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Mangrove Halophytes: A source of antiviral substances

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Abstract

Mangroves have been studied for their antiviral properties. The mangrove plants were sampled from Pichavaram in the southeast coast of India. The extracts of different portions of mangroves were tested *in vitro* against four RNA viruses *viz*., Newcastle disease virus, encephalomyocarditis virus, Semliki forest virus and human immunodeficiency virus and two DNA viruses - vaccinia virus and hepatitis B virus. A broad spectrum antiviral activity was exhibited in bark of *Rhizophora mucronata* and leaves of *Bruguiera cylindrica.* In general, plants belonging to the family - Rhizophoraceae are the source of potential antiviral substances.

Key words: mangrove, virus, antiviral, polysaccharides, lignin (not more than five)

Introduction

Mangrove plants are being used in folklore medicine for treatment of several diseases (KIRTIKAR and BASU, 1935; CHOPRA *et al.,* 1956; DATTA and DATTA, 1982). Although mangrove plants are known for medicinal use, specific reports on their antiviral activity are only a few (KIRTIKAR and BASU, 1935). Bearing this in mind, we have conducted a detailed study about antiviral activity of mangrove plants (PREMANATHAN 1991; PREMANATHAN *et al.,* 1992a,b; 1993b,c). This paper consolidates our previous reports on antiviral properties of mangrove halophytes.

Materials and methods

Extraction for antiviral substances: The mangrove plant samples were collected from Pichavaram mangrove forest (Lat. 11°27′N; Long. 79°47′E), Tamil Nadu, India. They were washed, shade-dried, and powdered. The samples were macerated with 70% aqueous ethanol followed by an exhaustive percolation of the material with the same solvent (VAN DEN BERGHE *et al.*, 1986). The solvent from the extract was removed under reduced pressure at 40°C. The solid obtained was used in an antiviral assay after dissolving in

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dimethyl sulphoxide (DMSO), taking into account that the maximum concentration of DMSO in the text solution should not exceed 1%. The solution was further diluted with glucosol solution for Newcastle disease virus (NDV) or phosphate buffer saline for hepatitis B virus (HBV) or maintenance medium [minimum essential medium (MEM) +2% fetal calf serum (FCS)] for vaccinia virus (VV), encephalomyocarditis virus (EMCV) and Semliki forest virus (SFV) or RPMI 1640 medium +10% FCS for human immunodeficiency virus (HIV).

Antiviral assay: Antiviral activity against NDV was carried out in chorioallantoic membrane (CAM) culture. For the assay, 1 ml of glucosol solution containing 75 µg of plant extract and 0.064 haemagglutination unit of NDV was poured into each tube containing CAM pieces and incubated horizontally at 37°C for 48 h. Then the medium was taken out and a haemagglutination titre was carried out. Inhibition of the haemagglutination by each extract was calculated as follows:

$$\text{Percent inhibition} = \frac{C - T}{C} \times 100$$

where C - virus control; T - treated with extract

Antiviral assay for VV, EMCV, SFV were conducted in CEF (chick embryo fibroblast), LM (mouse fibroblast) and Vero (monkey kidney fibroblast) cells, respectively, employing tissue culture microtiter plates. The cells in the growth medium (MEM +10% FCS) were seeded into microtiter wells of 100 µl volume and allowed to grow to a confluent monolayer. Once the monolayer was formed, the growth medium was changed to maintenance medium and challenged with VV (50 pock-forming units/ml) or EMCV and SFV (10 tissue culture infective dose 50/ml) and incubated at 37°C for 120 min (VV) or 90 min (EMCV and SFV) for adsorption of the viruses. Then the medium was changed to fresh maintenance medium with plant extracts and incubated further at 37°C for 72h (VV) or 49h (EMCV and SFV); then the monolayer was stained with crystal violet. The antiviral activity of the extracts was determined by the reduction of plaques formed by VV or inhibition of cytopathic effects (CPE) for EMCV and SFV, compared with controls as follows:

$$\text{Reduction of plaques (\%) or CPE inhibition (\%)} = \frac{C - T}{C} \times 100$$

