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In-vitro Callogenesis and Screening of Antimicrobial Activity of Callus and Seed of *Caesalpinia bonducella* F.: A Threatened Medicinal Plant of Western Ghats

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Authors' contributions

This work was carried out in collaboration among all authors. Author RS did the methodology writing and original draft. Author AR did the conceptualization and formal analysis. Author AK did the review and editing the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Caesalpinia bonducella* Flem is a dioecious scrambling woody liana of Caesalpinoideae, a subfamily of Leguminosae. The plant is threatened and distributed in the deciduous forests of the Western Ghats of India. Being an important medicinal plant *Caesalpinia bonducella F.* attracted many scientists to exploit various activities associated with a number of phytoconstituents. The present study was undertaken to evaluate the most suitable media and suitable concentrations of plant growth regulators for *in vitro* Callogenesis and screening of antimicrobial activity of callus and seed of *Caesalpinia bonducella*.

Materials and Methods: Callus was initiated from stem explants, on 1x and 0.5x MS medium plus supplements. The effects of plant growth regulators on callus cultures were studied and observations were made. The *in vitro* antibacterial activity was performed by using extracts of callus and seed of *Caesalpinia bonducella* in petroleum ether and methanol against multidrug resistance organisms. The organic extracts of seed and callus of the plant at concentrations of 0.02 mg/ml - 0.1 mg/ml were taken and their activities were measured.

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Results: The combination of 2.5 mgL^{-1} 2,4-D with 2 mgL^{-1} BAP, resulted in the highest frequency and the highest mean percentage of callus formation (2.35 ± 0.294) with yellow friable callus. The results revealed that all the extracts had a variable degree of antibacterial activity.
Conclusions: It was observed that 2,4-D at 2.5 mgL^{-1} in combination with BAP,2.0 mgL^{-1} BAP resulted in early initiation, highest induction percentage, with frequency highest mean percentage of callus formation, Antimicrobial tests with methanol and diethyl ether extract of *Caesalpinia bonducella* seed powder against the clinical isolates showed the zone of inhibition for all the pathogens tested with concentration of methanolic extract of *C. bonducella* seed powder.

Keywords: In vitro callogenesis; antimicrobial activity; Caesalpinia bonducella; phytoconstituents.

ABBREVATIONS

2,4-D - 2, 4-Dichlorophenoxyacetic acid; BAP -6-Benzylaminopurine; IBA -Indole 3- butyric acid.

1. INTRODUCTION

Caesalpinia bonducella Flem is a dioecious scrambling woody liana of Caesalpinoideae, a subfamily of Leguminosae. The plant has spiny stem and bipinnate leaves. The plant is threatened and distributed in the deciduous forests of the Western Ghats of India [1] Sri Lanka, etc In India it is especially found in tropical regions [2]. In the Ayurvedic system of medicine, the plant is popularly known as fever nut.The plant contains biologically active compounds like phenols, diterpenes, flavonoid, alkaloids and tannins. *Caesalpinia bonducella* is well known for its several medicinal properties. It has been reported that different parts of the plants are used for several ailments like diabetes [3,4,5], anti-inflammatory [6,7], anthelmintic [8], anti-estrogenic [9], antimalarial [10,11,12], and memory enhancer [13]. Overutilization of the plant for medicinal purposes and the destruction of natural habitat makes the species to be endangered [14]. In recent years, *In vitro* culture has achieved major industrial importance in the production of secondary metabolites, and callus cultures have been employed in the study of secondary metabolism. The *In Vitro* Callogenesis technique provides an appealing alternative for the production of valuable secondary metabolites, and they have been used throughout the years as a tool for the elucidation of the biosynthesis of metabolites [15].

There are reports of *in vitro* culture of *Caesalpinia bonducella* [16,17,18,19] dealing with callus studies as well as direct and indirect organogenesis using different explants. Since *Caesalpinia bonducella* has an immense medicinal value, the present paper attempted to derive *in vitro* callogenesis using stem explants. To evaluate the effect of different strength of Murashige and Skoog (MS) media (1x and 0.5x MS medium) and Plant growth regulators on callusing was the main objective of this study.

