

In vitro clonal propagation and evaluation of antibacterial activity of Benghal dayflower-*Commelina benghalensis* L.

Md. M. ISLAM^{1*}, Md. A. AL MAMUN², Md. F. ALAM³

¹Plant Biotechnology and Genetic Engineering Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Dhaka-1000, Bangladesh; monirul.rubd@gmail.com (*corresponding author)

²Agricultural Statistics Division, Bangladesh Rice Research Institute, Gazipur, Bangladesh; mamunru4777@gmail.com

³University of Rajshahi, Department of Botany, Rajshahi, Bangladesh; kalam.jes@gmail.com

Abstract

An efficient protocol for *in vitro* propagation of *Commelina benghalensis* was developed. Nodal segments were showed the superb explants in performance for shoot proliferation than other explants. On the other hand, BAP with auxin was better combination. The maximum (90.52) number of explants response and the highest (23.25) number of shoots per plant were obtained from nodal segments on MS medium fortified with 3.0 mg/l BAP+0.1 mg/l IBA. The highest shoot length (17.25 cm) was achieved on MS medium containing 3.0mg/l BAP+0.5mg/l IBA. *In vitro* proliferated shoots were transferred to full and half strength of MS media where 1.5 mg/l IBA on full strength of MS media was the best to fit for the maximum number (12.69) of roots formation per micro-shoot. Well rooted plantlets were transferred to soil and successfully acclimatized with 97% survival rate. Three extracts i.e. methanol, ethanol, Petroleum ether of *C. benghalensis* L and four concentrations of each extracts were used against five gram (+ve) and five gram (-ve) bacterial species for the screening of antibacterial activity. Ethanol extracts was the superior in performance. The susceptibility of tested pathogenic bacterial species was increasing compare with increasing of extracts concentration with few exceptions. The highest zone of inhibition was obtained against *S. aureus* (17.50 mm) and *P. aeruginosa* (17.44 mm) at 800 mg/l dose level of extracts. It was also noticed that Gram (+ve) bacterial species are more susceptible to Benghal dayflower crude extracts than Gram (-ve) bacterial species.

Keywords: bacterial strain; Benghal dayflower; nodal segment; organic solvent; plant extracts

Abbreviations: ALR: average length of roots; FSMS: full strength MS; HSMS: half strength MS; LS: length of shoots; PGR: plant growth regulators; R/M: roots per microshoot; RE: responsible of explants; RR/M: response of roots per microshoot; RP: responsible parameter; SPP: shoots per plant

Introduction

The species *Commelina benghalensis* L. ($2n = 22$) belongs to the family Commelinaceae includes large number of species that differ widely in their characteristics. Generally, it is usually known as “Benghal dayflower”. It has several scientific synonym names such as *C. kilimandscharica*, *C. obscura*, *C. pyrroblepharis*, *C. rufociliata* and *C. uncata*. This plant is a prostrate or scrambling ascending herb of about 1m in height. In the Kalangala district of Uganda, it is used as a wound ecbolic on skin or over a wound by crashing whole fresh

plant with mortar and pestle (Hamill *et al.*, 2003). Traditionally, the people of china use this plant as a diuretic, an anti-inflammatory agent. Hong and Defillipps (2000) Where Pakistani eat as a vegetable. Besides, according to Manandhar and Sanjay (2000) the people of Nepal eat the young leaves as a vegetable, use a paste of plant for burns and the fluid of roots is also use to treat indigestion. In Bangladesh, for the treatment of otitis media, burns and conjunctivitis, this plant is ethno-used. The plant is also used for skin disease such as eczema, acne, warts and scabies etc. But the responsible compounds of these medicinal properties have not been identified yet. It also contains alkanols, sterols, stigma sterol, beta-sito sterol and compesterol (Ghani, 2003). In these connections, plant tissue culture has a potential to introduce genetic variability in *Commelina* genotypes through somaclonal variants, somatic hybrids or transgenic plants. It's considered as a promising technology to get these subjects and it's also played a major role in the mass multiplication, conservation, secondary metabolites production and sustainable use of medicinal plants (Hasan and Roy, 2005). In the beginning of ethno-use of plants, people were used plants or plants derived substance to treat their health illnesses which is existing in modern health practices in almost all ancient civilizations, the Egyptian, the Chinese and even Greek and Roman civilizations (Aftab and Sial, 1999). However, although antibiotic considered as an important weapon for fighting against pathogen, it shows less efficiency on certain illnesses due to emergence of drug resistant bacteria. Plants can be used for various pharmacological purpose whereas it's known as a reservoir of secondary metabolites. Antibiotics is continuously decreasing its resistance power which is insufficient to control some pathogen due to non-prescribed use. In these circumstances, it is very urgent necessary to induce newer drug with high resistance (Sarkar *et al.*, 2003). In the present study, plant regeneration and antibacterial activity investigation using the extracts (methanolic, ethanolic and petroleum etheric) were done.

