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RESEARCH ARTICLE

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BIOACTIVE CONSTITUENTS AND ANTIMICROBIAL ACTIVITY OF CELL CULTURES OF *AZADIRACHTA INDICA*

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ABSTRACT

The present study describes the major content and antimicrobial activity of *Azadirachta indica* cell culture extracts. Major constituents of cell culture extracts were evaluated by HPLC and chromatographic analysis revealed that highest concentrations of azadirachtin (0.0069 % dry wt) synthesised by multiple shoot cultures. The results showed that multiple shoot cultures synthesised ~17-fold more azadirachtin compared to callus cultures. Cell cultures and plant materials were extracted with methanol, ethanol and petroleum ether that exhibited antimicrobial activity. Of the various solvent used, methanol extracts was exhibited better antimicrobial activity whilst petroleum ether extracts found ineffective. Bark and leaves extracts showed higher antimicrobial activity by minimum inhibitory concentration (MIC) compared to *in vitro* cell cultures extracts. Bark extracts showed maximum MIC against *Staphyloccocus aureus* followed by *Bacillus subtilis* whereas shoot culture extracts showed maximum MIC against *Pseudomonas aeruginosa* followed by somatic embryos and callus culture extracts. On the basis of these results we therefore suggested that *in vitro* cell cultures contain potential active antimicrobial components

KEWORDS

Azadirachta indica, azadirachtin, callus cultures, shoot cultures antimicrobial activity

INTRODUCTION

Azadirachta indica A. Juss (Neem*)* is an important medicinal plant and aqueous extract of it is widely used as a tonic, stimulant and against various ailments[1]. Biological activities of *A. indica* extracts have been investigated intensively. In general, extracts of neem fruit, seeds, seed kernels, twigs, stem bark and root bark have been shown to possess anti-tumour, anti-inflammatory and immune-stimulating activities[2-4]. Most of the active principles of *A. indica* belong to the group of tetranortriterpenoids, but biologically active diterpenoids, triterpenoids, pentanortriterpenoids and a small number of nonterpenoidal ingredients have also been isolated. More than 300 compounds have been isolated from various parts of the tree and a highly oxidized triterpenoid azadirachtin that occurs mainly in seed kernels is known for its insecticidal activity[5]. Along with azadirachtin, many other compounds like nimbin, nimbinin and nimbidin were also discovered[6].

Organized cultures are known to produce high levels of major bioactive compounds compared to unorganized callus cultures. The content of tropane alkaloids hyoscyamine and scopolamine were found to be higher in multiple shoot cultures in comparison with callus cultures of *Duboisia myoporoides*[7]. Fulzele et al., [8] demonstrated that organized cultures produced high levels of terpenoids than that of parent plants of *Artemisia annua*. The flavonoid content of shoot cultures was three times higher than callus cultures of *Plantago major* and *Nepeta septemcrenata*[9]. Srividya et al.,[10] reported that untransformed root and shoot cultures produced greater amount of azadirachtin and nimbin compared to callus cultures of *A. indica*. Secondary products of plant origin have a great demand in global market and therefore, it is essential to develop *in vitro* cultures system of *A. indica* for continuous production of azadirachtin. Higher yields ranging from 4 -189 µg g^{-1} dry weight azadirachtin were detected in callus cultures derived from wild trees of *A. indica* collected from Sri Lanka[11]. High yield of 64 µg/g^{-1} dry wt azadirachtin was achieved from callus cultures initiated from *A. indica* trees from Nicaragua and Togo[12]. Callus cultures using different chromatographic methods like HPLC, TLC and supercritical fluid chromatography (SFC) and found azadirachtin yields of 7 µg g^{-1} dry wt[13].