Microbes are responsible for many infectious diseases [20,21]. Although antibiotics are one of the most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life for a long time, their continuous use makes clinical pathogens drug-resistant [22,23]. The increasing clinical implications of drug-resistant fungal and bacterial pathogens has prompted antimicrobial drug research. Kachhawa et al. [24] suggested that there is a need to search for new antimicrobial agents from plants to control microbial infections. Among the potential sources of new agents, plants have long been investigated, as they contain many bioactive compounds that can be of interest in therapeutics. Literature states that plants are the natural reservoirs of many antimicrobial agents [25,26]. Shula et al. [27] observed *Caesalpinia bonducella* (defatted and non-defatted) extracts with different solvents possess potent antibacterial activity. They reported that acetone and ethanol extracts exhibited the highest antibacterial activity against all the tested bacteria used. They mentioned the antibacterial activity increased with the increased proportion of the extract concentration. Tambekar et al. [28] reported that methanol extract of *Caesalpinia bonducella* proved antibacterial to *Staphylococcus aureus, Shigella flexneri*, and *Enterobacter aerogenes*.

The difference in the antibacterial potentials of different extracts suggested that the solubility of various phytochemicals in various solvents made it different from the others. As the literature suggests *Caesalpinia bonducella* (defatted and non-defatted) extracts with different solvents

possess potent antibacterial activity. Therefore, in the present study, an attempt has also been made to test the antimicrobial activity of callus and seed powder of *Caesalpinia bonducella* against gram-positive and gram-negative bacteria.

2. MATERIALS AND METHODS

2.1 Induction of Callus in *Caesalpinia bonducella*

2.1.1 Plant material

The plant material was collected from foot hill of Matheran in Raigad District, Maharashtra, India in the month of September and was maintained in the medicinal plant garden of Smt. C.H.M. College, Ulhasnagar from Thane district of Maharashtra, India.

2.1.2 Surface sterilization of explants

2.1.2.1 Surface sterilization of explants

Internodal segments of the stem were used as explants for callus induction. After the collection, the stem were excised into small segments (1 to 1.5 cm). These explants were kept under running tap water for 30 minutes and then rinsed with Dettol for 10 minutes. Tween-20 (a detergent and antiseptic) was used as a surfactant for 10 minutes. Bacillocid at a concentration of 1% (v/v) and Bavistin (1% w/v) were used for 15 minutes as antibacterial and antifungal agents respectively. The explants were washed thrice with sterilized distilled water, followed by treatment with 70% alcohol for 30 seconds. The explants were swirled for 1-2 minutes in 0.07% $HgCl_2$ (w/v) followed by repeated washings with sterilized distilled water. The explants were trimmed. Surface-sterilized explants were blot dried with sterilized tissue paper. Such explants were aseptically inoculated onto Murashige and Skoog(MS 1962) mediums supplemented with different combinations of auxins and cytokinins.

2.1.3 *In vitro* callus induction and proliferation

Callus initiation experiments were carried out using internodal segments of the stem. The media used for induction studies was Murashige and Skoog (MS 1962) [29], and half-strength MS media. Plant growth regulators used in the present study are 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzylamunopurine (BAP), indole-3-butyric acid (IBA), and 6-furfuryladenine (Kinetin) at different concentrations (1.0 mgL^{-1},2.0 mgL^{-1} and 3.0 mgL^{-1}) and combinations either singly or in combinations. Sucrose (3%) was used as a carbon source. The gelling agent was 0.8% (w/v) Agar (Bacteriological Grade, Qualigens, Mumbai). The pH of the medium was adjusted to 5.7 with 0.1N NaOH or 0.1N HCl as per requirement and autoclaved. The cultures were incubated at 25 ± 2^0C under 16 hrs photoperiod with cool, white fluorescent tube light (2000 Lux) with 60 ±10 % relative humidity.

After callus induction; the calli were transferred to the fresh medium of the same combination. Every 15 days of incubation the cultures were maintained for further proliferation. The inoculated culture tubes were observed at an interval of 3 to 4 days and the results were recorded. Callus was sub-cultured at regular intervals in the appropriate medium for further studies.

The chemicals for these experiments were used of analytical grade and procured from HiMedia Pvt Ltd Mumbai. Sigma Chemical Co., USA, and E-merck, (India).