Materials and Methods

Explants collection and sterilization

The juvenile explants of *Commelina benghalensis* L. were collected from Rajshahi university campus, Rajshahi, Bangladesh. Shoot tips, nodes, internodes and leaf segments were used. Explants were washed thoroughly under running tap water for 15 minutes and then washed with continuous agitation in a few drops Savlon™ containing water for 15 minutes. The pre-treated explants were then treated with 0.1% HgCl₂ for 5 minutes under laminar air flow to disinfect them. Finally, explants were washed 3 to 5 times with sterile distilled water.

Culture media preparation and inoculation

Explants were cultured on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations and combination of cytokinin i.e. BAP (6- benzyl amino purine), KIN (kinetin) and auxin i.e. IBA (Indole-3 butyric acid), IAA (Indole-3 acetic acid) and NAA (Naphthalene acetic acid) for axillary shoot proliferation. The pH of the medium was adjusted to 5.80 ± 2 before autoclaving at 121 °C for 20 minutes at 1.2 kg/cm² pressure. For the root formation different types of auxin were used with half strength and full strength of MS media. *In vitro* elongated shoots (5-6 cm) bearing at least 3-4 internode were excised from the mass of proliferated shoots and transferred to rooting medium. The explants in all experiments were sub cultured at 2-week intervals.

Hardening

Rooted plantlets were carefully washed with tap water and transferred to polycups containing sterile soil and vermiculite (1:1) and covered with plastic bag to maintain humidity. Finally, plantlets were acclimatized under field condition.

Data analysis

The data pertaining to number of inoculated explant response, number of multiple shoots per plant, length of shoots per microshoot and rooting were subjected to analysis of variance (ANOVA) test. Mean separation was done using Duncan's Multiple Range Test (DMRT) ($P < 0.05$).

Sample collection and culture media preparation

In the present investigation, five-gram (+ve) bacteria (*Staphylococcus aureus*, *Bacillus cerus*, *Streptococcus phaemolytica*, *Bacillus rubbitis*, *Sarcina lutea*) and five-gram (-ve) bacteria (*Klebsiella strain*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella dysentriae*, *Klebsiella pneumoniae*) were used to investigate the antibacterial activity of the plant extracts of *Commelina benghalensis* L. Those bacteria were collected from the international Centre for Diarrhoeal Diseases Research of Bangladesh (ICDDR, B). In this study, nutrient LB (Laury broth) medium was used for antibacterial screening. For bacterial growth 28 g nutrient was taken into two separate 500 ml autoclaved conical flask and the media were properly dissolved with distilled water then sterilized. Sterilized media were allowed to cool and poured into the autoclaved petri discs in laminar airflow cabinets.

Bacterial suspension preparation and plant extract preparation

10 ml of distilled water was taken into the screw capped test tube and pure colony of freshly cultured bacteria which earlier prepared were added into the tube and vortexed. The OD (optical density) was measured with the colorimeter and microbial population was also confirmed to be within in 10^7ml^{-1} to 10^8ml^{-1} . This suspension was used as inoculums. On the other hand, properly drying plant materials were pulverized into a coarse power. 30 g of studied plant powder was weighted with the electric balance and 60 ml each of the solvents (ethanol, methanol, petroleum ether) was added in each conical flask. The samples with solvents were placed in water bath shaker for 5-6 hours. The extracts (ethanol, methanol, petroleum ether) were filtered using filter paper than air dried after filtration to concentrate. The experiment was performed with three replications. Three concentrations i.e. 400 mg/l, 600 mg/l, and 800 mg/l) of plant extracts were used on paper disc to create zone of inhibition which was measured in terms of millimetre.