Antimicrobial agents of plant origin have been studied from various plant extracts with the objective of developing novel antimicrobial agents[14,15]. Bioassays are adaptable for the purpose of screening and testing plant extracts and initial screening for biological activity may be used for identification of active ingredients[16]. Extracts of *A. indica* have been used in traditional medicine as antitumour, antiinflammatory, antipyretic, analgesic, immunostimulant, diuretic, hypoglycaemic, cardiovascular, antimicrobial, antiviral, antimalarial and anthelmintic agents[17,18]. Residual extracts of leaves of *A. indica* was found to be active against poliomyelitis, herpes virus, small pox, chickenpox, polio virus etc., [19-21]. Recently, Gonzaler-Garza and co-workers[22] have reported that methanol extracts of *A.indica* showed *in vitro* stimulatory activity in stem cell reproduction to improve health. Extracts of *in vitro* cell cultures have been employed earlier for antimicrobial activity[23,24]. However, none of the previous research papers have focused on the

comparison of antimicrobial activity of *in vivo* and *in vitro* cell cultures of *A. indica*. The aim of the present paper is to study antimicrobial and antifungal activity of *in vivo* and *in vitro* cell and organ culture extracts of *A. indica* and azadirachtin production.

MATERIALS AND METHODS

Plant materials and tissue cultures

Plant materials leaf and bark of *Azadirachta indica* L. Juss (Neem) were collected from experimental field station, Bhabha Atomic Research Centre, Trombay, Mumbai. For initiation of callus cultures, cotyledons of immature seeds of *A. indica* were used. Seeds were initially washed with running tap water for 30 min and treated with Dettol for 5 min. The explants were disinfected with 70% (v/v) alcohol for 2 min followed by $HgCl_2$ solution (0.1% w/v) for 3 min and subsequently washed 4-5 times with sterile distilled water. The cotyledons were removed from the surface-sterilized seeds and incised into two pieces. Disinfected explants were cultured on 50 ml test tubes containing 20 ml of Murashige and Skoog's[25] medium supplemented with 3% sucrose and growth regulators. The medium was adjusted to pH 5.8 prior to the addition of 0.25% gelling agent Phytagel and autoclaved at 121^0C at 15 lbs for 20 min. Cultures were kept on culture racks in the culture room at 26 ± 1^0C and maintained under 16 h light and 8 h dark period (50 μmol m^{-2} S^{-1}, white fluorescent light). Cultures were regularly subcultured after every three weeks on medium of the same composition and maintained under similar cultural conditions.

Callus and Multiple shoot cultures

Cotyledons from immature seeds of the *A. indica* were used for initiation of cell cultures. Cotyledons were cultured on Murashige and Skoog's medium supplemented with growth regulators such as picloram (10.35 μM) and benzyl aminopurine (BAP) (0.44 μM) for callus cultures, indole-3-acetic acid (IAA) (0.57 μM), a-napthaleneacetic acid (NAA) (26.85 μM) and kinetin (Kn) (9.30 μM) for multiple shoot cultures and BAP (8.90 μM) and IAA (0.57 μM) for somatic embryos with 3% sucrose. Established cell and organ cultures were harvested and determined the major contents and antimicrobial activity.

Microorganisms

Human pathogenic microbial strains were used for antimicrobial activities included Gram-positive and Gram-negative bacteria namely *Bacillus pumilus*, *Bacillus subtilis*, *Staphyloccocus aureus, S. pyogenes Pseudomonas* aeruginosa, *Eschrichia coli*, *Proteus vulgaris and* fungal species *Aspergillus niger* and *Rhizoctonia oryzae.* These microorganisms were obtained from the Department of Life Sciences, Devi Ahilya Vishwavidyalaya, Indore, India.

Preparation of extracts for HPLC and antimicrobial activity

The harvested biomass (cell cultures, leaf and bark) were dried in an oven at 55^0C for 16 h. Dried biomass was powder with the help of Wiely Mill (Model No. 4276, Thomas Scientific, USA). The dried powder material (50 gm) was extracted with methanol, ethanol and petroleum ether in a Soxhlet apparatus for 8 hr with ~12 cycles. Thereafter, samples were evaporated under reduced pressure by BÜCHI Rotavapor (RI 111, Switzerland) at 40^0C. The residues were dissolved in dimethyl sulfoxide (DMSO) and tested for antimicrobial and antifungal activities. For HPLC, residual extracts were transferred into polypropylene micro-centrifuge (Eppendorf) tubes and centrifuged at 12000x*g* for 5 min. The supernatant was transferred to clean glass vials and applied directly onto High Performance Liquid Chromatography (HPLC).

