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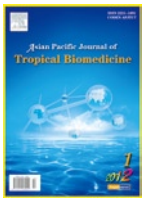

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Antiviral, antioxidant and toxicological evaluation of mangrove plant from South East coast of India

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ABSTRACT

Objective: To identify the antiviral, antioxidant and toxicological evalution of the mangrove plant from South East coast of India. **Methods:** *In vitro* antiviral analysis such as RT inhibition assay, HBsAg binding assay, HBV DNA polymerase Inhibitory assays were carried out with the leaf extract of *Avicennia marina, Avicennia officinalis, Rhizophora mucronata* and *Rhizophora apiculata* mangrove plants. Further, the *in vitro* antioxidant assays and *in vivo* toxicological assay was also carried out with the most potent *Avicennia marina* leaf extract. **Results:** Of the selected 4 mangrove plants, *Avicennia marina* leaf extract showed the minimum IC_{50} values with 403.91, 489.39 and 372.09 μg/mL concentrations against reverse transcriptase inhibition assay, HBsAg binding assay, HBV DNA polymerase Inhibitory assays, respectively. The antioxidant IC_{50} values were identified as 12.80±0.93, 640.06±34.93, 19.91±3.93 and 142.06±17.93 μg/mL concentrations against SOD, LPO, NO and DPPH assays, respectively. The phytochemical analysis of *Avicennia marina*leaf extract showed the presence of reducing sugars, polyophenols, flavonoids and tannin. The LD_{50} value was 2 500 mg/kg bodyweight and the sub acute toxicity analysis did not show any significant changes in serum and haematological parameters when compared with the control animals. **Conclusions:** It can be concluded from the present findings that, the leaf extract of *Avicennia marina* can be further used as potential antiviral drug after completing the clinical trials.

1. Introduction

Viral diseases has become a global health problem causing a millions of death every year apart from reducing the quality of life and causing damaged productivity for millions of people around the world[1]. Acute viral infection is extremely common in our country leading to a significant loss of man due to its morbidity. It is considered to be a self limiting disease, caused by the infected virus, but sometimes majority of viral diseases are ending up with serious complications. So, a faster restoration is desirable for every patient, even though they suffer from a self limiting disease. Viral replications and its complications still do not have appropriate drugs in modern medicine. Mangrove and mangrove associate plants are proved to have rich of high value secondary metabolites *viz.*, saponins, alkaloids, polyphenols[2-7] which show antibacterial, antifungal, antiplasmodial and hepatoprotective[8,9] activities. But, the studies related with the antiviral activities from mangrove plants are too limited. Hence, the present study was made an attempt to identify the antiviral activities from mangrove plants against hepatitis B virus and human immuno deficiency virus infections.

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2. Materials and methods

2.1. Collection and preparation of extracts

Fresh matured leave samples from *Avicennia marina*

(*A. marina*), *Avicennia officinalis*, *Rhizophora mucronata*, *Rhizophora apiculata* (*R. apiculata*) were collected during the spring season from Pichavaram mangrove forest (Lat. 11° 20′ N; Long. 79° 47′ E), South East coast, Tamil Nadu, India. The specimen sample was authenticated by Prof. K. Kathiresan, Centre of Advanced Study in Marine Biology, Annamalai University, Porto Novo, Tamilnadu in India. The voucher specimen samples 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 dried in room temperature. Coarsely powdered sample (500 g) was defatted with petroleum ether (60-80 ℃) and then extracted with 1 L of 95% (V/V) ethanol and water mixture by percolation method. The extract was concentrated under vacuum to the 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[10].

2.2. In vitro reverse transcriptase (RT) inhibition assay

The HIV-1 RT inhibitory assay was performed by using standard HIV-RT ELISA kit (Roche Diagnostics). In briefly, 10 μL of various concentrations (100, 200, 300 400, 500, 600, 700, 800, 900, 1 000 μg/mL) of different mangrove extracts were serially diluted with the 0.2 units of 10 μL of RT enzyme, 60 μL of reaction mixture [25 mm of DNA primer/RNA template complex, 50 mM tris buffer solution and dTTP solution (NEN, NET520A, 90-120 Ci/mmol)] and incubated at 37 ℃ for 1 h. Control plates were also maintained without the addition of the mangrove 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:

$$\text{Percentage of inhibition} = 100 - \text{mean CPM}_{\text{test}} \times 100 / \text{mean CPM}_{\text{negative control}}$$

2.3. In vitro HBsAg binding assay

Various concentration (100, 200, 300 400, 500, 600, 700, 800, 900, 1 000 μg/mL) of different mangrove extracts were serially mixed individually with equal volume of HBsAg positive plasma and the mixture was incubated for 1 h at 20 ℃. The mixture was assayed directly for HBsAg Ausria II ELISA kits (Abbott). Binding activity was expressed as the decrease in the absorption of the test sample compared to that of the control composed of 1:1 (v/v) mixture of surface antigen positive serum and PBS (PBS=0.01 M sodium phosphate/0.85% NaCl, pH 7.2.) [11].