Disc preparation, incubation and measurement

In vitro antibacterial activities of the extracts were measured by employing standard agar disc diffusion method (Bauer *et al.*, 1966). In this method, the filter paper was punched with the punching machine for preparing disc and sterilized. The discs were placed aseptically over the bacterial culture which was earlier prepared by striking with sterile transfer loop on nutrient agar plates and incubated at 37 °C temperature for 20 h. After incubation, the zone of inhibition around the disc was measured by millimetre scale. Discs were impregnated with each treatment and control was assayed on duplicate agar medium plate for all bacterial species. Blank paper discs were impregnated with only solvent (petroleum ether, methanol and ethanol) and used as negative control and Tetracycline (30 µg/disc) contained paper disc were used as positive control each time. In the present study, it was determined following the serial dilution technique according to Reiner (1982).

Results and Discussion

The present investigation was carried out to standardize some of the aspects of *in vitro* plant regeneration from the excised tissues and antibacterial screening of Benghal dayflower. For easy understanding and precise assessment of the results, the present investigation has been carried out under two parts.

Micro propagation

The experiments were conducted with four different types of explants viz. shoot tips, nodes, internodes and leaf segments. Differences were taken into account regarding to regeneration system, explants type and response to nutrient media, growth regulator type and concentration as well as other chemical and physical factors, in regenerating the complete plantlets of Benghal dayflower subsequently, tested for antibacterial activity against a number of bacterial strains. For the best explant's selection, different concentration of cytokinin like, BAP or KIN either alone or in combination with auxin i.e. IAA, NAA or IBA were used on MS basal medium and observed on morphogenetic response of field grown surface sterilized juvenile explants (shoot tips, internodes, leaf segments and nodal segments) of *C. benghalensis*. Observing phenomena denoted that nodal segments were found to be the best source of multiple shoot buds' inductions. This result similar with other medicinal plants *Holarthema antidysenterica* (Ahmed *et al.*, 2001) and *Enicostemma hyssopifolium* (Seetharam *et al.*, 2002). It also indicates that single node explants elicited more numbers of multiple shoots as compared to shoot tip explants in cytokinin media. Similar results were also noted in *Zehneria scabra* (Anand and Jeyachandran, 2004) and *Eclipta alba* (Borthakur *et al.*, 2000). Plant proliferation by *in vitro* process from nodal segments is simple and it is also economically more efficient technology. In this study, we also found that shoot buds' induction and auxiliary shoots proliferation were more effective and better on MS media supplemented with BAP than others cytokinin as also reported earlier *Zehneria scabra* (Anand and Jeyachandran, 2004), *Alternanthera sessilis* (Boro *et al.*, 1998).

Table 1. Effect of cytokinin (BAP & KIN) of Benghal dayflower with different concentrations and combinations of auxin (IBA, IAA & NAA) on axillary shoot proliferation from the nodal explants