Antimicrobial activity assay

Twenty-hour-old culture suspension (100 µl) of the tested microorganisms containing 10^8 CFU/ml of bacteria and 10^4 spore/ml of fungal strains were seeded in molten sterile nutrient agar (NA) and potato dextrose agar (PDA) medium, respectively. After the medium solidified, a sterile cork borer (5 mm diameter) was used to bore equidistant wells into the agar plate. About 10 mg residues were dissolved in 1 ml of DMSO. Thereafter, each cup was filled with 50 µl of different extracts. Negative control was prepared by putting 50 µl of DMSO in one of bored hole. Ampicillin was used as positive reference standards to determine the sensitivity. One hour pre-diffusion time was allowed, after which the plates were incubated at 37°C for 24 h for bacteria and 30^0C for 72 h for fungi. The zones of inhibition were then measured in millimetre. Studies were performed in triplicate, antimicrobial activity was evaluated by measuring the developed clear zone of inhibition around the well measured in mm and compared with antibiotic used.

Determination of the minimum inhibitory concentration (MIC)

MIC values, which represent the lowest extract concentration that preventing visible growth of microorganisms, were determined as described previously[26]. Petri dishes of NA and PDA containing various extracts of cell cultures and plant materials were inoculated with 100 µl bacterial inoculums adjusted to10^8 cfu/ml of bacteria cells and 10^4 spores/ml of fungal strains respectively. All tests were performed in NA and PDA. Bacterial strains were cultured overnight in NA at 37^0C whilst fungal strains were incubated 72 hr in PDA at 37^0C. Then, they were incubated 24 h at 37^0C for bacteria strains and 72 h at 30^0C for fungal strains. Control without tested samples were essayed simultaneously. All tests were carried out for three sample replications and the results were averaged.

HPLC conditions for quantification of azadirachtin

An Isocratic analytical HPLC was performed on a JASCO (900 Series Japan) system equipped with a Jasco AS-950 auto injector with a 20µl sample loop. The HPLC column was a HI-Q sil C-18 (particle size 5µm, 100 A^0, 4x150 mm ID) packed column. The mobile phase for alkaloid elution was Acetonitrile: Water (60:40) at a flow rate of 1ml/min and UV detection at 227 nm. Data collection and integration were accomplished using BORWIN software. A retention time of azadirachtin was 5.77 min. Peak identification was carried out on the basis of an authentic sample of azadirachtin (SIGMA, USA). Co-chromatography of the extracts was also performed with standard sample of azadirachtin. This method is sensitive and accurate with good reproducibility. Validation of quantitative method was performed with samples for five times. The results of the five injections from the same samples at the five concentrations (0.1µg–0.5µg) showed similar retention time.

Standard solutions were prepared by dissolving azadirachtin in methanol (1 mg/10 ml methanol). Further calibration levels were prepared by diluting the stock with methanol (correlation coefficient R2=0.99877).

Statistical analysis

The influence of various treatments on antimicrobial activities and azadirachtin content was carried out by one-way analysis of variance (ANOVA). Values are mean of three replicates from the experiment was performed thrice. The data was analysed statistically by analysis of variance (ANOVA) and difference between means of the samples was analysed by the least significant difference (LSD) at a probability levels of 0.05.