2.4. HBV DNA polymerase Inhibition Assay

HBV DNA polymerase inhibition assay was performed by the radiometric method[12]. 10 μL of various concentrations (100, 200, 300 400, 500, 600, 700, 800, 900 and 1 000 μg/mL dissolved in 5% DMSO) of different mangrove extracts were serially diluted with 100 μL of reaction mixture [100 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 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 h. Control plates were also maintained without the addition of the mangrove extracts. After 3 h, 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 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 given below:

$$\text{Percentage of inhibition} = 100 - \text{mean CPM}_{\text{test}} \times 100 / \text{mean CPM}_{\text{negative control}}$$

2.5. Determination of in vitro antioxidant assay

Various concentrations (1.9 to 500 μg/mL) *A. marina* leaf extract and vitamin C (Positive control) was used for the determination of DPPH assay[13], nitric oxide (NO) radical scavenging assay[14], lipid peroxide (LPO) radical scavenging assay[15] and superoxide radical (SOD) scavenging assay[16] by standard protocols.

2.6. Calculation of IC_{50}

Statistical calculations such as IC_{50} values and SD values calculated with office XP/SDAS add-ins program.

2.7. Toxicity studies

2.7.1. Experimental animals

Albino rats weighing 180-230 g 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 Institutional Animal Ethics Committee, Madurai Kamaraj University, Madurai.

2.7.2. 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 24 h. 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.* 300 mg/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.

2.7.3. 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/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.

2.8. Estimation of ash content

2.8.1. 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.

2.8.2. 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.

2.8.3. 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.

2.8.4. 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.

2.8.5. Elemental analysis

About 100 mg of the *A. marina* 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 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.

3. Results

The results of the present study suggest that, the minimum concentration of IC_{50} value was identified as 489.39, 372.09 and 403.91 μg/mL concentrations, respectively in HBsAg, DNA polymerase inhibition assay, RT inhibition assay of *A. marina* leaf extract. Further, the leaf extract of *R. apiculata* did not show any inhibitory activity against the HBsAg and RT inhibition assay (Table 1). The results of the antioxidant assays showed that, minimum concentration of the IC_{50} value was identified in the SOD [(12.80±0.93) μg/mL] inhibitory assay and nitric oxide [(19.91±3.93) μg/mL] inhibitory assay, but the vitamin C (positive control) showed minimum [(2.87 ±1.26) μg/mL] concentration of IC_{50} value in DPPH radical

Table 1.
Percentage inhibitory activity of inhibition assays.

Plant species	IC_{50} (μg/mL)		
	RT inhibition assay	HBsAg binding assay	DNA polymerase inhibition assay
A. marina	403.91	489.39	372.09
Rhizophora mucronata	<1 000	702.91	<1 000
R. apiculata	–	–	<1 000
Avicennia officinalis	–	<1 000	619.64

Table 2.
IC_{50} values of *A. marina* leaf extract and vitamin C with various antioxidant activities (μg/mL).

Parameter	*A. marina* leaf extract	Vitamin C (Positive control)
DPPH scavenging assay	142.06±17.93	2.87±1.26
Nitric oxide scavenging assay	19.91±3.93	4.98±1.28
Lipid peroxide scavenging assay	640.06±34.93	31.79±1.21
Superoxide radical scavenging	12.80±0.93	24.31±0.71

Values are mentioned with ± SD values of three replicates.

Table 3.
Content of physical properties and element contents in *A. marina* leaf extract.

Heavy metals/ Trace metal parameters	Observed level (ppm/g)	Permissible limits (ppm/g) as per the WHO guidelines per gram sample
Iron	3.779	30
Copper	0.766	150
Zinc	0.835	20
Chromium	3.858	2
Manganese	1.397	30
Nickel	0.118	30
Arsenic	0.046	5
Lead	0.022	10

Table 4.
Effect of various concertinos of *A. marina* leaf extract in LD_{50} analysis of Wistar albino rats.

Concentration of the extract (mg/kg)	Behavioural changes	Mortality rate
500	–	–
1 000	–	–
1 500	Hyperactivity, rigidity, jumping and loss of traction	2(20%)
2 000	Hyperactivity, rigidity, irritability, jumping, loss of traction abnormal secretion	4(40%)
2 500	Hyperactivity, irritability, jumping, loss of traction, abnormal secretion	7(70%)

Values in the parenthesis indicates the percentage of mortality.

Table 5.
Effect of *A. marina* leaf extract on the serum parameters in treated and control rats.