PGR (mg/l)					RP		
BAP	KIN	IBA	IAA	NAA	RE (%)	SPP (no.)	LS (cm)
2.0	-	0.1	-	-	89.46 ab	20.18 abc	15.34 abcd
2.0	-	0.5	-	-	89.71 ab	18.05 bc	15.27 abcd
3.0	-	0.1	-	-	90.52 a	23.25 a	16.59 ab
3.0	-	0.5	-	-	88.91 abc	19.58 abc	17.25 a
2.0	-	-	0.1	-	87.86 abc	20.20 abc	16.21 abc
2.0	-	-	0.5	-	87.86 abc	22.08 ab	13.30 abcdef
3.0	-	-	0.1	-	85.32 abcde	17.26 cde	14.59 abcde
3.0	-	-	0.5	-	86.92 abcd	16.32 cdefg	15.29 abcd
2.0	-	-	-	0.1	85.19 abcde	17.80 cd	14.51 abcde
2.0	-	-	-	0.5	83.13 bcdef	19.83 abc	15.22 abcd
3.0	-	-	-	0.1	82.69 cdef	16.83 cdef	13.51 abcdef
3.0	-	-	-	0.5	83.18 bcdef	17.77 cd	14.35 abcde
-	2.0	0.1	-	-	82.48 cdef	13.83 defgh	13.21 abcdef
-	2.0	0.5	-	-	80.97 def	12.21 h	12.55 bcdef
-	3.0	0.1	-	-	79.94 efg	13.14 fgh	10.82 def
-	3.0	0.5	-	-	77.74 fghi	10.44 h	11.82 cdef
-	2.0	-	0.1	-	79.97 efg	13.42 efgh	11.38 def
-	2.0	-	0.5	-	79.00 efgh	12.88 fgh	12.06 bcdef
-	3.0	-	0.1	-	76.51 fghi	11.69 h	9.35 f
-	3.0	-	0.5	-	73.18 hi	13.16 efgh	10.19 ef
-	2.0	-	-	0.1	73.78 ghi	11.43 h	11.00 def
-	2.0	-	-	0.5	73.17 hi	12.89 gh	9.58 f
-	3.0	-	-	0.1	72.09 i	11.35 h	11.72 cdef
-	3.0	-	-	0.5	72.50 hi	12.45 gh	9.13 f

In a column, the figures with similar letter (s) do not differ significantly by DMRT (Duncan's multiple range test) at $p < 0.05$

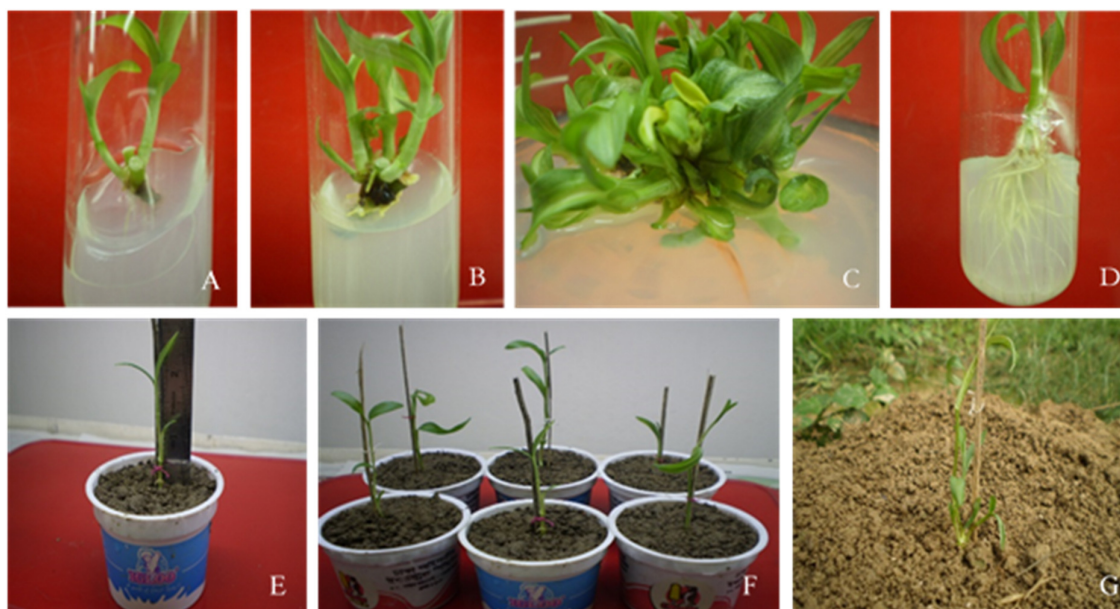


Figure 1. Micropropagation of *Commelina benghalensis* L.