where C - virus control; T - treated with extract

The binding activity of HBsAg (hepatitis B virus surface antigen) was assayed following VENKATESWARAN *et al.* (1987). Plant extracts were mixed separately with an equal volume of sera positive for HBsAg, and the mixture was incubated for one hour at 37°C. The mixture was assayed directly for HBsAg using the Auzyme II ELISA Kit (Abbott, USA). Binding activity was expressed as the percentage decrease in the adsorption of the test sample compared to that of the controls composed of 1:1 (vol/vol) mixture of HBsAg

positive serum and PBS.

Antiviral assay for HIV was carried out in MT-4 cells using the MTT method. Briefly, MT-4 cells were suspended at 3×10^5 cells per ml and infected with HIV at a multiplicity of infection (MOI) of 0.01. The HIV-infected or mock-infected MT-4 cells were placed in 96 well microtiter plates (200 µl/well) and incubated at 37°C in a CO_2 incubator in the presence of the test substance. After 5 days, cell viability was quantified by the MTT assay, as described previously (PAUWELS *et al.,* 1987), from which 50% cytotoxic concentration (CC_{50}), 50% effective concentration (EC_{50}) and selectivity indices ($SI = CC_{50}/EC_{50}$) were calculated.

Results and Discussion

Mangrove plant samples were extracted in aqueous ethanol and their antiviral activities were evaluated. The results of the overall *in vitro* screening are summarized in Table 1. The extracts which showed more than 50% of antiviral activity *in vitro* are described in detail.

Anti-NDV activity: Plant extracts were tested *in vitro* against NDV in CAM culture and their gross antiviral activity was evaluated in terms of percent inhibition of haemagglutination and is shown in Table 2. Of the 51 extracts, 10 were found effective when treated along with the virus, by inhibiting the haemagglutination (HA) titer within a range from 60 to 100%. Bark and stilt root *Rhizophora mucronata* showed minimum and maximum HA inhibition, respectively.

Anti-VV activity: Plant extracts were tested *in vitro* in CEF cell culture and their antiviral activity was evaluated in terms of reduction in number of plaques by the extracts and the 50% effective concentration (EC_{50}) of each test sample was calculated. The results are shown in Table 3. Of the 51 extracts, only four showed anti-VV activity. The EC_{50} ranged from 18.2 µg/ml in bark of *Lumnitzera racemosa* to 48.1 µg/ml in leaf of *Ceriops decandra.*

Anti-EMCV activity: Plant extracts were tested *in vitro* against EMCV in LM cells and their antiviral activity was evaluated in terms of inhibition of cytopathic effect (CPE) and the results are shown in Table 4. Of the 51 extracts, 13 showed anti-EMCV activity when assayed along with the virus. The CC_{50} ranged from 32.3 µg/ml in hypocotyl of *Rhizophora apiculata* to 326 µg/ml in leaf of *Avicennia marina.* The EC_{50} ranged from 6.1 to 50 µg/ml in bark and flower of *R. mucronata*, respectively. The SI ranged from 2.0 in leaf of *Salicornia brachiata* to 15.4 in bark of *R. mucronata.*

The extracts which were found effective when treated along with the virus, were tested further for their prophylactic activity against EMCV. The LM cells were first treated with plant extracts, washed after 24 h and then subjected to viral infection. The prophylactic activity was evaluated in terms of inhibition of CPE and the results are shown in Table 5. Of the 13 extracts, four were effective with the range of CPE inhibition. The EC_{50} ranged from 7.5 µg/ml in bark of *R. mucronata* to 21 µg/ml in leaf of *Bruguiera cylindrica.*

The extracts which were found effective when treated along with the virus, were tested