2.1.4 Antimicrobial studies of callus

2.1.4.1 Preparation of extract

The seed powder and callus of *Caesalpinia bonducella* was used to screen their antibacterial potential. The extracts were prepared by cold infusion method, suggested by Handa [30]. Five grams of callus was homogenized in a minimum quantity of methanol and diethyl ether at a concentration of 40%. It was kept for 48 hours. The volume was made to 50 ml using respective solvents. After 48 hours, the extracts were filtered using Whatman grade 1 filter paper. The extracts were evaporated to dryness. Prior to use, the residue were dissolved in known quantity of solvents (stock solutions) so as to maintain the concentrations (Table A1). To get the seed extract, the seeds of *Caesalpinia bonducella* were separated from the testa and were sun-dried. The kernels of seeds were ground into fine powder. A procedure which was used for preparation of callus extract was followed in a similar way to get the extract from seed powder. Similarly, methanol and diethyl ether were used as organic solvents.

2.1.4.2 Bacterial strains

The Gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus* and Gram-negative

bacteria *Escherichia coli*, *Klebsiella pneumonia* were selected because of their highly pathogenic nature. The required bacterial cultures were obtained from Department of Pharmacology, Bombay college of Pharmacy, Kalina, Santacruz, (Mumbai). These clinical isolates as pure cultures were maintained by regular sub culturing on nutrient agar slants. The bacteria were sub cultured 48 hours prior to use so as to get vigorously growing microorganisms.

2.1.5 Antibacterial bioassay

Antibacterial activity of callus of *Caesalpinia bonducella* was studied by the agar well diffusion method [31]. The Methanolic and Diethyl Ether extracts were tested in dose levels of 0.02 to 0.1 mg/ml. Stock solutions of extracts were prepared in Saline solution (Table A1). The Mueller-Hinton's [32] agar medium was prepared. The suspension was prepared from 48-hour old cultures. One ml of culture suspension added in sterile molten agar butts of MH media, having an approximate temperature of 45^{o}C. The butts were mixed thoroughly and the media was poured into sterile petri plates and inoculated with one ml of culture suspension of the above-cited test organisms. The Mueller-Hinton agar medium was allowed to set at room temperature for about 10 minutes. The plates were left undisturbed for media to set and allowed to solidify in a refrigerator for 30 minutes. With the help of sterile cork borer wells of 6mm diameter were made in each petri plate. Wells were filled with 20µl of different concentrations of each extract. The petri plates were incubated for 48 hours at 37°C. After 48 hours, the zone of inhibition was observed and results were recorded by measuring the diameter in millimeter. Inhibition zones are the average values calculated from three replicate. The results were tabulated. Positive control, negative control, and medium control were also maintained as per Table A2. All the experiments were carried out in triplicate.

Table A1. Dilution table for the preparation of different concentrations of extract

Sr. No.	Extract (ml)	Saline (ml)	Total Volume (ml)	Percentage (%)
1	0.02	4.08	5	0.2
2	0.04	4.06	5	0.4
3	0.06	4.04	5	0.6
4	0.08	4.02	5	0.8
5	0.1	4.00	5	1.0

Table A2. Results for positive control, negative control and medium control

Sr. No.	Controls	Culture	Incubation time and temperature	Results
1.	Positive Control (Sterile MH agar butt with 1 ml culture poured in sterile petriplate)	*E. coli* *K. pneumoniae* *B. subtilis* *S. aureus*	37^{o} C for 24 hours	+ + + +
2.	Negative Control (Sterile MH agar butt with 1ml culture poured in sterile petriplate)	*E. coli* *K. pneumoniae* *B. subtilis* *S. aureus*	4^{o} C for 24 hours	- - - -
3.	Medium Control (Sterile MH Agar poured in sterile petriplate)	No microorganisms	37^{o} C for 24 hours	-

3. RESULTS AND DISCUSSION

3.1 Induction of Callus

In this study, the two different media composition were used. Murashige and Skoog (MS) and half-strength MS and the result indicated that both MS and half Strength MS with auxins alone, and in combination with cytokinins responded for induction of callus using intermodal segment of stem.

3.1.1 Effect of auxins on stem internode as explants

The two auxins used to analyse the potency for callus induction are as follows.

3.1.1.1 Effect of 2, 4-D on stem internode as explants

Results indicated that explants dedifferentiated and initiated calli at the cut surfaces. Within 7 to 12 days of inoculation calli initiated. After four weeks of inoculation culture has proliferated. For this MS with different concentrations of 2, 4-D (1.0 to 3.0 mgL^{-1}) used, among the different concentrations used in MS +3.0 mgL^{-1} 2, 4-D showed early initiation of calli. The initiation of callus took place on 6th day of inoculation and 70 % of explants responded for callus formation and after four weeks of inoculation, 0.43 ± 0.0412 g of callus formation was recorded, (Fig. 1) with creamish white, brittle calli. At a lower concentration, 1.0 mgL^{-1}, 2, 4-D initiation of callus took place on 9th day, with only 40%, explants responded for callus initiation, and 45 ± 0.0531g of callus formed (Table 1).

