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Antiviral, antioxidant and toxicological evaluation of mangrove associate from South East coast of India

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ABSTRACT

Objective: To identify the antiviral antioxidant and toxicological evaluation of marine halophyte. **Methods:** Mangrove associates such as *Salicornia brachiata*, *Clerodendron inerme*, *Rhizophora lamarckii*, *Suaeda maritima* were collected. *In vitro* antiviral studies such as HBsAg binding assay, DNA polymerase inhibition assay, RT inhibition assay were carried out. Moreover, antioxidant properties, ash content, elemental analysis, LD_{50} analysis were measured for the *S. maritima* leaf extract which was the most potent. **Results:** *S. maritima* leaf extract showed minimum concentration of IC50 value with HBsAg binding assay, DNA polymerase inhibition assay, RT inhibition assay as 325.98, 843.09 and 587.32 μg/ml concentrations respectively. Antioxidant properties of *S. maritima* leaf extract showed the minimum concentration (23.64±5.27 μg/ml) of IC_{50} value with the nitric oxide scavenging assay, followed by DPPH assay (112.03±18.39 μg/ml). The ash content of *S. maritima* leaf extract was varied between 8.05% to 87.30% concentrations. The elemental analysis of *S. maritima* showed the values within the limits of WHO guidelines. The lethal dose of *S. maritima* leaf extract was identified as 3000 mg/kg/body weight. The sub acute toxicity was not showed any significant differences with organ weights between control and extract treated animals. Biochemical parameters such as SGOT, SGPT, ALP, sugar and urea were not showed any significant variations between control and extract treated animals. But, the results of haematological parameters such as WBC (6600±234.90 cells/cumm), lymphocytes (69±14.09), polymorphs (38±9.38), eosinophils (02±0.00) were found significantly increased with extract treated animals. Phytochemical analysis of *S. maritima* leaf extract showed the presence of various phytochemical constituents such as reducing sugars, polyophenols, flavonoids and tannins with the leaf extract. **Conclusions:** The results of the present findings pave the way for the identification of novel molecules for the possible utilization of antiviral and antioxidant drugs from *Suaeda maritima* leaf.

1. Introduction

Viruses induce several diseases in human beings representing a major health threat to the public. Controlling of viral infections is a major unachieved goal due to the emerging of intracellular replicative and mutating nature of the viral genomes [1]. The use of natural products from terrestrial and marine sources is widely used for curative agents for many human and animal ailments during ancient years [2]. Many of the medicinal plant materials and its chemical constituents are widely used as antiviral agents [3]. In that, marine plants such as mangroves and mangrove associates are scientifically proved to have antibacterial, antifungal, antiplasmodial, hepatoprotective, antiviral, spermicidal and mollusicidal activities [4-8]. The studies related to the antiviral activities by using marine halophyte plants are too limited. In this connection, the present study was made an attempt to identify the antiviral and antioxidant properties of the marine halophyte plants.

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2. Materials and methods

2.1. Collection and preparation of extracts

Fresh matured leafs from *Salicornia brachiata*, *Clerodendron inerme*, *Rhizophora lamarckii*, *Suaeda maritima*, were collected during the spring season from Pichavaram mangrove forest (Lat. 11° 20′ N; Long. 79° 47′ E), Tamil Nadu, India. The specimen sample was authenticated by Prof. K. Kathiresan, Centre of Advanced Study in Marine Biology, Annamalai University, Porto Novo, Tamil Nadu in India. The voucher specimen (AUOCAS038 and AUOCAS041) was also maintained in the herbarium cabinet facility, sponsored by Indian Council of Medical Research, New Delhi at School of Marine Sciences, Department of Oceanography and Coastal Area Studies, Alagappa University, Thondi Campus, Thondi, India. The collected parts were washed thrice with distilled water to remove the contaminants and air dried in shade at room temperature. Coarsely powdered sample (500 g) was defatted with petroleum ether (60-80 ℃) and then extracted with 1L of 95% (V/V) ethanol and water mixture by percolation method. The extract was concentrated under vacuum to obtain solvent free residues. Preliminary phytochemical analysis such as phenolic group, alkaloids, flavonoids, catachin, triterpenoids, tannins, and anthroquinones were assessed for the most potent antiviral extract by following standard protocol[5].