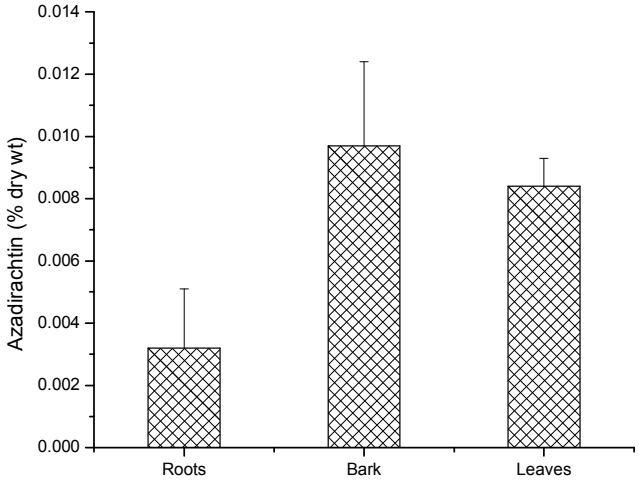
RESULTS AND DISCUSSION

Evaluation of azadirachtin in A. indica plant material

Different plant parts of *A. indica* grown field were harvested and determined the azadirachtin content. Figure 1 depicts the quantification of azadirachtin in different parts of naturally grown plants. HPLC chromatogram revealed that azadirachtin was one of the major constituent and highest amount was accumulated in bark (0.0097% dry wt), which is ~4-fold more than that of the roots of same plants.

Figure 1

Distribution of azadirachtin in different plant parts of A. indica plant. Results are the mean of three replicates ± SD



Evaluation of azadirachtin by callus and multiple shoot cultures

Callus proliferation was obtained on all MS medium supplemented with growth regulators but found variation in callus morphology, colour and texture. Of the four different combinations tested for initiation of callus cultures, MS medium supplemented with picloram (10.35 µM) and BAP (0.44 µM) proved better for callus induction. Culture on picloram + BAP showed sustained and massive callus proliferation. However, cultures grown in presence of NAA (26.85 µM) combination with BAP (0.44 µM) or KN (0.46 µM) showed moderate growth and produced low levels of azadirachtin. Replacement of NAA by picloram into the culture medium did not influence the production of azadirachtin (Fig. 2). The volumetric yield of azadirachtin was increased constantly during cultivation period when callus cultures were grown on picloram (10.35 µM) and BAP (0.44 µM) and maximum azadirachtin (0.0042 % dry wt) production was achieved on day 20.

Shoots formation was achieved directly from cotyledons cultured on MS medium supplemented with IAA (0.57-2.85 µM) and KN (2.32-9.30 µM). Numerous shoots were developed in four weeks of cultivation. Improved shoot multiplication with increased concentrations of KN fortified with decreased concentrations of IAA. Figure 3 shows that

highest azadirachtin (0.0069 % dry wt) production was obtained from shoots cultured on MS medium supplemented with IAA (0.57 µM) and KN (9.30 µM).

Plant cell cultures offer balanced exogenous growth regulators for dedifferentiation and volumetric increase of production of bioactive compounds. Combinations and concentrations of auxin and cytokinins in culture medium produced best callus induction in *Crocus sativus*[27]. In the present study, picloram and BAP enrich medium produced sustained and massive callus growth and high levels of azadirachtin production on day 20. It was found that cell cultures often synthesized low levels of bioactive compounds compared to intact plants[28,29]. However, organized cell cultures are known to synthesised high concentrations of bioactive compounds. Several reports have demonstrated that organized cell cultures produced high levels of bioactive compounds in comparison with de-differentiated cell cultures[7,9]. Similarly, our present results demonstrated that shoot cultures produced maximum azadirachtin production compared to callus cultures.

Figure 2

Accumulation of azadirachtin in callus cultures of A. Indica established on MS medium supplemented with different growth regulators. The experiment was performed thrice and values are expressed as mean ± SD of three experiments.