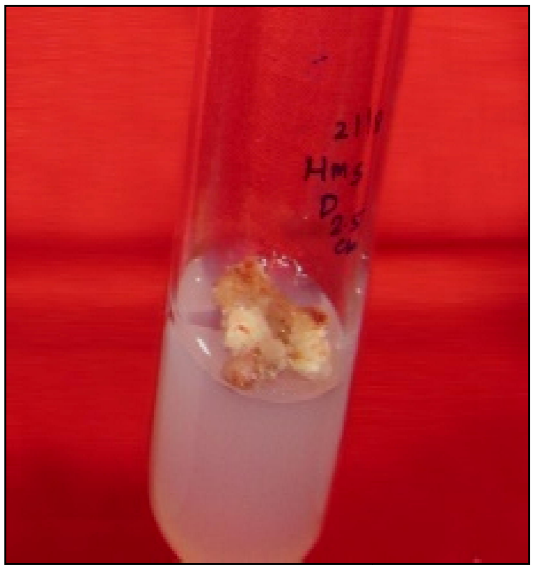

Fig. 1. Callus formation on 3.0 mgL-1 2,4-D in *C. bonducella*

In the case of Half Strength MS fortified with 2, 4-D (1.0 to 3.0 mgL^{-1}) initiation of calii took place on 7th day, At MS +2.0 mgL^{-1} 2, 4-D 60 % cultures responded for callus formation and after four weeks of inoculation, 1.24 ± 0.0570 g of callus formation is recorded with creamish white, brittle calli. At lower concentration, half-strength MS +1.0 mgL^{-1} 2, 4-D 45% explants responded.

Table 1. Effect of 2,4-D on callus growth of *C. bonducella* when supplemented to MS and half-strength MS media

Sr. No.	Media	Conc. of 2,4-D (mgL^{-1})	Callus induction (In no. of Days)	Callus formation% response	Callus formation in (g)	Morphology Callus
1	MS	1.0	9	40	1.45 ± 0.0531	Creamish white, hard
2		2.0	8	40	1.45 ± 0.0450	Creamish white hard
3		3.0	6	70	0.94 ± 0.1007	Creamish white, brittle
4	MS Half Strength	1.0	7	45	0.43 ± 0.0412	Creamish white, brittle
5		2.0	7	60	1.24 ± 0.0570	Creamish white, brittle
6		3.0	7	30	1.17 ± 0.0613	Creamish white, brittle

**Values represent mean ± standard error of 18 replicates per treatment in three repeats*

3.1.1.2 Effect of IBA on stem internode as explants

For study of effect of IBA on stem, MS with different concentrations of IBA (1.0 to 3.0 mgL^{-1}) were used. At all the concentrations, only 20 to 30 % explants responded for callus initiation. Maximum callus formation took place in 3.0 mgL^{-1} of IBA, and 0.48 ± 0.0368 g of calli formed (Table 2).In the case of Half Strength, MS fortified with IBA (1.0 to 3.0 mgL^{-1}) initiation of calii took place on 9th day, At MS +2.0 mgL^{-1} IBA, 60 % explants responded for callus formation and after four weeks of inoculation, 2.08 ± 0.0519 g of callus formation was recorded with creamish white, hard calli.

Out of these two media used half strength MS medium with both 2,4-D and IBA responded for induction of callus in stem internode, however MS medium was found to be better than half strength MS culture medium.

3.1.2 Effect of Cytokinins (BAP, Kinetin) on stem internode as explants

3.1.2.1 Effect of BAP on stem internode as explants

Effect of varying concentrations of BAP (1.0 mgL^{-1}, 2.0 mgL-1 and 3.0 mgL^{-1}) in MS media when supplemented with 2.0 mgL^{-1} BAP showed 80% callus induction. Initiation of callus started on 8th day. The callus obtained was creamish white and brittle. BAP at 3.0 mgL-1 concentrations in the media showed callus initiation on 7th day with 60% response. The callus obtained was greenish-white color and was brittle. Half strength MS medium with 3.0 mgL^{-1} BAP produced callus but the response was delayed as callus was initiated after 18 days and the growth of the callus was also slow. The obtained callus was greenish-white and compact. The other two concentrations, 1.0 mgL^{-1} and 2.0 $mgL^{-1}$of BAP induced moderate callusing and the response was 40% with initiation at 15th and 17th day respectively. When MS media was supplemented with 2.0 mgL^{-1} BAP initiation of callus was observed on 8th day with a good response (80%). The callus obtained was white colored and brittle.

