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SCREENING FOR BIOACTIVE PHYTOCHEMICALS AND CALLUS PRODUCTION IN *ALSTONIA VENENATA R.BR.*

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Alstonia venenata R.Br. is a small sized evergreen tree belongs to the family Apocynaceae. It has many medicinal properties. In the present study micropropagaion, biochemical analysis, phytochemical screening and antimicrobial activity of *Alstonia venenata* were done. A suitable proportion of media has been standardized for the callus production of *Alstonia venenata* R.Br. in MS medium using different hormonal combinations like 2, 4-D + Kinetin, 2, 4-D + BAP, NAA + kinetin, NAA + BAP, IAA + Kinetin, IAA + BAP, IBA + Kinetin and IBA + BAP. Leaf and internodal segments were used as explants. Biochemical and phytochemical analysis of both stem bark powder and fresh callus indicated the presence of phenols, carbohydrates, protein, chlorophylls, free aminoacids, alkaloids, glycosides, terpenoids. Metabolites were separated through thin layer chromatography (TLC), fractionation by column chromatography. Each fraction were collected and then subjected to phytochemical screening and bioactivity test, isolated compounds coded as C_1, C_2 and C_3 also showed remarkable results. Regarding antibacterial activity, significant results were observed at a concentration of 15.4 mg/disc against all test organisms, total inhibition of fungal growth at 154mg/ml in the antifungal studies comparable to standards.

Keywords : *Alstonia venenata* R. Br; Antimicrobial activity; Biochemical analysis; Callus production; Methanol extract; Phytochemical screening.

Abbreviations: BAP: 6 - Benzyl aminopurine, 2, 4 D: 2, 4 Dichlorophenoxy acetic acid, NAA: α- Naphthalene acetic acid, IAA: Indole - 3- acetic acid, IBA: Indole- 3- butric acid, MS: Murashige and Skoog.

Introduction

Alstonia venenata R.Br is a small tree up to 6.0m in height. Stem bark are grayish, rough, hard, lenticellate, exudes latex when injured. Leaves are glabours, oblong to lancelolate[1]. Flowers white, in terminal subumbellate pedunculate cymes. Seeds are flattened, linear oblong. The ripe fruit is tonic; it is used in syphilis, insanity and epilepsy. Roots are bitter, astringent, thermogenic, depurative, febrifuge and anodyne[2]. Major compounds isolated are alsovenine, venalstonine, venalstomidine, venenatin[3] etc. It is a medicinally important threatened plant hence it require conservation. Micropropagation is a useful method of multiplication of this plant. In the present study micropropagaion, biochemical analysis, phytochemical screening and antimicrobial activity of *Alstonia venenata* were done.

Material and Methods

In the present study explants for *in vitro* culture (healthy young leaves and internodes from young saplings), mature stem bark were taken from *Alstonia venenata* R.Br. brought from inferior forest of Ponmudi hills. Explants for tissue culture were planted in the green house of the Department of Botany, University College, Thiruvananthapuram. Systematic position was confirmed by taxonomical studies[4]. Sample has been documented. The collected mature stem bark were weighed; oven dried at 39°C, weighed and powdered, extracted in methanol for 8 hours continuously to obtain bioactive principles from the bark. Crude extract was concentrated, weighed and made up to 100ml. It is kept in sterilized bottle under refrigerated condition until use (Table 1).

Callus production of Alstonia venenata R. Br-Tissue cultures of *Alstonia venenata* were initiated in MS medium[5]. Phytohormones used in the medium were auxins such as IAA, IBA, NAA and 2, 4-D and Cytokinins namely BAP and Kinetin in either singly or in combinations. Leaves and internodes were taken as explants. The surface sterilized material was inoculated in culture tubes and inoculated tubes were then taken into incubation room. Growth characteristics and other features of the callus were

Table 1. Quantitative data of *Alstonia venenata* R.Br.

