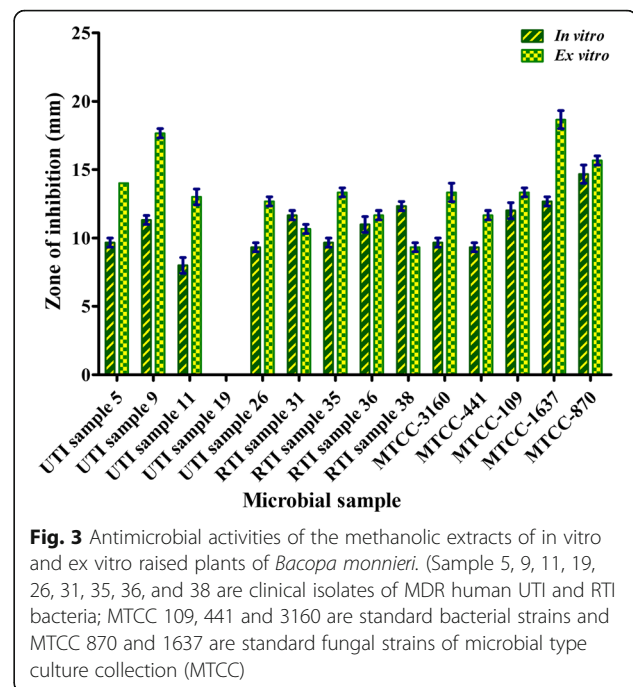


Fig. 3 Antimicrobial activities of the methanolic extracts of in vitro and ex vitro raised plants of *Bacopa monnieri*. (Sample 5, 9, 11, 19, 26, 31, 35, 36, and 38 are clinical isolates of MDR human UTI and RTI bacteria; MTCC 109, 441 and 3160 are standard bacterial strains and MTCC 870 and 1637 are standard fungal strains of microbial type culture collection (MTCC))

and till the research is continuing for improvement of these protocols [2, 3, 26–30]. So far, to our present knowledge, the present manuscript is the first report describing the promoting role of spermidine on micropropagation of *B. monnieri*. The addition of spermidine along with the optimum concentration of BAP has proven helpful in increasing the adventitious shoot bud induction rate of *B. monnieri*. Chi et al. [31] had also described the enhancing role polyamines in the shoot regeneration of *Brassica campestris*. Polyamines have been found to act as a growth stimulant and sometimes as the enhancer of the action of PGRs [32]. Polyamines play crucial role in the growth and development of higher plants by influencing cell division, stem elongation, root growth and many other functions [33]. Present findings showed the exogenous application of spermidine had not only enhanced the shoot induction rate, but also had significantly improved the growth and elongation of the adventitious shoots in *B. monnieri*. The similar optimistic role of polyamines, especially spermidine, on the growth and development of plants was previously reported by several authors [25, 34].

The antimicrobial property of *B. monnieri* was previously reported by few researchers against several standard bacterial strains [10–12, 35]. Only Hema et al. [13] had described the antimicrobial property of *B. monnieri* against clinical isolates of human pathogenic strains, but these are not MDR strains. Antibacterial resistance has become one of the grave public health concerns, globally, over the last two decades [14, 15]. Particularly, MDR is a major concern in present day medical science.

In this study standard laboratory microorganisms as well as MDR-UTI and MDR-RTI pathogens are found to be susceptible to various extracts of *B. monnieri*. The wonder plant *B. monnieri* is pharmaceutically very significant as it contains many secondary metabolites viz. alkaloids, flavonoid, phenolic, glycosides, and other substances like stigmastanol, stigmasterol and β -sitosterol, triterpene oligoglycosides, bacomosaponins A and B, bacomosides A, B1, and B2 [4, 9]. A group of secondary metabolite compounds has effective antimicrobial properties [36]. Mainly, methanol extract contains more antioxidant as because of this it might be used as folk medicine [37]. It was also reported that the methanol extract of *B. monnieri* exhibited the more significant inhibitory effect against *Klebsiella pneumoniae*, *Staphylococcus aureus* which corroborates with our findings [38, 39]. Similar to our findings against *Candida albicans* Canli et al. [40] also reported that the ethanolic extract exhibited best anti-fungal activity among all other solvent extracts against *Lycoperdon lividum*.

The study of in vitro and ex vitro plants exhibited the antimicrobial activity was better in ex vitro field grown plants than in vitro plants (Fig. 3). With a similar approach, a comparative study was conducted by few researchers in recent past [41–44]. Some of them reported the in vitro plants exhibited more antimicrobial activity as compared to ex vitro plants [42, 43], whereas the majority of them reported ex vitro plants are superior or more or less comparable as compare to in vitro plants [41, 44]. The in vitro plants, producing secondary metabolites at a primary stage of growth, provides an opportunity for fast production of pharmacologically important compounds that can be utilized for medicinal purposes [41].

Bacterial resistance against existing drugs has directed to severe health concerns all over the world [45]. Our present findings confirm that *B. monnieri* is very effective against MDR-UTI and RTI clinical isolates, where majority of the commercially available antibiotics are unable to treat those. Similarly, in very recent times few researchers had reported that *B. monnieri* proved to be effective against clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae* [13] and *Staphylococcus aureus* [46]; although their pathogens are not MDR strains. Because of *B. monnieri* contains many bioactive metabolite, the crude extract shows more effectiveness rather than commercial antibiotics. In addition, the herbal formulation have no side effect, which is an important advantage over commercial antibiotics. The determination of minimum lethal concentration (MLC), also known as the MBC or MFC, is the most common estimation of bactericidal or fungicidal activity [16]. Overall, present study exhibited a substantial bactericidal activity of the tested extracts of *B. monnieri* against the clinical isolates of MDR-UTI and RTI strains as indicated by the MBC/MIC ratio less than “32” [47].

Conclusion

The present manuscript described a very reliable method for large scale multiplication of *B. monnieri*. In addition, present findings show the methanolic extract of *B. monnieri* have potential antimicrobial activity against clinical isolates of MDR-UTI and MDR-RTI bacterial strains. In future this plant may be used to treat these infectious diseases. Though in vitro plants show slightly lesser antimicrobial potency as compared to ex vitro plants, they can be produced in large quantities using very little space throughout the year. Present protocol can be commercially implemented by pharmaceutical industries to address cumulative market demand of *B. monnieri*.

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Authors' contributions

SMH performed all the plant tissue culture related experiments and write up a part of manuscript. AC screened the antimicrobial activities of plant extracts and write up a part of manuscript. DD collected the clinical isolates and checked their response against multidrug. SM and SN was involved in result interpretation and made necessary correction in the write up. Critical revision of the article was done by SM, SN and BG. Conception, experiment design, overall monitoring and final approval of the article was done by BG. All authors read and approved the final manuscript.

Competing interests

All authors declare that they have no competing interests.

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