Table 1 . Results of antiviral screening of extracts of mangrove plants*

Plant name	Part	NDV[a]	VV[b]	EMCV[c]	SFV[c]	HBV[d]	HIV[c]
Acanthaceae							
Acanthus ilicifolius (L.)	calyx	++	+	-	NT	NT	-
	corolla	-	-	-	NT	+++	-
	fruit	++	-	-	NT	+++	+
	leaf	-	-	-	NT	NT	++
	root	++++	+	++	++	NT	+
Aizoaceae							
Sesuvium portulacastrum L.	leaf	-	-	-	NT	+++	-
Avicenniaceae							
Avicennia marina (Forssk.) Vierh.	bark	-	-	-	NT	NT	-
	flower	-	-	-	NT	NT	-
	fruit	-	-	+	-	+++	+
	leaf	-	-	++++	+	+++	+
	Pneumatophore	-	-	-	NT	-	NT
Avicennia officinalis L.	bark	-	+	+	-	NT	+
	leaf	-	-	+	-	NT	+
	Pneumatophore	-	-	-	-	-	NT
	stem	-	-	-	NT	NT	NT
Chenopodiaceae							
Salicornia brachiata Roxb.	leaf	+	-	+++	++	+++	+
	stem	-	-	-	NT	NT	NT
Suaeda maritima (L.) Dumort	leaf	-	-	-	NT	NT	-
Suaeda monoica Forssk.	leaf	-	-	+	NT	NT	-
Combretaceae							
Lumnittzera racemosa Willd.	bark	++++	+++	+++	++	NT	+
	leaf	-	-	++	+	NT	-
	stem	-	-	-	NT	NT	NT
Euphorbiaceae							
Excoecaria agallocha L.	flower	+	-	++	+	NT	NT
	fruit	+++	-	++++	++	NT	++
	leaf	-	+	-	-	-	++++
Myrsinaceae							
Aegiceras corniculatum (L.) Blanco	fruit	++++	-	-	+++	NT	++
	leaf	+++	-	-	-	+++	++++
Rhizophoraceae							
Bruguiera cylindrica (L.) Blume	bark	++++	++	+++	++	++++	+
	fruit	+++	+	+++	++	+++	NT
	hypocotyl	-	-	-	NT	NT	NT
	leaf	+++	+++	+++	++++	++++	++
Ceriops decandra (Griff.) Ding Hou	bark	++	++	+	+	++++	++
	fruit	+	++	-	-	++++	NT
	hypocotyl	+	++	-	-	NT	NT
	leaf	++	+++	-	++++	++++	++++
Rhizophora apiculata Blume	bark	+	+	++	+	++++	++
	flower	-	-	+++	++	++++	NT
	fruit	-	++	-	-	++++	NT
	hypocotyl	++	+	+++	+	+++	NT
	leaf	+	++	+	+	NT	++++
	stilt root	-	+	-	-	NT	++
Rhizophora lamarckii Montr.	flower	++	++	++	+++	++++	NT
	leaf	+	++	++++	++	++++	++++
	stilt root	++	-	-	-	NT	++
Rhizophora mucronata Poir.	bark	+++	+++	++++	++++	++++	++++
	flower	++	-	+++	++	++	NT
	fruit	-	-	+	+	+++	NT
	hypocotyl	+	-	++	+	++++	NT
	leaf	+	-	+	+	+++	+++
	stilt root	++++	++	++++	+++	++++	+
Sonneratiaceae							
Sonneratia apetala B. Ham.	leaf	-	-	-	NT	NT	-

\- No activity
\+ 1-24% activity
++ 25-49% activity
+++ 50-74% activity
++++ 75-100% activity
NT Not tested

a% Inhibition of haemagglutination
b% Plaque reduction
c% Inhibition of cytopathic effect
d% Binding inhibition

* data taken and modified from Premanathan *et al.,* 1992

Table 2. *In vitro* gross anti-NDV activity of mangrove plants*

Plant name	Part	HA inhibition (%)
Acanthus ilicifolius	root	90
Aegiceras corniculatum	fruit	85
	leaf	72
Bruguiera cylindrica	bark	90
	flower	70
	leaf	62
Excoecaria agallocha	fruit	70
Lumnitzera racemosa	bark	76
Rhizophora mucronata	bark	60
	Stilt root	100