(A, B & C: Shoot proliferation on 1 week, 2 weeks and 3 weeks after inoculation; D: Rooted plantlet; E & F: 2 weeks and 3 weeks hardening plantlets; G: Acclimatization)

As shown in Table 1 the maximum (90.52%) response of explant and number of shoots (23.25) per plant were showed from nodal segments on MS medium fortified with BAP 3.0 mg/l + IBA 0.1 mg/l but the highest length (17.25 cm) of shoots were obtained whilst IBA 0.5 mg/l was applied with BAP 3.0 mg/l. These results disparity with Sarwar *et al.* (2009) have reported that BAP 1.0 mg/l with NAA 0.5 mg/l, 75 % and 70% shooting response was observed respectively from shoot tip and nodal segments of *Commelina benghalensis* L. These contrast results may be occurred due to strength of MS and media combinations. On the other hand, the lowest (72.09%) explant response, minimum number (10.44) of shoots per plant and the lowest (9.13 cm) length of shoot were found at NAA 0.1 mg/l, IBA 0.5 mg/l and NAA 0.5 mg/l with BAP 3.0 mg/l respectively. For root induction three types of auxin i.e IBA, IAA and NAA were used where four concentration of each auxin (0.5-2.0 mg/l) and two strength of MS media were tested as shown in Table 2.

Table 2. Effect of auxin for adventitious root formation from *in vitro* proliferated shoots

PGR mg/l	FSMS			HSMS			
	RR/M (%)	R/M (no.)	ALR (cm)	RR/M (%)	R/M (no.)	ALR (cm)	
IBA	0.5	94.37 abc	10.15 abc	4.99 abc	88.97 abc	7.52 abcd	4.09 abc
	1.0	96.08 ab	11.06 abc	6.32 a	93.17 a	8.30 ab	5.77 a
	1.5	99.12 a	12.69 a	5.91 ab	90.44 ab	9.04 a	4.91 ab
	2.0	94.70 abc	11.98 ab	4.62 abcd	88.17 bcd	7.75 abcd	4.39 abc
NAA	0.5	93.37 abcd	11.99 ab	3.79 bcd	83.64 def	8.63 ab	2.96 bc
	1.0	89.90 bcd	12.85 a	5.36 ab	86.56 bcd	8.21 abc	3.71 abc
	1.5	91.16 abcd	11.12 abc	4.15 abcd	84.61 cde	4.36 e	3.50 abc
	2.0	89.21 bcd	9.12 bc	3.79 bcd	85.14 cde	5.69 cde	3.26 bc
IAA	0.5	93.49 abcd	10.03 abc	4.50 abcd	87.12 bcd	5.49 de	3.92 abc
	1.0	87.50 bcd	10.39 abc	2.42 d	81.63 ef	3.97 e	2.36 c
	1.5	86.34 cd	8.18 c	2.68 cd	81.86 ef	6.39 bcde	3.55 abc
	2.0	85.12 d	8.42 bc	2.42 d	79.36 f	4.53 e	2.45 c

In a column, the figures with similar letter (s) do not differ significantly by DMRT at $p < 0.05$

In this study, two types of MS strength i.e. full strength and half strength of MS were used. Among these media, we found that full strength of MS media was better for root formation than half strength of MS media. The maximum (99.12%) response of micro-shoot and highest (12.69) number of roots per micro-shoot were obtained on full strength of MS medium having 1.5 mg/l IBA. The highest (6.32 cm) average length of roots was found at IBA 1.0 mg/l. For acclimatization, well rooted plantlets were gently removed from the test tubes and thoroughly washed with running tap water to remove traces of medium and transferred to plastic pots having soil and compost (1:1). Through this process of acclimatization, regenerated plantlets were established under *ex-vitro* conditions. About 97% of plantlets survival under *ex vitro* environment was observed after hardening of *C. benghalensis* as shown in Figure 1.

Evaluation of antibacterial activity

For the assessment of antibacterial activity of *C. benghalensis*, three types of organic solvent i.e. methanol, ethanol and petroleum ether were used to prepare plant extract.