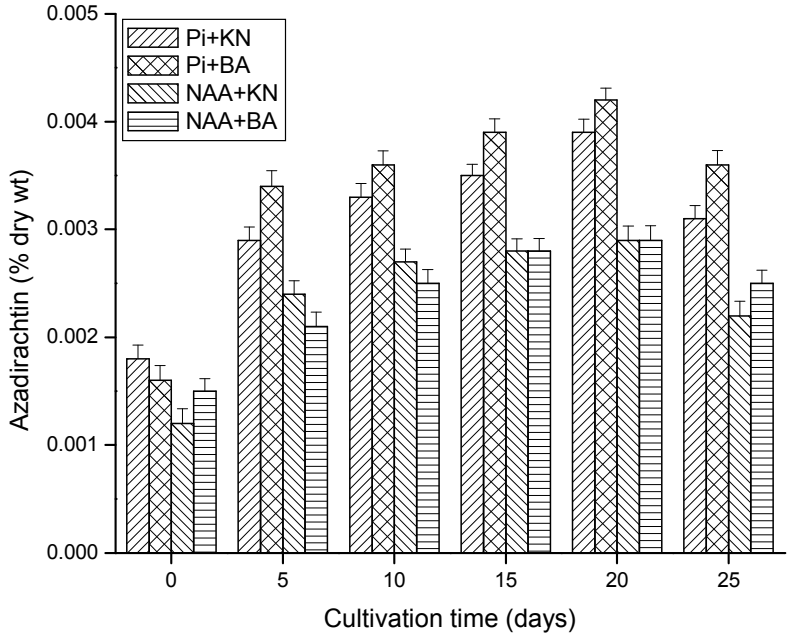
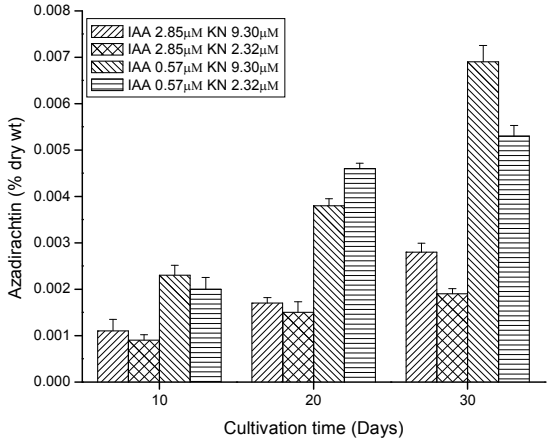


Figure 3

Production of azadirachtin by shoot cultures of A. Indica established on MS medium supplemented with different growth regulators. The experiment was performed thrice and values are expressed as mean ± SD of three experiments.



Antimicrobial activity

Different solvent extracts of leaf and bark of *A. indica* were tested against gram negative, gram positive bacteria and fungi. Results of antibacterial activity of all the extracts and their efficacies as compared to standard are shown in Table 1. The quantitative screen of activity was determined by minimum inhibitory concentration[30]. Qualitative analysis revealed that methanol extracts has better antimicrobial and antifungal activities compared to other solvent extracts. Irrespective of plant materials used, bark extracts showed greater antimicrobial activity against *S. aureus*, the commonest urinary tract infection causing bacteria[31]. Bhuiyan et al.,[32] reported an appreciable antimicrobial effect of bark extracts *A. indica* on the growth of *Streptococcus sobrinus*, a causative agent for dental caries in human being. Leaf extracts showed maximum antimicrobial activity against *B. subtilis,* which are encountered in deteriorating many food products, thus shortening their shelf life[33]. Interestingly, leaf and bark extracts did not influence the antimicrobial activity against *E. coli.* Similar observations were reported from *Rauvolfia tetraphylla* and *Physalis minima* leaf and callus extracts[34].

Various growth regulators were used for induction of cell cultures and production of azadirachtin. Major constituents accumulated steadily during cultivation period and antimicrobial activity was performed with cell cultures which produced highest amount of azadirachtin. Callus cultures grown on MS medium supplemented with picloram (10.35 µM) + BAP (0.44 µM) harvested on day 20, whereas shoot cultures showed maximum production levels on day 30 grown on IAA (0.57 µM) + KN (9.30 µM) were used for antimicrobial and antifungal activity. It was found that callus and shoot cultures exhibited a broad spectrum of inhibitory activity to gram-positive, gram-negative bacteria and fungi tested. No inhibitory activity was observed for *E. coli*, which is common cause of diarrhea and urinary tract infection[35,36]. Compared to this, shoot cultures showed greater antimicrobial and antifungal activities. Methanol extract of multiple shoot cultures showed significant anti-fungal activity against *A. niger,* which is found more frequently in burn wounds[37].

Methanol and ethanol extracts of somatic embryos and multiple shoot cultures demonstrated maximum activity against *S. pyogenes*, which caused wound infection[35]. On the basis of these results concluded that *in vitro* cell cultures possess activity inhibitory to gram-positive, gram-negative bacteria and fungi which cause various infections (Table 2). It indicated that the potential antimicrobial components present in the cell cultures which exhibited the activity against bacteria and fungi. Therefore, it is possible that the presence of triterpenoid azadirachtin or other components in cell culture as reported earlier[15] are likely to be responsible for antimicrobial and antifungal activity.