*data taken from Premanathan *et al.,* 1993a

Table 3. *In vitro* anti-VV activity of mangrove plants*

Plant name	Part	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Lumnitzera racemosa	bark	68.5	18.2	3.8
Bruguiera cylindrica	leaf	102.3	33.3	3.1
Ceriops decandra	leaf	189.3	48.1	3.9
Rhizophora mucronata	bark	87.1	18.5	4.7

*data taken from Premanathan *et al.,* 1994a

Table 4. *In vitro* anti-EMCV activity of mangrove plants*

Plant name	Part	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Avicennia marina	leaf	326.0	46.7	7.0
Bruguiera cylindrica	bark	111.0	14.6	7.6
	fruit	82.4	25.0	3.3
	leaf	115.0	23.1	5.0
Excoecaria agallocha	fruit	132.0	16.7	8.0
Lumnitzera racemosa	bark	72.5	8.4	8.5
Rhizophora apiculata	flower	78.9	28.6	2.8
	hypocotyl	32.3	10.0	3.2
Rhizophora lamarckii	leaf	119.0	30.0	4.0
Rhizophora mucronata	bark	92.5	6.1	15.4
	flower	162.0	50.0	3.2
	stilt root	88.5	18.7	4.7
Salicornia brachiata	leaf	62.1	30.8	2.0

*data taken from Premanathan *et al.,* 1994b

Table 5. Prophylactic anti-EMCV activity of mangrove plants*

Plant name	Part	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Bruguiera cylindrica	leaf	115	21.0	5.5
Rhizophora mucronata	bark	93	7.5	12.3
	Stilt root	88	17.9	4.9
Lumnitzera racemosa	bark	76	8.0	9.4

*data taken from Premanathan *et al.,* 1994b

Table 6. *In vitro* anti-SFV activity of mangrove plants*

Plant name	Part	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Bruguiera ccylindrica	leaf	139	14	10
Ceriops decandra	leaf	209	18	11
Aegiceras corniculatum	fruit	112	18	6
Rhizophora lamarrckii	flower	143	36	4
Rhizophora mucronata	bark	110	6	18
	Stilt root	111	15	8

*data taken from Premanathan *et al.,* 1995a

further for their therapeutic activity against EMCV. The LM cells were treated with virus for 6 h and then were treated with extracts. Of the 13 extracts, only the bark of *R. mucronata* was found effective with 70% inhibition of CPE and with the EC_{50} value of 8.57 μg/ml (PREMANATHAN *et al.,* 1994b).

The extracts which were effective in prophylactic condition were tested *in vivo* in Swiss albino mice at the rate of 31.25 mg/kg animal/day (0.5 mg/mouse/day). Only the extract of bark of *R. mucronata* showed 30% protection and 3.2 days increase in average survival time (AST) (PREMANATHAN *et al.,* 1994b).

Anti-SFV activity: Plant extracts were tested *in vitro* against SFV in Vero cells along with the virus. The antiviral activity was evaluated in terms of inhibition of CPE and the results are shown in Table 6. Of the 36 extracts, six showed anti-SFV activity. The EC_{50} ranged from 6 μg/ml in bark of *R. mucronata* to 36 μg/ml in flower of *Rhizophora lamarckii.*

The extracts which were effective *in vitro* were tested *in vivo* in Swiss albino mice at the rate of 31.25 mg/kg animal/day (0.5 mg/mouse/day). Bark of *R. mucronata* protected the mice to an extent of 40% with 5.8 days increase in AST. The stilt root of *R. mucronata* could protect only 10% with 1.7 days increase in AST (PREMANATHAN *et al.,* 1995a).

Anti-HBV activity: Plant extracts were mixed at a concentration of 3 mg/ml with surface antigen of HBV (HBsAg) and then allowed to react with antibody to HBsAg (anti-HBs). The binding activity of the extracts was determined in terms of percent decrease in absorbance at 492 nm as compared to control. The results are shown in Table 7. Of the 28 extracts, 24 exhibited binding activity with HBsAg and inhibited the reaction of HBsAg with anti-HBs. The maximum and minimum inhibition was observed in fruit of *C. decandra* and fruit of *B. cylindrica*, respectively.