Half strength modified MS media when supplemented with 3.0 mgL^{-1} BAP initiated callus on 5th day. The response was 60% and the appearance of callus was white and brittle. The concentrations 1.0 mgL^{-1} and 2.0 mgL^{-1} of BAP did not show significant results, as the response was 30% and 20% respectively with moderate callus induction. It was observed that the hard callus did not proliferate. However, soft callus proliferated at much faster than rate.

3.1.2.2 Effect of kinetin on stem internode as explants

Effect of varying concentrations of Kinetin (1.0 mgL^{-1}, 2.0 mgL^{-1} and 3.0 mgL^{-1}) with MS media when supplemented with 3.0 mgL^{-1} Kinetin showed 60% callus induction. Initiation of callus started on 12th day (Table 4). The callus obtained was white and hard. BAP at 2.0 mgL^{-1}concentrations in the media showed callus initiation on 12th day with only 10% response. The callus obtained was white and was hard.

Table 2. Effect of IBA on callus growth of *C. bonducella* when supplemented to MS and half-strength MS media

Sr. No.	Media	Conc. of IBA (mgL^{-1})	Callus induction (In no. of Days)	Callus formation % response	*callus formation in (g)	Morphology Callus
1	MS	1.0	10	20	0.42 ± 0.0207	Creamish white, hard
2		2.0	12	20	0.25 ± 0.0250	Creamish, hard
3		3.0	12	30	0.48 ± 0.0368	Creamish, hard
4	MS Half Strength	1.0	9	20	1.01 ± 0.0754	Creamish, hard, compact
5		2.0	9	60	2.08 ± 0.0519	Creamish white, hard
6		3.0	9	40	1.15 ± 0.04	Creamish white, hard

**Values represent mean ± standard error of 18 replicates per treatment in three repeated*

Table 3. Effect of BAP on callus growth of *C. bonducella* when supplemented to MS and half-strength MS media

Sr. No.	Media	Conc. of BAP (mgL^{-1})	Callus induction (In no. of Days)	Callus formation% response	Morphology Callus
1	MS	1	9	10	White, cottony
2		2	8	80	Cremish white, brittle
3		3	7	60	Greenish white, brilttle
4	MS Half Strength	1	15	40	Yellow white, compact
5		2	17	40	White, hard
6		3	18	30	Greenish white, compact

Table 4. Effect of Kinetin on callus growth of *C. bonducella* when supplemented to MS and half-strength MS media

Sr. No.	Media	Conc. of Kinetine (mgL^{-1})	Callus induction (In no. of Days	Callus formation% response	Morphology Callus
1	MS	1.0	--	--	--
2		2.0	12	10	Whitish, hard
3		3.0	12	60	Whitish, hard
4	MS Half	1.0	--	--	--
5	Strength	2.0	12	10	Creamish, hard
6		3.0	10	60	Whitish cream, hard

In the case of half-strength MS medium with 3.0 mgL^{-1} Kinetin produced callus but the response was 60% and callus was initiated after 10 days, the obtained callus was whitish cream and hard. Out of these two media used half-strength MS medium with both BAP and Kinetin responded for induction of callus in stem internode but MS medium was found to be better than MS half strength culture medium.Out of cytokinins used, BAP was found to be more suitable than Kinetin ,in terms of early initiation and percentage of explant in callus formation.