In this study, ethanol and methanol extract were more active because of consistent more antimicrobial activity against the gram (+ve) bacteria than on gram (-ve) bacteria. This is in agreement with previous reports (Vlietinck and Vanden, 1991; Rabe and Van, 1997). The experimental data was obtained from the disc diffusion method (Table 3) indicate that all of three extracts exhibited a variable degree of antibacterial activity by producing zone of inhibition with few exceptions on different tested bacterial strains. These extracts of leaf and stem of studied plant were shown zone of inhibition at three concentrations i.e. 400 mg/ml, 600 mg/ml, and 800 mg/ml against all tested bacterial stain except *E. coli* and *S. typhi* (Table 3). These results indicate that plant extracts were showed its activity against tested gram-positive and gram-negative bacteria that may lead to active with broad spectrum. It's also denoted that it would be performance against multidrug-resistant organisms for the probability of developing therapeutic substances. Range of zone of inhibition was found from 08.05 mm to 15.75 mm at all tested concentrations of petroleum ether extract of studied plant. Highest zone of inhibition was produced against *S. aureus* (12.28, 14.08 and 15.75) mm and *Pseudomonas aeruginosa* (12.75, 13.84 and 15.05) mm at all tested dose levels respectively. *S. β-pharmolytica* bacterial species was showed minimum 08.05 mm zone of inhibition at 400 mg/ml concentration level. On the other hand, minimum (10.55 and 11.75) mm zone of inhibition was obtained at 600 mg/ml and 800 mg/ml concentration against *S. lutea* and *B. subititis*. From these observations, it was indicated that the producing zone of inhibition was higher against gram (+ve) bacteria than gram (+ve) bacteria. This result is similar with (Jigna and Sumitra, 2006) as reported earlier.

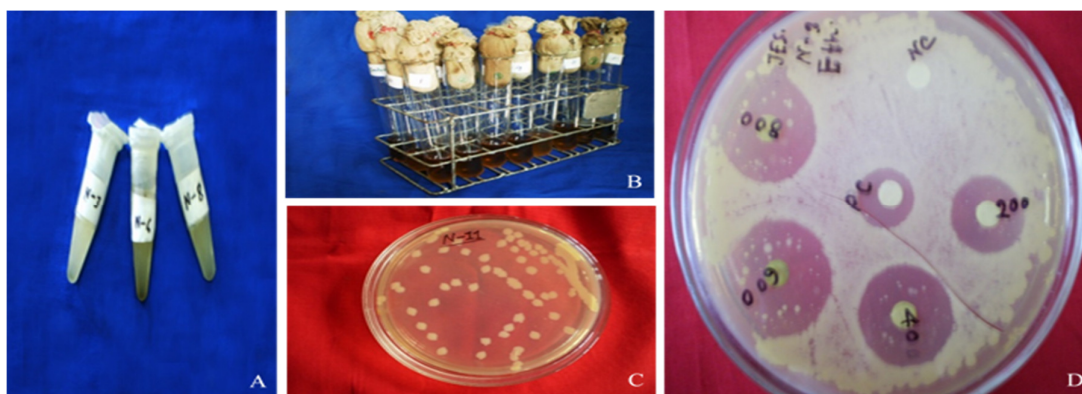


Figure 2. Antibacterial activity of crude extracts of *Commelina benghalensis* L.

(A: bacteria stock; B: Bacterial suspension; C: Bacteria with single colony; D: Ethanolic extracts showing inhibition zone in four concentration i.e. 200 mg/l; 400 mg/l; 600 mg/l; 800 mg/l with positive and negative control)

Table 3. Antibacterial screening of *Commelina benghalensis* against pathogenic bacteria on different extracts