2.2. In vitro HBsAg binding assay

Various concentration (5, 2.5, 1.25, 0.62 and 0.312 mg/ml) of different halophytic extracts were mixed individually with equal volume of HBsAg positive plasma and the mixture was incubated for 1 hr at 20℃. The mixture was assayed directly for HBsAg Ausria II ELISA kits (Abbott). Control was maintained with 1:1 (v/v) mixture of surface antigen positive serum and PBS. (PBS = 0.01 M sodium phosphate/0.85% NaCl, pH 7.2.) Binding activity was expressed as the decrease in the absorption of the test sample compared to that of the control [9].

2.3. In vitro RT inhibition assay

The HIV-1 reverse transcriptase (RT) inhibitory assay was performed by using standard HIV-RT ELISA kit (Roche Diagnostics). In brief, 10 μl of various concentrations (5, 2.5, 1.25, 0.62 and 0.312 mg.ml^{-1}) of different halophytes were serially diluted with the 0.2 units of 10 μl of RT enzyme, 60 μl of reaction mixture (25 μl of DNA primer/RNA template complex, 50mM tris buffer solution and dTTP solution (NEN, NET520A, 90-120 Ci/mmol)) and incubated at 37℃ for 1 hr. Control was maintained without the addition of the plant extracts. The radioactivity was measured using the liquid scintillation analyzer (TRICARB 2100TR, Packard Instrument Company, USA). The mean of the triplicate counts was used to calculate the percentage of inhibition according to the formula given below,
Percentage of inhibition= 100- Mean CPM test X 100 /Mean CPM negative control

2.4. HBV DNA polymerase Inhibition Assay

HBV DNA polymerase inhibition assay was performed by the radiometric method (3). 10 μl of various concentrations (5, 2.5, 1.25, 0.62 and 0.312 mg.ml^{-1} dissolved in 5% DMSO) of halophytic extracts and were serially diluted with 100 μl of raction mixture (100 mM Tris-HCl (pH 8.0), 20 mM MgCl2, 200 mM KCl, 10 mM dATP, 10 mM dCTP, 10 mM dGTP and 0.5 μCi 3H-dTTP (70 Ci/mmol, Amersham) and 25 μl of viral preparation and the mixture was incubated at 37 ℃ for 3 hr. Control plates are also maintained without the addition of the plant extracts. After 3 hr, 10 μl of 0.2M EDTA was added and then spotted on a Whatman DEAE cellulose (DE-81) paper disc. The disc was dried and washed with 5% TCA for 3 times, twice in 95% ethanol. Finally, the discs were dried and immersed in the vial containing 5 ml scintillation fluid. The radioactivity was measured using the liquid scintillation analyzer (TRICARB 2100TR, Packard Instrument Company, USA). The mean of the triplicate counts was used to calculate the percentage of inhibition according to the formula,
Percentage of inhibition= 100- Mean CPM test X 100 /Mean CPM negative control

2.5. Determination of in vitro antioxidant assay

Various concentrations (1.9 μg/ml to 500 μgml) of the most potent *S. maritima* leaf extract and vitamin C (Positive control) was used for the determination of DPPH assay, nitric oxide radical scavenging assay, lipid peroxide radical scavenging assay and superoxide radical scavenging assay by following standard protocols[8]. Statistical calculations such as IC_{50} values and SD values were calculated with office XP/SDAS add-ins program.