Parameters	Normal control	Treatment (250 mg/kg.day. bw)
SGOT (IU/L)	167.33±13.27	178.93±15.98
SGPT (IU/L)	60.67±5.39	58.39±9.62
ALP (IU/L)	135.27±9.67	119.63±14.89
Total WBC (cells /cu. mm)	4 800.00±120.34	6 300.00±140.98
Polymorphs (%)	54.00±5.89	65.00±6.98
Lymphocytes (%)	45.00±4.05	32.00±3.62
Eosinophils (%)	1.00±0.00	3.00±0.00
Blood sugar (mg/dL)	40.00±3.45	52.00±8.76
Blood urea (mg/dL)	21.00±2.76	20.00±3.65

Values are mentioned with ±SD values of three replicates.

Table 6.
Effect of *A. marina* leaf extract on the organ parameters in treated and control 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 (250 mg/kg)	0.42±0.17	3.92±0.07	0.59±0.12	0.58±0.06	2.56±0.18

Values are mentioned with ±SD values of three replicates.

scavenging activity assay (Table 2). Physical properties such as total ash, water soluble ash, acid insoluble ash, ethanol soluble ash were identified as 87.10%, 8.95%, 12.65% and 12.95% respectively. The results of the elemental analysis showed that, all results of the heavy and trace metal analysis were within the limitations of WHO guidelines (Table 3). The LD_{50} analysis of the *A. marina* leaf extract showed lethal concentration as 2 500 mg/kg body weight. In addition no behavioural changes and mortality rate was observed with 500 and 1 000 mg/kg body weight concentrations, but, the maximum (2 500 mg/kg body weight) concentration of the leaf extract showed behavioural changes such as hyperactivity, irritability, jumping, loss of traction, 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 *A. marina* leaf extract treated animals (Table 5). In addition the results of biochemical parameters such as SGOT, SGPT, ALP, sugar and urea did not show any significant variations between control and extract treated animals. But, the results of haematological parameters such as WBC, lymphocytes, polymorphs, eosinophils were found significantly increased with extract treated animals (Table 6). The results of preliminary phytochemcial analysis revealed the presence of various phytochemical constituents such as reducing sugars, polyophenols, flavonoids and tannins with the *A. marina* leaf extract.

4. Discussion

Development of herbal drugs for the newly emergence

of unknown pathogens and continuous development of antibiotic resistant pathogens have gained top priority in recent years. Simultaneously, drug that can inhibit viral infections and cardiovascular degenerative process and help to restore normal functions in the aging nervous system are still in great demand. In recent years, extracts from mangroves and mangrove associated plants are proved to have potential biological activity against human, animals and plant pathogens but few studies were done with viral diseases[17]. In view of this, the present study was carried out to find out the effectiveness of the mangrove plant leaf extracts against the HBsAg and the results suggested that, the minimum concentration of inhibitory activity was identified with the *A. marina* leaf extract and this results can be directly used to block the surface antigen binding efficiency of viral particles to the hepatocytes[12]. Further, the leaf extract of *A. marina* also showed inhibitory activity against DNA polymerase and reverse transcriptase enzyme and this can be directly used as inhibitory agent of the viral polymerase enzyme to incorporate to the deoxy nucleotides in to the viral replicative intermediates present within viral core particles[12]. The inhibitory activity of the *A. marina* leaf extract might be due the presence of unique phytochemcial constituents such as flavonoids, phenol compounds and tannins or the combination of the phytochemcial constituents[4]. Flavonoids and phenolic compounds are proved to have potential viral inhibitory activities[18,19]. Similar reports of HBsAg inhibitory activity was also identified with the *Aporosa lindleyana* root extract[11]. Moreover, the replication of the viruses in the host system may generate the large amount of reactive oxygen species and this may damage the other organs[19]. To reduce the effect of the this free radical production the present study was also made an attempt to identify the antioxidant properties with the *A. marina* leaf extract and the results showed that the potential free radical scavenging properties and this free radical scavenging properties can be directly used to prevent the viral inception in the host cells and the progression of the disease[19]. The results of the element analysis and ash content of the *A. marina* leaf extract showed the values within the limits of WHO guidelines and this results further supports the future development of drugs from *A. marina* leaf extract. According the report of Mukinda and Syce[20] the toxicity analysis of the substances can be classified in to two groups (LD_{50} values between 500 to 5 000 mg/kg can be considered as slightly toxic and 5 000 to 15 000 mg/kg can be considered as non toxic). Based on the hypothesis, the LD_{50} value of present findings comes under the category of slightly toxic group. The results of hematological parameters such as WBC, platelets (in sub acute toxicity analysis) not showed any significant changes between the control and *A. marina* extract treated animal groups and this directly indicates the non toxicity effect of leaf extract to the blood cells circulation[20]. Further, the results of SGOT, SGPT, ALP and urea parameters did not show 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[21]. In conclusion, the leaf samples of *A. marina* collected from the Pichavaram mangrove forest has a potential inhibitory activities against the hepatitis B surface antigen, DNA polymerase inhibitory activity and reverse transcriptase inhibitory activity and phytochemcial constituents of the leaf extract can be further used as a potential antiviral agents after completing the successful clinical trials.

Conflict of interest statement

We declare that we have no conflict of interest.

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