Anti-HIV activity: Plant extracts were tested *in vitro* against HIV in MT-4 cells and their

Table 7. Effect of mangrove plant extracts at 3 mg/ml on the binding of HBsAg to anti-HBs *in vitro*[*]

Plant name	Part	Inhibition of binding (%)
Acanthus ilicifolius	corolla	61.2
	fruit	56.8
Aegiceras corniculatum	leaf	58.5
Avicennia marina	fruit	56.1
	leaf	63.8
Bruguiera cylindrica	bark	91.7
	fruit	53.4
	leaf	83.3
Ceriops decandra	bark	92.2
	fruit	93.0
	leaf	67.7
Rhizophora apiculata	bark	84.3
	flower	89.2
	fruit	87.5
	hypocotyl	60.6
Rhizophora lamarckii	flower	89.0
	leaf	84.3
Rhizophora mucronata	bark	85.4
	fruit	70.8
	hypocotyl	91.3
	leaf	54.0
	Stilt root	91.0
Salicornia brachiata	leaf	68.0
Sesuvium portulacastrum	leaf	68.4

[*]data taken from Premanatham, 1991

antiviral activity was evaluated in terms of inhibition of cytopathic effects (CPE) as measured by MTT assay and the results are shown in Table 8. Of the 34 extracts, seven showed anti-HIV activity. The EC_{50} ranged from 7.3 μg/ml in leaf of *Excoecaria agallocha* to 492.3 μg/ml in leaf of *R. mucronata.* The SI ranged from 1.6 in leaf of *R. mucronata* to 16.2 in leaf of *C. decandra.* The active plant samples were further extracted in different solvents and tested for anti-HIV activity. Alkaline extracts were found effective (PREMANATHAN *et al.,* 1995b).

The overall screening of the mangrove plants reveals that plants from the family Rhizophoraceae were more effective than the plants from other family. Fourteen plant extracts were effective against any one of the viruses. Eight were effective against any two viruses. The most promising extracts were bark of *R. mucronata* and leaf of *B. cylindrica,* which were effective against six and five viruses, respectively. Leaf of *C. decandra,* stilt root of *R. mucronata* were effective against four viruses. Bark and fruit of *B. cylindrica,* leaf of *R. lamarckii,* bark of *L. racemosa* and leaf of *Aegiceras corniculatum* were effective

Table 8. *In vitro* anti-HIV activity of mangrove plants*

Plant name	Part	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Aegiceras corniculatum	leaf	48.3	29.3	1.6
Ceriops decandra	leaf	216.5	13.4	16.2
Excoecaria agallocha	leaf	77.8	7.3	10.7
Rhizophora apiculata	leaf	998.2	108.5	9.2
Rhizophora lamarckii	leaf	331.5	139.2	2.4
Rhizophora mucronata	bark	38.8	7.6	5.1
	leaf	798.4	492.3	1.6

*data taken from Premanathan *er al.,* 1996

against three viruses. In general, plants belonging to the family Rhizophoraceae are the source of potential antiviral substances. To our knowledge, none of the aforementioned extracts have yet been reported by others as antivirals in literature. Our work reports for the first time that mangrove halophytes have antiviral activity.

We have partially purified the active substances from the bark of *R. mucronata* and leaf of *R. apiculata* and identified the active substances are acid polysaccharides (PREMANATHAN *et al.,* 1999; PREMANATHAN *et al.,* unpublished data). These acid polysaccharides blocked the virus binding to cells as mechanism of action for its anti-HIV activity (PREMANATHAN *et al.,* 1999). Lignins were identified as active substance from the leaf of *C. decandra* for its anti-HIV activity (PREMANATHAN *et al.,* unpublished data).

Indigenous drugs for the treatment of HIV infection are urgently needed as currently available synthetic anti-HIV drugs are very expensive and limited in supply. Natural substances from plants are widely used in medicine in India. It is, however, essential that the mangrove halophytes as source of new antiviral leads should be explored further.

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