3.1.3 The combined effect of BAP and 2,4-D on callogenesis

The combined effect of BAP with both auxins was also evaluated, The BAP fortified media which responded optimal for callus induction and proliferation, MS +2.0 mgL^{-1} BAP combined with both 2,4-D/IBA as shown in Table 5. The observations were recorded after four weeks of inoculation of explants. The combined effect of BAP (2.0 mgL^{-1}) and 2,4-D at different concentrations were studied. Morphologically the callus was greenish-white or cremish and brittle at all the concentrations. It was observed that 2,4-D at 2.5 mgL^{-1} in combination with BAP,2.0 mgL^{-1} resulted in early initiation, highest induction percentage, with highest mean percentage of callus formation 2.35 ± 0.2941 (Table 5). This observation was also recorded by Meena K C, *et al* [17]. According to them, the addition of 1.0 mgL^{-1} 2, 4-D, or NAA in combination with BAP crucial for callus induction in the same species. Meena K. Cheruvathur *et al* [33] obtained optimum callus induction when the pulvini were cultured on MS medium fortified with 6.0 mgL^{-1} 2,4-D and 1.0 mgL^{-1} BAP. Santosh Kumar S.E *et al* [18] found that callogenic media for *Caesalpinia bonducella* consisted of a range of 0.25 mgL^{-1} to 3.0 mgL^{-1} 2,4-D and 0.1 to 0.5 mgL^{-1} BAP. It was also found that a lower concentration of 2,4-D with BAP showed a lower percentage of callus formation. In the case of the combined effect of BAP with different

concentrations of IBA, at 1.0 mgL^{-1} resulted in 1.75 ± 0.1140, the highest mean percentage of callus formation with creamish colored callus (Fig.3).

Fig. 2. Callus formation on MS + 2.5 mgL^{-1} 2,4-D in combination on 2.0 mgL^{-1} BAP

Table 5. Effect of BAP and auxins (2,4-D/IBA) on callus growth of *Caesalpinia bonducella* when supplemented to MS media

Sr. No.	PGR's	Concentration of PGR (mgL^{-1})	Callus induction (In no. of Days	Callus formation% response	*Fresh Weight (g)
1.	BAP + 2,4-D	2.0 + 0.5	08	45	0.32 ± 0.0161
2.		2.0 + 1.0	08	40	1.42 ± 0.0573
3.		2.0 + 1.5	07	60	1.61 ± 0.0658
4.		2.0 + 2.0	07	69	1.40 ± 0.0905
5.		2 .0 + 2.5	06	85	2.35 ± 0.2941
6.		2.0 + 3.0	06	70	1.78 ± 0.0787
7.	BAP + IBA	2.0 + 0.5	09	35	1.45 ± 0.0534
8		2.0 + 1.0	07	48	1.75 ± 0.1140
9		2.0 + 1.5	07	53	1.69 ± 0.3059
10		2.0 + 2.0	08	30	1.75 ± 0.1459
11		2.0 + 2.5	08	33	0.78 ± 0.0492
12		2.0 + 3.0	08	45	0.97 ± 0.0602

**Values represent mean ± standard error of 18 replicates per treatment in three repeated*

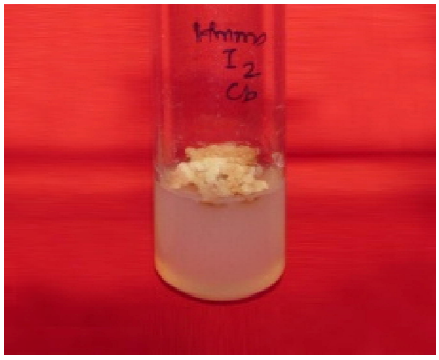

Fig. 3. Callus formation on MS + 1.0 mgL^{-1} IBA in combination with 2.0 mgL^{-1} BAP

3.2 Antimicrobial Activity of Callus and Seed of *Caesalpinia bonducella*

The effect of methanolic and diethyl ether extracts from the callus of *C. bonducella* on four pathogens is depicted in Table 6. All the bacterial cultures were found to be susceptible to the methanol extract of *C. bonducella* except *S. aureus*. It was observed that no inhibition zone was obtained at lower concentrations (0.2 mg/ml) for *E. coli* and *K. pneumonia*. Rest of the concentrations such as 0.04 ml, 0.06 ml 0.08 ml, and 0.1ml were found to be effective with increasing antimicrobial activity for *E. coli*. Hence, it can be interpreted that the higher concentration inhibited the growth of the microbial culture. *K. pneumoniae* and *B. subtilis* showed 13 mm zone of inhibition at 0.1mg/ml concentration (Fig. 4). It was observed that *E. coli* and *K. pneumonia* both Gram-negative bacteria were susceptible to the *C. bonducella* callus in methanol extract. For Gram-positive bacteria only *B. subtilis* showed zone of inhibition (0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml). *S. aureus* did not get affected at any concentrations of the extracts used. The extract in diethyl ether did not show any inhibitory effect against the selected pathogens (Table 6).