About 80% of available drugs are obtained from medicinal plants and in industrialized countries plants make up the raw material for chemical processes, which synthesize pure bioactive molecules[38]. Various solvent extracts from *A. indica* bark showed better inhibiting activity on disease causing gram-negative bacteria, gram-positive bacteria and fungi, the most inhibited being *Staphylococcus aureus*, *Bacillus subtilis, S. aeruginosa, Bacillus pumilus*. This is particularly interesting from a medical point of view because these microbes are responsible for severe opportunistic infections[39]. Our findings on antibacterial activity of *A. indica* could justify some ethnopharmacological uses such as against urinary tract infection, diarrhea and dysentery because we demonstrated good activity of this plant against some pathogens. Eloff[40] and Cowan[41] reported that methanol was more efficient than any other solvents in extracting phytochemicals from plant materials. The absence of antibacterial activity of petroleum ether extracts of *A. indica* indicates the insolubility of the active ingredients in these solvents. In the present investigations the ethnobotanical efficacy of methanol extracts of *A. indica* shows greater antimicrobial activity compared to all tested solvent extracts.

In comparison to *in vivo,* the *in vitro* extracts depicted reduced antimicrobial activity. HPLC profile showed that cell cultures synthesized low amount of azadirachtin than that of bark and leaves of the same plant. These findings are in good agreement with earlier studies wherein antimicrobial potential of *in vivo* and *in vitro* against various pathogenic bacteria and fungi. Jana and Shekhawat[42] reported that cell cultures of *Anethum graveolens* showed decreased antimicrobial activity. It is not surprising that there are differences in the antimicrobial activities of the *A. indica* plant materials and cell culture extracts. From this observation it is evident that secondary products are responsible for the antibacterial activities exhibited by the extracts (Fig. 4). Moreover, unorganized cell cultures have a constant expression pattern in a specialized set of cells even after their rapid division and differentiation into callus form.

Table 1

Antimicrobial activity of A. indica leaves and bark extracts by well-in-agar diffusion method

Type	Microorganisms	Zone of Inhibition (mm ± S.E.M)[a]					
		Ethanol extracts		Petroleum ether extracts		Methanol extracts	
		Leaves	Bark	Leaves	Bark	Leaves	Bark
Gram positive bacteria	*B. pumilus*	24.2±0.98	23.4±1.06	--	4.1±0.87	21.8±1.14	24.2±1.13
	B. subtilis	25.4±1.09	20.5±0.83	--	4.5±1.02	26.7±0.93	26.5±0.87
	S. aureus	19.8±1.17	20.1±0.85	--	5.1±1.25	25.5±0.88	28.5±1.16
	S. eruginosa	4.8±0.91	6.5±1.21	--	--	20.5±0.90	25.4±0.95
Gram negative bacteria	*P. pyogenes*	18.5±2.01	16.3±1.73	3.3±0.92	2.9±1.33	19.8±1.89	18.3±0.92
	E. coli	--	--	--	--	--	--
	P. vulgaris	20.7±1.20	18.8±1.21	4.2±1.56	2.9±0.93	23.8±0.82	21.9±1.10
Fungi	*A. niger*	8.9±0.97	11.2±0.98	5.6±0.93	4.2±1.24	17.8±1.32	17.2±1.56
	R. oryzae	2.5±1.45	3.2±1.29	--	--	3.7±0.91	4.8±0.82
Antibiotic Zone		26.7±1.34	27.1±1.10	25.2±1.32	27.2±1.02	28.3±1.43	26.2±1.09
Control (DMSO)		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[a]*Average of three replicates; -- no inhibition. The experiment was performed thrice and values are expressed as mean ± SD of three experiments.*