2.6. Toxicity studies

2.6.1. Experimental animals

Albino rats weighing 180-230g (either sex) animals were housed in large spacious cages, maintained in controlled environment of temperature, humidity and light/dark cycles. They were fed with standard pelleted diet obtained from Sai Durga Feeds and Foods, Bangalore, India and water ad libitum. All the animal model studies and experimental protocols have been approved by International Research And Review Board (Irb), Ethical Clearance (EC), Biosafety and Animal Welfare Comittee, Madurai Kamaraj University, Madurai, Tamilnadu, India.

2.6.2. Acute toxicity studies

Female albino rats were used for acute toxicity study. Ten animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose of 250 mg/kg body weight and observed for 24h. If the mortality was observed in six out of nine animals, then the dose administered was assigned as toxic dose. If mortality was observed in three animals, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher

dose i.e. 300mg/kg body weight. One tenth of the maximum dose of the extract tested for acute toxicity was selected for evaluation of sub acute toxicity analysis.

2.6.3. Sub-acute toxicity studies

Fifteen albino rats were taken and divided into two groups. Group I served as control and group II received drug extract of 200 mg/kg/body weight/ day. This dose was given to animal for 28 days. All the animals were sacrificed at 29th day. Blood sample was collected from all the animals and serum was separated by centrifugation at 2,500 rpm for 10 min. Organs such as liver and kidney were then processed to study histological changes adopting paraffin method. The blood serum sample was used for the determination of the SGOT, SGPT, ALP, urea and sugar level. The cell counts were calculated by using the haemocytometer.

2.7. Estimation of ash content

2.7.1. Total Ash

One gram of the extract was taken in preweighed silica crucible which were previously ignited, cooled and weighed. It was incinerated by gradually increasing the heat not exceeding dull red heat (450℃) until free from carbon, cooled and weighed. The percentage of ash was calculated with reference to air-dried drug. The procedure was repeated to get the constant weight.

2.7.2. Water Soluble Ash

The total ash was boiled with 25 ml water and filtered through ash free filter paper (Whatmann 4.1). It was followed by washing with hot water. The filter paper was dried and ignited in the silica crucible, cooled and the water insoluble ash was weighed. The water-soluble ash is calculated by subtracting the water insoluble ash from the total ash.

2.7.3. Acid insoluble ash

To total ash obtained was boiled for 5 minutes with 25 ml of (10% w/v) dilute hydrochloric acid and filtering through ash free filter paper (Whatmann 4.1). The filter paper was ignited in the silica crucible, cooled and the water insoluble ash was weighed.

2.7.4. Ethanol Soluble Ash

The total ash obtained was boiled for 5 minutes with 25 ml of (10% w/v) ethanol and filtering through ash free filter paper. The filter paper was ignited in the silica crucible, cooled and insoluble ash was weighed. The ethanol-soluble ash is calculated by subtracting the ethanol insoluble ash from the total ash.

2.8. Elemental Analysis

About 100 mg of the *S. maritima* leaf extract was weighed and 5-10 ml of concentrated sulphuric acid was added to it. The acid digestion was further initiated by heating up to 400℃ using a digesdahl apparatus. The samples were made free from organic matter and the resulting solution was made colorless by adding 5-10 ml of H_2O_2. The digested material was made up to 100 ml for analysis in an AAS. Hg and As

Table 1

Antiviral properties of marine halophytes against various inhibitory assays

Plant species	HBsAg inhibition assayIC_{50} (μg/ml)	DNA polymerase inhibition IC_{50} (μg/ml)	RT inhibition assayIC_{50} (μg/ml)
S. brachiata	<1000	879.09	-
C. inerme	-	>1000	679.90
R. lamarckii	>1000	-	-
S. maritima	325.98	843.09	587.32

Table 2

Antioxidant properties (IC_{50}) of the most effective marine plant *S. maritima* leaf extract

Parameter	*S. maritima* leaf extractIC_{50} (μg/ml)	Vitamin C IC_{50} (μg/ml)
DPPH radical scavenging assay	112.03±18.39	2.87±1.26
Nitric oxide (NO) scavenging assay	23.64±5.27	4.98±1.28
Lipid peroxide radical scavenging assay (LPO)	604.33±46.49	31.79±1.21
Super oxide radical scavenging assay (SOD)	127.36±23.98	24.31±0.71