Plant part	Fresh weight (g)	Dry weight (g)	Powder weight (g)	Quantity (g)	Weight 10ml crude(g)	Material used for bacterial study (mg/disc)	Material used for fungal study (mg/ml)
Stem bark of *A.venenata*	1000	260	260	90	3.08	15.4	154

Table 2. Biochemical studies on stem bark powder and fresh callus.

Parameters	Stem bark powder (mg/g)	Fresh callus (mg/g)
Total Phenol	0.024	0.009
Total Carbohydrate	24.0	15.40
Total Protein	10.186	1.581
Free Aminoacids	0.592	0.066
Reducing sugar	0.124	0.057
Tannin	2.0	Nil
Cellulose	0.163	Nil
Chlorophyll a	0.007219	0.000851
Chlorophyll b	0.000856	0.002482
Total chlorophyll	0.008044	0.003355
Carotenoid	0.024520	0.004072

Table 3. Distribution of Secondary metabolites in methanol extract of stem bark and fresh callus.

Phytochemicals	Stem bark extract	Callus extract
Reducing sugar	+	+
Glycosides	+	+
Flavanoids	-	-
Tannins	+	-
Terpenoids	+	+
Steroids	-	-
Phlobatannins	-	-
Alkaloids	+	+
Coumarins	+	+
Saponins	+	+
Anthraquinones	-	-
Iridoids	-	-

noted. Sub culturing was done at four week intervals[5].

Biochemical Analysis-Dried stem bark powder and fresh callus were used for analysis of total phenols (Folin Ciocalteau method), reducing sugar (Dinitrosalycylic acid method), total carbohydrates (Anthrone method), total protein (Lowry's method) , chlorophyll (Arnon's method), amino acids (Ninhydrin method), tannin (Schanderl's method) and cellulose (Updegroff's method)[6,7].

Phytochemical Screening -The phytochemicals like reducing sugar (Fehling's test), glycosides (Keller Killani test), flavanoids (Shinoda test), alkaloids (Dragendroff's method), tannins, steroids, Terpenoids (Libermann Burchard method), coumarins, saponins, anthraquinones, phlobatannins and iridoids were tested[6,7]. The stem bark extract and callus extract (Fresh callus was grind with distilled water) were used for phytochemical screening[7].

Table 4. Phytochemical screening of methanol extract by TLC.

Extract	Solvent system	Ratio	Observation	Compounds	Rf Value
Methanol	Benzene: Chloroform: Methanol	2:3:5	Green, Orange Reddish Orange Yellow Brown Grey	Glycosides Alkaloids Terpenoids Phytosterols Phenolic compounds Phenolic compounds	0.98 0.94 0.92 0.80 0.70 0.60
	Methanol: Benzene	1:1	Green Orange Yellow Brown	Glycosides Alkaloids Terpenoids Phenolic compounds	0.98 0.94 0.92 0.70

Table 5. Response of internodal and leafy explants of *Alstonia venenata* in MS medium.

No.	Hormone combinations	Concentration of hormone combinations (mg/l)	Callus from leaves of *A.venenata*	Callus from internodes of *A.venenata*	Remarks of Callus
1.	2,4-D&BAP	1.5& 2 2.5&2	++++*	+++	Brownish white
2.	2,4-D&Kinetin	1.5& 2 2.5&2	++++	+++	White callus
3.	NAA&BAP	1.5& 2 2.5&2	+++	++	Greenish white
4.	NAA&Kinetin	1.5& 2 2.5&2	+++	++	Greenish white
5.	IAA&BAP	1.5& 2 2.5&2	++	Nil	White callus
6.	IAA&Kinetin	1.5& 2 2.5&2	+	Nil	White callus
7.	IBA&BAP	1.5& 2 2.5&2	++++	++	White callus
8.	IBA&Kinetin	1.5& 2 2.5&2	+++	++	Brownish white

*Callus growth is indicated by + marks
\+ : meager callus growth (callus fresh weight<0.5 g after 4 weeks)
++ : average callus growth (callus fresh weight 0.5g to <1g after 4 weeks)
+++ : profuse callus growth (callus fresh weight 1g to <1.5 g after 4 weeks)
++++ : Maximum growth (callus fresh weight 1g to <1.5 g after 4 weeks)

Thin layer chromatography -Qualitative chemical analysis of crude extract was performed by Thin layer chromatography by different solvent systems indicated the presence of steroids, flavanoids, phenolic compounds and Terpenoids[8].