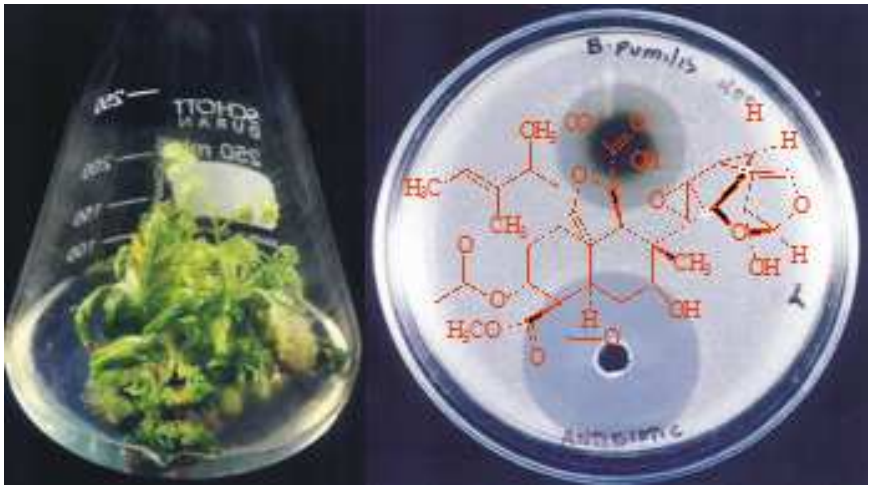
Table 2.

Antimicrobial activity of A. indica cell cultures by well-in-agar diffusion method

Type	Strain	Zone of Inhibition (mm ± S.E.M)[a]								
		Ethanol extracts			Petroleum ether extracts			Methanol extracts		
		Callus	Somatic Embryos	Multiple Shoots	Callus	Somatic Embryos	Multiple Shoots	Callus	Somatic Embryos	Multiple Shoots
Gram positive bacteria	*B. pumilus*	14.8±1.26	9.6±0.98	16.6±0.85	_	_	_	13.1±1.27	11.6±0.92	13.5±1.32
	B. subtilis	10.8±0.95	9.5±1.22	10.8±1.38	_	_	_	11.6±0.88	12.9±1.22	14.5±1.58
	S. aureus	11.3±1.34	9.6±0.92	13.2±0.79	_	2.2±0.95	2.5±0.79	9.5±1.74	13.5±1.25	14.4±0.95
Gram negative bacteria	*S. aeruginosa*	10.6±1.56	12.1±1.10	13.5±1.15	_	_	2.9±1.29	12.8±1.78	14.6±1.48	17.5±1.28
	P. pyogenes	8.4±1.55	11.3±0.94	15.8±1.77	_	_	_	7.6±0.94	13.3±0.98	15.0±1.33
	E. coli	_	_	_	_	_	_	_	_	_
	P. vulgaris		7.4±1.25	13.8±0.87	_	2.8±1.25	3.2±1.05	9.4±1.76	8.4±1.05	16.2±0.85
Fungi	*A. niger*	5.3±0.98	7.5±1.20	8.9±0.79	_	_	3.4±1.45	9.2±0.85	11.3±1.66	11.5±1.83
	R. oryzae	3.5±1.63	3.1±1.83	4.2±1.27	_	_	_	4.1±1.19	3.2±0.93	4.8±1.54
Antibiotic Zone		28.5±1.03	25.2±1.25	27.2±1.11	27.5±1.29	28.3±1.60	27.1±1.59	28.9±1.16	24.2±1.25	26.6±1.19
Control (DMSO)		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[a]*Average of three replicates; -- no inhibition. The experiment was performed thrice and values are expressed as mean ± SD of three experiments.*

Figure 4
.Multiple shoot cultures of A. indica and zone of inhibition showing antimicrobial activity of cell culture extracts



CONCLUSION

In vitro cell culture demonstrated antimicrobial activity against pathogenic microbes used for study. This study further revealed that besides leaf and bark of *A. indica*, the cell cultures also possess antimicrobial property. It was observed that shoot cultures contained higher levels of azadirachtin which showed maximum inhibitory activity. Shoot cultures also showed greater antimicrobial and antifungal activity in comparison with callus and somatic embryos. In conclusion, cell cultures produce potential antimicrobial components that may be of use for the development of phytomedicine for the treatment of various infections.

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