		Zone of inhibition (mm)								
		Methanol extracts			Ethanol extracts			Petroleum ether extracts		
		400 mg/ml	600 mg/ml	800 mg/ml	400 mg/ml	600 mg/ml	800 mg/ml	400 mg/ml	600 mg/ml	800 mg/ml
Gram Positive	<i>Staphylococcus aureus</i>	13.58 a	14.67 a	16.44 a	14.47 a	15.52 a	17.50 a	12.28 a	14.08 a	15.75 a
	<i>Bacillus cereus</i>	12.89 ab	13.85 ab	15.96 ab	13.18 ab	14.05 abc	15.58 bc	10.85 ab	13.39 ab	13.98 ab
	<i>Streptococcus β-phaemolytica</i>	12.15 ab	10.87 c	14.34 b	12.69 ab	13.53 bc	14.84 c	08.05 c	12.65 ab	13.35 ab
	<i>Bacillus subtilis</i>	11.24 b	11.66 bc	15.36 ab	11.05 c	13.85 c	16.03 abc	10.35 ab	11.74 b	11.75 c
	<i>Sarcina lutea</i>	10.05 c	13.25 ab	12.05 c	12.83 ab	13.61 c	14.75 c	10.45 ab	10.55 c	13.50 ab
Gram Negative	<i>Pseudomonas aeruginosa</i>	13.34 a	14.75 a	16.21 a	14.25 a	15.42 a	17.44 a	12.75 a	13.84 a	15.05 a
	<i>Escherichia coli</i>	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
	<i>Salmonella typhi</i>	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
	<i>Shigella dysenteriae</i>	10.75 bc	13.62 ab	13.45 bc	12.15 b	14.35 abc	16.75 ab	10.15 ab	11.25 bc	12.65 b
	<i>Klebsiella pneumoniae</i>	12.88 ab	11.84 b	14.98 ab	12.60 ab	14.78 ab	16.31 abc	09.75 b	13.38 ab	14.15 ab
	Average (+ve) control.	14.23	15.29	17.28	15.75	17.01	18.35	13.29	15.39	16.95

In a column, the figures with similar letter (s) do not differ significantly by DMRT (Duncan's multiple range test) at $p < 0.05$; Nd: Not detected zone of inhibition

In case of methanol extract it produces the maximum zone of inhibition was showed against *S. aureus* (13.58, 14.67 and 16.44) mm and *Pseudomonas aeruginosa* (13.34, 14.75 and 16.21) mm at all concentration levels respectively. *S. lutea* bacterial species was showed lowest (10.05 and 12.05) mm zone of inhibition at 400 mg/ml and 800 mg/ml dose level whilst 10.87 mm zone of inhibition was found at 600 mg/ml against *S. β phaemolytica* tested bacteria. As shown in (Table-2), Ethanol soluble fraction of leaf and stem of Benghal dayflower was found the highest inhibitory activity under *in vitro* condition against tested bacterial species comparing the methanol and petroleum ether extract due to allow the extraction of chemical components that were showed activity against those bacteria. Highest zone of inhibition was obtained against *S. aureus* (14.47, 15.52 and 17.50) mm and *Pseudomonas aeruginosa* (14.25, 15.42 and 17.44) mm at all concentrations respectively. Besides, *S. aureus* was the most sensitive bacterial strain with the strongest inhibition zones (17.50 mm) at 800 mg/ml dose level followed by *K. pneumoniae* (16.31 mm), *P. aeruginosa* (17.44 mm), *S. dysenteriae* (16.75 mm), *B. cereus* (15.58 mm), *S. β -phaemolytica* (14.84 mm), *B. subtilis* (16.03 mm) and *S. lutea* (14.75 mm). Negative control (disc containing only solvent) exhibited no zone of inhibition against all tested bacterial strain. But positive control (disc containing tetracycline antibiotic) exhibited zone of inhibition against almost all bacteria. Besides, these data indicate that zone of inhibition were increasing compare with increasing of dose level of extracts against all studied bacterial stain except *S. dysenteriae*, *K. pneumoniae* bacterial stain on methanol extract and *S. β -phaemolytica* on methanol and petroleum ether extracts. It may be occurred due to exist inadequate active components in crude extracts as also reported earlier (Taylor *et al.*, 2001). Comparatively, Gram-negative bacteria exhibited low susceptibility to the extract which is in accordance with the fact that those have high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier, and it is highly resistant even to synthetic drugs. Considering the three extracts of Benghal dayflower ethanol extract showed the best antibacterial activity.

Conclusions

From the experiment of *in vitro* propagation of *C. benghalensis* L. it can be duly inferred that the BAP fortified with IBA is the excellent media for Commercial growth of *C. benghalensis* L. We also conclude that the research platform may be formed from this screening experiments lead to isolation of the therapeutic antibacterial and further pharmacological evaluation.

Authors' Contributions

MMI designed the methodology of the experiments and executed the study activities. MAM analyzed the data improving the methodology. MFA supervision of the study, revised and improved English language. All of the authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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