Chromatographic fractionation- On chromatographic fractionation several fractions of 20ml each were collected and their homogeneity was monitored by using T.L.C

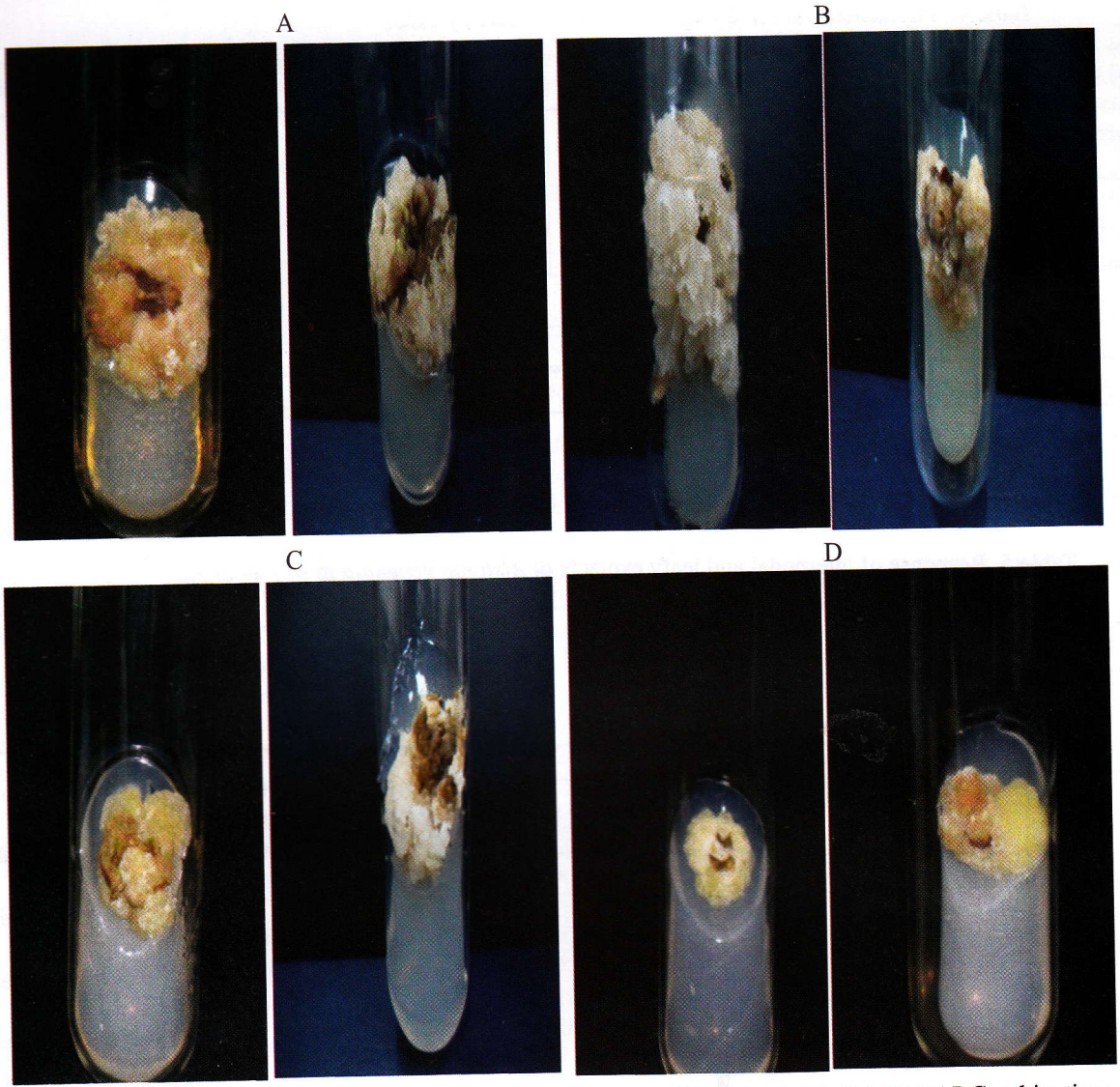

Fig.1. *In vitro* callus production of *Alstonia venenata* R.Br. A: Callus induction in 2,4-D+BAP Combination; B: Callus induction in 2,4-D+Kinetin Combination; C: Callus induction in NAA+BAP Combination; D: Callus induction in NAA+ Kinetin Combination.

behavior. Identical fractions were pooled together to get single compound or mixtures. Twelve compounds obtained each of them washed with respective solvents and alcohol. Final products were allowed to crystallize. All the compounds were subjected to antibacterial activity and antifungal activity. Effective bioactive compounds only were selected and coded as C_1, C_2, C_3. bioactivity and chemical analysis of these compounds were performed[10].

Bioactivity assays by in vitro methods-

• *Antibacterial activity assay:* Crude stem bark extract and callus extracts were subjected to their antibacterial property against pathogenic and industrially important strains of bacteria by disc diffusion method[10]. Bacteria were provided from microbiology lab of Medical College, Trivandrum.

• *Antifungal activity assay:* Conducted antifungal activity studies against common phytopathogenic forms of fungi with crude stem bark extract in methanol at two different concentrations by incorporating crude extract in the media[11]. All the strains of fungi used here were procured form Agricultural College, Vellayani, Trivandrum.

Results and Discussion

Callus growth of *Alstonia venenata* in different hormone combinations shown in Table 5 (Fig 1.A to H). Biochemical and phytochemical analysis on both stem bark powder and fresh callus were shown in Table 2, 3 and 4. Twelve different compounds were obtained through column chromatography (Table 6). All the twelve compounds were subjected to preliminary phytochemical