Table 3

Content of elements present in *S. maritima* leaf extract

Heavy Metal/Trace metal	Observed level (ppm/g)	Permissible limits as per the WHO guidelines per gram sample (ppm/g)
Iron	2.278	30
Copper	0.731	150
Manganese	1.361	30
Zinc	0.749	20
Chromium	2.491	2
Lead	0.022	10
Arsenic	0.076	5
Nickel	1.98	30

Table 4

Effect of various concertinos of *S. maritima* leaf extract in LD_{50} analysis of Wistar albino rats

Concentration of the extract (mg/kg)	Behavioural changes	Mortality rate
500	Loss of traction	–
1000	Loss of traction, hyperactivity, jumping	–
1500	Loss of traction, hyperactivity, jumping	–
2000	Loss of traction, hyperactivity, jumping	3 (30)
2500	Loss of traction, hyperactivity, jumping	4 (40)
3000	Loss of traction, hyperactivity, jumping, abnormal secretion, jumping	7 (70)

Values in the parenthesis indicates the percentage of mortality

Table 5

Effect of *S. maritima* leaf extract on the organ weight (g) in the control and treated rats

Group	Weight of the Organs (mg/100g body weight)				
	Heart	Liver	Spleen	Kidney	Testis
Control	0.38 ± 0.13	3.94 ± 0.26	0.46 ± 0.08	0.46 ± 0.04	2.52 ± 0.12
Treatment (300 mg/kg/ body weight)	0.40 ± 0.06	3.42 ± 0.05	0.53 ± 0.15	0.74 ± 0.04	2.66 ± 0.13

Values are mentioned with ± SD values of three replicates

was estimated using a hydride generator attached to the AAS. Results were calibrated using standard linear calibrations and the mean of triplicate values were tabulated.

Table 6

Effect of *S. maritima* leaf extract on the serum parameters in treated and control rats

Parameters	Normal control	Treatment
(300 mg/Kg/day/ body weight)		
SGOT(IU/L)	167.33±13.27	177.93±12.89
SGPT(IU/L)	60.67±5.39	65.90±8.60
ALP(IU/L)	135.27±9.67	129.79±12.90
Total WBC (cells /cu. mm)	4,800.00±120.34	6600±234.90
Polymorphs (%)	54.00±5.89	69±14.09
Lymphocytes (%)	45.00±4.05	38±9.38
Eosinophils (%)	01.00±0.00	02±0.00
Blood sugar (mg/dl)	40.00±3.45	46±6.78
Blood Urea (mg/dl)	21.00±2.76	20±3.09

Values are mentioned with ± SD values of three replicates

3. Results

Minimum concentration of IC50 value was identified with *S. maritima* leaf extract with HBsAg, DNA polymerase inhibition assay, RT inhibition assay as 325.98, 843.09 and 587.32 μg/ml concentrations respectively (Table 1). Further, the results of antioxidant properties of *S. maritima* leaf extract showed that, the minimum concentration (23.64±5.27 μg/ml) of IC_{50} value was identified with the nitric oxide scavenging assay, followed by DPPH assay (112.03±18.39 μg/ml) but the results of the vitamin-C showed the minimum concentration (2.87±1.26 μg/ml) of IC_{50} value with the DPPH assay (Table 2). The total ash, water soluble ash, acid insoluble ash and ethanol soluble ash content of *S. maritima* leaf extracts were identified as 87.30%, 8.05%, 12.25% and 12.60% respectively. The results of the elemental analysis showed that, all the heavy and trace metal analysis showed the results within the limitations of WHO guidelines (Table 3). The lethal does of *S. maritima* leaf extract was identified as 3000 mg/kg/body weight. In addition no mortality rate was observed with 500 to 1500 mg/kg/body weight concentrations, but, the maximum (3000 mg/kg body weight) concentration of the leaf extract was showed the behavioural changes such as loss of traction, hyperactivity, jumping and abnormal secretion during the time of treatment (Table 4). Further, the results of the sub acute toxicity analysis showed that, there were no significant differences between organs weight of control and *S. maritima* leaf extract treated animals (Table 5). In addition the results of biochemical parameters such as SGOT, SGPT, ALP, sugar and urea were not showed any significant variations between control and extract treated animals. But, the results of haematological parameters such as WBC (6600±234.90 cells/cumm), lymphocytes (69±14.09), polymorphs (38±9.38), eosinophils (02±0.00) were found significantly increased with extract treated animals (Table 6). The results of preliminary phytochemcial analysis showed that, the *S. maritima* leaf extract showed the presence of various phytochemical constituents such as reducing sugars, polyophenols, flavonoids and tannins with the leaf extract.