The results for antimicrobial tests with methanol and diethyl ether extract of *Caesalpinia bonducella* seed powder against the clinical isolates are depicted in Table 7. Highest concentration (0.1mg/ml) of methanolic extract of *C. bonducella* seed powder showed the zone of inhibition for all the pathogens tested (*E. coli*, *K. pneumonia, B. subtilis, and S. aureus*) Fig. 5. The lower concentrations such as 0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml and 0.08 mg/ml did not show any significant activity. It was observed that *B. subtilis* was the most susceptible amongst all the tested pathogens. The zone of inhibition was found to be 18mm diameter. The extracts with diethyl ether also inhibited the growth of *B. subtilis* by producing a zone of inhibition (15 mm in diameter at 0.1mg/ml concentration) (Fig. 6).

Table 6. Effect of *Caesalpinia bonducella* callus extracts on Gram-positive and Gram-negative bacteria

Extracts	Conc. (mg/ml)	Zone of inhibition (mm)			
		Gram-negative		Gram-positive	
		E. coli	*K. pneumoniae*	*B. subtilis*	*S. aureus*
Methanol	0.02	Nil	Nil	Nil	Nil
	0.04	**9**	**10**	Nil	Nil
	0.06	**12**	**11**	**9**	Nil
	0.08	**13**	**12**	**11**	Nil
	0.1	**15**	**13**	**13**	Nil
Diethyl Ether	0.02	Nil	Nil	Nil	Nil
	0.04	Nil	Nil	Nil	Nil
	0.06	Nil	Nil	Nil	Nil
	0.08	Nil	Nil	Nil	Nil
	0.1	Nil	Nil	Nil	Nil

Nil: No inhibition

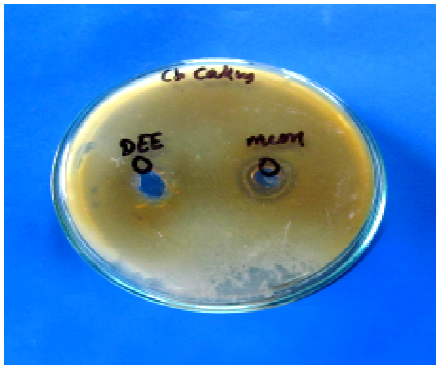

Fig. 4. Effect of methanolic callus extract of *C. bonducella* on *B. subtilis*

However, other concentrations were not found to be effective. Similarly, Parekh and Chanda [34] reported the maximum inhibitory activity by methanol extract of *Caesalpinia pulcherrima* against *E. coli*, *S. aureus*, *E. aerogenes*, *B. cereus*, and *K. pneumoniae*. Among them, *K. pneumoniae* was found to be most susceptible to the plant extract.

Bushra and Ganga, [35] reported that the solvents like ethanol, hexane, and methanol are used to extract plant chemicals and most of them can exhibit inhibitory effects on both gram-positive and gram-negative bacteria. Taraquzzaman et al. [36] observed that there was no significant activity with methanol extract of *Pterospermum semisagittatum* against gram-positive and gram-negative bacterial cultures. Contrary to this several reports are suggesting the methanol extracts have antibacterial properties. Kachhawa et al. [12] reported that *Pterocarpus marsupium*, a member of the family Leguminosae, showed potent antibacterial activity with methanolic extract against *E. coli*. Kamraj et al. [37] reported significant antimicrobial activity of *Acacia karroo* with the methanolic extract. Similarly, Parekh and Chanda [34] also reported the potent antibacterial activity with methanol extract compared to the aqueous extract of *Caesalpinia pulcherima*. Praveena and Suriyavathana [38] reported the methanolic extract of *Toddalia asiatica* showed antibacterial activity against *S. aureus, K. pneumonia, E. coli, P. vulgaris, P. aeruginosa, B. anthracis,* and *B. subtilis.* The zone of inhibition obtained was comparable with the standard antibiotics.