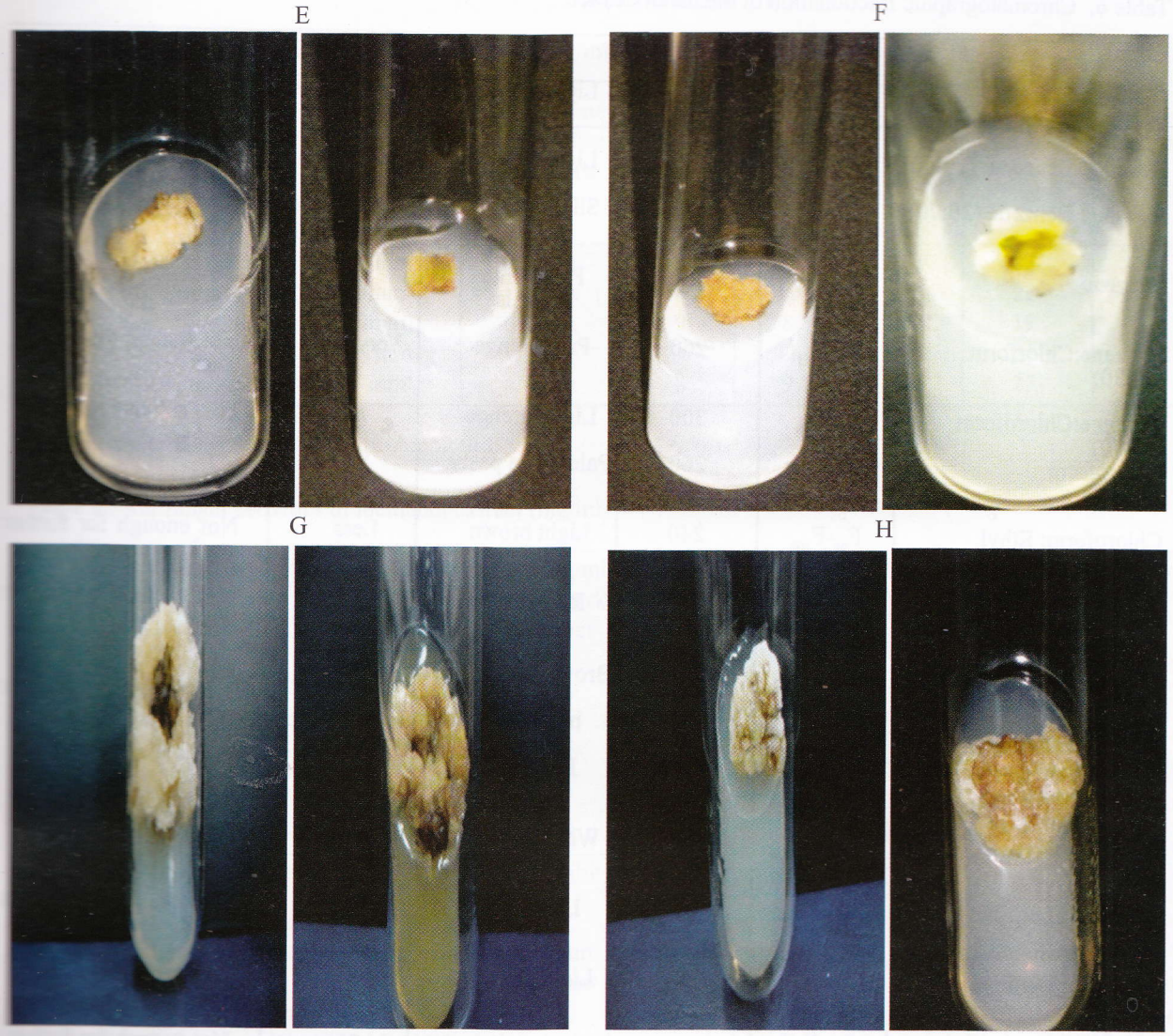

Fig2. *In vitro* callus production of *Alstonia venenata* R.Br. E: Callus induction in IAA+BAP Combination; F: Callus induction in IAA+ Kinetin Combination; G: Callus induction in IBA+BAP Combination; H: Callus induction in IBA+ Kinetin Combination.

studies. Antibacterial and antifungal activity of crude extract and compounds c_1, c_2, c_3 were shown in Table 7 and 8.

Reports regarding bioactive properties in several members of the family Apocynaceae strongly support present study isolation, structure and bioactivities of several compounds from different members of the family[9]. Formulation of a natural medium for the induction of callus and their bioactivity in *Alstonia venenata* R.Br. were reported[3]. Phytochemical screening of leaf extract of *Alstonia venenata* R.Br. showed the presence of alkalodis, terpenoids, steroids tannins etc.[13]. Phytochemical activity of leaves of *Alstonia scholaris* R.Br. was investigated. The different solvent extracts showed the presence of Iridoids, alkaloids, coumarins, flavonoids, leucoanthocyanins, reducing sugars, simple phenolics, steroids, saponins and tannins[14,15]. Antibacterial effect of the crude leaf and stem bark extracts of *Alstonia venenata* in solvent systems like hexane, benzene, isopropanol, ethyl acetate, methanol, and water were investigated[10].

From the results of present study, it was concluded that-

Stem bark and fresh callus of *Alstonia venenata* R.Br showed the presence of various phytochemicals which showed antimicrobial properties against phytopathogens.

- The use of plant extract provides a potential alternative to antibiotics against phytopathogens and the compounds C_1, C_2, C_3 were as potent as standards.

Hence, stem bark extract of *Alstonia venenata* R.Br deserve further investigation.

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Table 6. Chromatographic fractionation of methanol extract.