4. Discussion

In recent years, a number of plant parts and extracts have been found to inhibit a variety of viruses [10-12]. However, marine plants as a source of antiviral agents have not been extensively studied [13,14]. Therefore the present study was carried out to identify the antiviral activity of certain marine and coastal plant species against HIV and HBV viruses. The results suggested that, the maximum inhibition of HBsAg and DNA polymerase inhibition activity was identified with the *S. maritima* leaf extract and the antiviral properties of the leaf extract might be due to the presence of unique chemical constituents such as flavonoids, phenolic compounds, tannins or the synergistic activity of the phytochemcial constituents[4]. Traditionally, the leaf from *Suaeda maritima* is known to use as a medicine for hepatitis [15] and scientifically it is reported to have antiviral activity and hepatoprotective properties [5,16] with the presence of triterpenoids, sterols [17] and the inhibitory effect of the *S. maritima* leaf extract can be directly used as an blocking agent of the viral surface antigen or inhibitory agent of viral polymerase enzymes involving in DNA replication [3]. Similarly Venkataraman [9] also reported that, the HBsAg

inhibitory activity with the *Aporosa lindleyana* root extract. Moreover, the multiplication of the viral cells in the host cell system may produce large amount of free radicals [18] and this free radicals are highly reactive oxygen molecules which includes with hydroxyl radical, super oxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides [19]. All the radicals are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage [20]. To neutralize the activity of the free radicals, the present study was also made an attempt to identify the antioxidant properties of the *S. maritima* leaf extract and the results showed that, the potential radical scavenging activities and this can be directly used as a preventive agent for the neutralization of the free radicals in the viral infected host system [21]. The results of element analysis of *S. maritima* leaf extract showed the values within the limits of WHO guidelines and this result further supports the future development of drugs from *S. maritima* leaf extract. Biological substances can be classified into two major groups (LD_{50} values between 500 to 5000 $mg.kg^{-1}$ can be considered as slightly toxic and 5000 to 15,000 $mg.kg^{-1}$ can be considered as non toxic) based on Mukinda and Syce [22] hypothesis, the LD_{50} value of present findings comes under the category of slightly toxic group. The hematological parameters such as WBC, platelets (in sub acute toxicity analysis) not showed any significant changes between the control and *S. maritima* extract treated animal groups and this directly indicates the non toxicity effect of leaf extract to the blood cells circulation [22]. Further, the results of SGOT, SGPT, ALP and urea parameters were not showed any changes between control and extract treated animals and this directly indicates the non toxicity effect of leaf extract to the vital organs such as liver, spleen, kidney, testis and heart tissues [23]. It can be concluded from the present findings that, the leaf samples of *Suaeda maritima* collected from the Pichavaram mangrove forest, has potential inhibitory activity against the hepatitis B surface antigen and DNA polymerase inhibitory activity. Further studies are highly warranted to identify the active principles to establish the *in vivo* antiviral activities.

Conflict of interest statement

We declare that we have no conflict of interest.

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