Table 7. Effect of *Caesalpinia bonducella* seed extracts on Gram-positive and Gram-negative bacteria

Extracts	Conc. (mg/ml)	Zone of inhibition (mm)			
		Gram-negative		Gram-positive	
		E. coli	***K. pneumoniae***	***B. subtilis***	***S. aureus***
Methanol	0.02	Nil	Nil	Nil	Nil
	0.04	Nil	Nil	Nil	Nil
	0.06	Nil	Nil	Nil	Nil
	0.08	**13**	Nil	**12**	**10**
	0.1	**17**	**13**	**18**	**10**
Diethyl Ether	0.02	Nil	Nil	Nil	Nil
	0.04	Nil	Nil	Nil	Nil
	0.06	Nil;	Nil	Nil	Nil
	0.08	Nil	Nil	Nil	Nil
	0.1	Nil	Nil	**15**	Nil

Nil: No inhibition

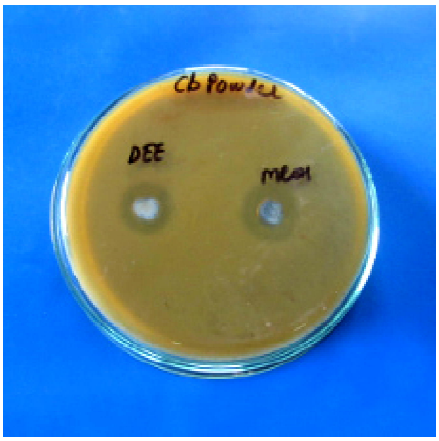

Fig. 5. Effect of methanolic seed extract of *C. bonducella* on *E. coli*

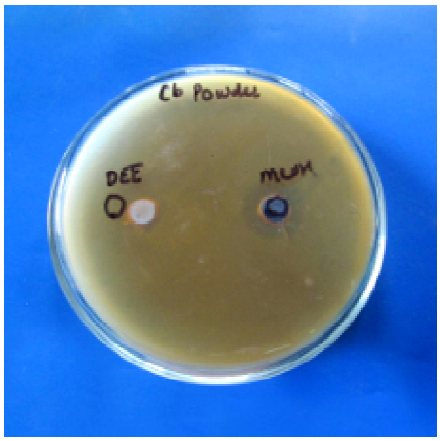

Fig. 6. Effect of methanolic seed extract of *C. bonducella* on *B. subtilis*

Similar way, the leaves are reported as a potent antimicrobial agent when anti-microbial studies were carried out by Pingale et al. [39] indicating methanolic extracts exhibited larger zones of inhibition against bacterial species like *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella aerogenes*.

There are several reports in the literature indicating that Gram-positive bacteria are more susceptible to plant extracts as compared to Gram-negative bacteria (Linn et al. [40,41], suggested that these differences may be attributed to fact that the cell wall in Gram-positive bacteria is of a single layer, whereas the Gram-negative cell wall is a multilayered structure. Nostro et al. [42] and Hodges [43] explained the reason for the differences in sensitivity of Gram-positive and Gram-negative bacteria towards plant extracts. They suggested that it is due to the morphological constitutions between the organisms. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipo-polysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances. The gram-positive bacteria on the other hand have only an outer peptidoglycan layer which is an effective permeability barrier. Therefore, the cell walls of gram-negative organisms which are more complex than the gram-positive ones act as a diffusional barrier and make them less susceptible to antimicrobial agents than the gram-positive bacteria. This may be the reason in the present study as the zone of inhibition was high in Gram-positive bacteria than the Gram-negative bacteria.

Several reports are indicating multidrug resistance in bacteria, among them *E. coli* is the most prominent [44,45]. It can be concluded that *C. bonducella* callus possesses antibacterial properties with methanolic extract. Most of the methanolic extracts inhibited the growth of *E. coli* and *B. subtilis*. Hence; callus obtained from *Caesalpinia bonducella* can be a good source of antimicrobial agent.

4. CONCLUSION

This investigation reports that, among MS and half strength MS culture medium was found suitable for callus induction in *Caesalpinia bonducella*.In the case of all tested concentrations and combinations of plant growth regulators, It was observed that 2,4-D at 2.5 mgL^{-1} in combination with BAP,2.0 mgL^{-1} BAP resulted in early initiation, highest induction percentage, with highest mean percentage of callus formation of 2.35 ± 0.2941g in 12 days for stem explants. These studies again proved medium containing both auxin and cytokinin was capable of inducing the callus formation. Hence the production of secondary metabolites from *Caesalpinia bonducella* on large scale by callogenesis is possible. The results for antimicrobial tests with methanol and diethyl ether extract of *Caesalpinia bonducella* callus show that the bacterial cultures were found to be susceptible to the methanol extract of *C. bonducella* except for *S. aureus*. The results for antimicrobial tests with methanol and diethyl ether extract of *Caesalpinia bonducella* seed powder against the clinical isolates that a high concentration of methanolic extract of *C. bonducella* seed powder showed the zone of inhibition for all the pathogens tested.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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