Eluent	Fraction	Volume (ml)	colour	yield	Bioactivity*
Hexane	F_1-F_{10}	220	Light yellow	Very less	Not enough for further work
Hexane : Benzene (70:30)	F_{11}-F_{22}	240	Light yellow	67	(-) (-)
Hexane : Benzene (30:70)	F_{23}-F_{34}	240	Slight yellow	Very less	Not enough for further work
Benzene	F_{35}-F_{49}	300	Pale white crystals	97	(+) (+) coded as C_1
Benzene:Chlorform (70:30)	F_{50}-F_{63}	260	Pale orange	Very less	Not enough for further work
Benzene:Chloroform (30:70)	F_{64}-F_{78}	300	Light Orange	79	Less active
Chloroform	F_{79}-F_{95}	260	Pale white pasty	99	(+) (+) coded as C_2
Chloroform: Ethyl acetate (70:30)	F_{96}-F_{108}	240	Light brown soln	Less quantity	Not enough for further work
Chloroform: Ethyl acetate (30:70)	F_{109}-F_{122}	260	Brown soln	Very less	Not enough for further work
Ethyl acetate	F_{123}-F_{136}	280	Brown wax like	91	(-) (-)
Ethyl acetate:Methanol (70:30)	F_{137}-F_{143}	280	Greenish brown soln	Very less	Not enough for further work
Ethyl acetate:Methanol (30:70)	F_{149}-F_{160}	240	Pale white granule	93	(-) (-)
Methanol	F_{161}-F_{173}	240	White granules	96	(+) (+) coded as C_3
Methanol:Distilled Water (70:30)	F_{174}-F_{183}	240	Light white	Very less	Not enough for further work
Methanol:Distilled Water (30:70)	F_{184}-F_{192}	300	Light granules	Very less	(-) (-)
Distilled Water	F_{193}-F_{215}	240	White colour	91	Not enough for further work
Distilled Water:NaCl (70:30)	F_{216}-F_{225}	240	Very light white	79	(-) (-)
Distilled Water:NaCl (30:70)	F_{226}-F_{234}	260	Light white	Very less	(-) (-)
NaCl	F_{235}-F_{242}	280	Colour less	Very less	Not enough for further work
NaCl:Oxalic acid (70:30)	F_{243}-F_{256}	240	Colour less	72	(-) (-)
NaCl:Oxalic acid (30:70)	F_{257}-F_{235}	250	Colour less	91	Not enough for further work
Oxalic acid	F_{236}-F_{245}	220	Colour less	Very less	(-) (-)

*Bioactivity 1. against *Bacillus subtilis*, 2. against *Rhizopus nigricans* (+) active, (-) not active

Table 7. Antibacterial activity of methanol extract (mg/ml).

No.	Test Organisms	Zone of inhibition in mm						
		225mg/disc Callus extract	15.4mg/disc Stem bark extract	C_1 1mg disc	C_2 1mg disc	C_3 1mg disc	*Standard	Control
1	*Bacillus subtilis*	10mm	14mm	9	11	12	23	-
2	*Bacillus brevis*	8mm	12mm	8	12	14	22	-
3	*Escherichia coli*	9mm	10mm	9	8	9	19	-
4	*Bacillus mycoides*	11mm	15mm	10	11	14	21	-
5	*Bacillus coagulans*	9mm	13mm	8	9	11	23	-

(-) No zone of inhibition, *Standard-Ampicillin

Table 8. Antifungal activities of methanol extract (mg/ml).

No.	Test Organisms	77mg/ml Stem bark extract	154mg/ml Stem bark extract	C1 5mg/disc	C2 5mg/disc	C3 5mg/disc	Control	*Standard
1	*Candida tropicalis*	+	+	+	+	+	-	+
2	*Phytophthora infestans*	+/-	+	+/-	+	+	-	+
3	*Pencillium italicum*	+	+	+/-	+	+	-	+
4	*Mucor* sps.	+	+	+	+	+	-	+
5	*Rhizopus nigricans*	+	+	+	+	+	-	+

(-) No inhibition, (-/+) Partial inhibition, (+) Total inhibition, *standard-Miconazol

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