Phytochemical profiling, characterization, and antiviral evaluation of hydro alcoholic bark extract of *Alstonia scholaris* (L.) against DENV-2

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Abstract:
No antiviral drug or very few drugs are available to treat arboviral disease, dengue. The present study focuses on the design, extraction, and antiviral evaluation of hydroalcoholic bark extract of *Alstonia scholaris linn.* Hydroalcoholic extract of bark was prepared using Soxhlet apparatus. Phytochemical analysis confirmed the presence of alkaloids, glycosides, flavonoids, and steroids. UV-Visible spectroscopy exhibited characteristic absorption peaks at 200 nm, while FTIR analysis revealed functional groups corresponding to hydroxyl, carbonyl, and alkene groups, confirming the presence of stigmasterol. Quantitative HPTLC profiling using toluene: ethylacetate: formic acid (5:4:1) demonstrated an Rf value of 0.849 with a stigmasterol content of 0.2038 mg/100 mg extract.
The cytotoxicity profile of the bark extract (BE) was determined on Vero CCL-81 cells using the MTT assay, showing a CC_{50} value 105.5 µg/mL at 120 h, indicating time-dependent cytotoxicity. Antiviral studies against Dengue virus serotype-2 (DENV-2) demonstrated moderate inhibitory activity during pre, co and post-treatment conditions. These findings suggest that hydroalcoholic bark extract possesses potential antiviral activity against DENV-2 and can serve as a promising natural source for further pharmacological exploration.

Keywords: *Alstonia sholaris* Linn. Stigmasterol, MTT assay, Antiviral (Dengue)
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Introduction

*Alstonia scholaris linn.*known as “Devil’s Tree" or "Saptaparni," have Apocynaceae family. This plant is used in traditional alternative medicine systems, Ayurvedic, Unani, Homeopathy, and Siddha/Tamil against various diseases such as asthma, malaria, fever, dysentery, diarrhea, epilepsy, skin diseases, snake bites, and other.[1]

The important role in cure treating human disease such as cancer, Immunomodulatory, diabetes and infectious and viral diseases.[2] Therapeutic phytochemical compounds and various secondary metabolites such as steroids, flavonoids, alkaloids, glycosides etc. has a different bio-potential of medicinal plants. Medicinal plant resources to maintain biodiversity and ensure continued availability for therapeutic purposes[3].The identification of bioactive compounds from medicinal plants using a various instrumentation techniques like UV-VIS, FT-IR and HPTLC study[4].

The bark of *Alstonia scholaris* contains various phytochemicals which can be analyzed using HPTLC. This method is a reliable and efficient technique used for the identification, fingerprinting, and quantification of bioactive compounds in plant extracts.

The hydroalcoholic extract of *Alstonia scholaris linn.* bark contains diverse bioactive compounds which contribute to its pharmacological properties. This extract has potential therapeutic applications and serves as an important natural source for drug development[5,6].

Stigmasterol, a plant-derived sterol (phytosterol) which shows different action such as

1. Antiviral Activity: Studies suggest that phytosterols like stigmasterol can inhibit viral replication by interfering with viral protein synthesis. It may block the entry of the dengue virus into host cells, preventing infection.
2. **Anti-inflammatory Activity**: Dengue infection leads to excessive inflammation and cytokine storm. Stigmasterol modulate the immune response, reducing inflammation and minimizing severe symptoms like haemorrhagic fever.
3. **Hepatoprotective Effects**: Dengue often affects the liver, leading to liver damage. Stigmasterol has Hepatoprotective properties, reducing liver toxicity and damage caused by viral infection.
4. **Antipyretic and Analgesic Effects**: Reduces fever and body aches, which are common symptoms of dengue fever.

These are diseases that can be protected or treated by using these secondary metabolites among other are the disease which caused by occurring of oxidation on cell or human tissue such as cancer and other degenerative diseases. To prevent of occurring of oxidation can be

used antioxidant. There are many secondary metabolites having antioxidant activity.

Dengue and their co- infections have emerged as major public health threats in tropical and sub-tropical regions. With the lack of licensed vaccines and antiviral, natural compounds present an exciting possibility to explore because of their expected low side effects, high accessibility in nature and cost effectiveness[7]. There have been several studies on the antiviral activity of various phytochemicals against DENV including various alkaloids ,flavonoids, polysaccharides, sterols , terpenoids, terpenes, etc[8]. Studies have shown that hydroalcoholic bark extract medicinal plants provide better anti-DENV activity as compared to their synthetic analogues[9]. Hence the objective of the present study was to test hydroalcoholic bark extract of *Alstonia Scholaris linn*. as an active ingredientfor the *in-vitro* antiviral activity against DENV-2[10,11]. The antiviralactivity was investigated using focus forming unit (FFU) assay.

2. Materials and methods:

Ethanol purchased from Sahyadri research Lab, Islampur. Stigmasterol purchased from Yucca Enterprises. Antiviral work was done at ICMR-NIV, Pune.

Plant Material collection and authentication:

Alstonia scholaris (L.) bark were collected from Ashta Naka, Islampur Dist. - Sangli, in January 2023.The plant was authenticated by Botanical survey of India, Pune.

Plant sample extraction: [12,13]

The bark was dried at ambient temperature in the shade for 7 days. Approximately 200 g of the dried bark were obtained. From a small portion of the bark was carefully ground with a grinder for fine powder. 100g of dried bark powder was soxhlet extracted with hydro alcoholic solvents (70% ethanol-30%water) for about 12-24h. The sample extract were collected in air tight container for further analysis. Representative images of the performed plant extract is presented in Fig. 1

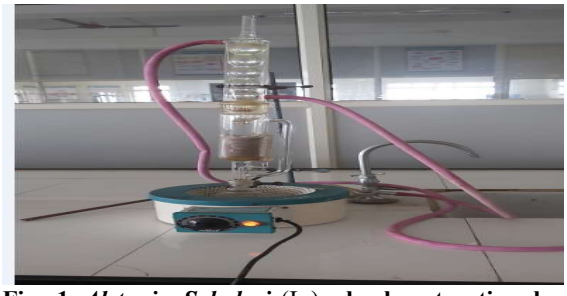


Fig. 1 *Alstonia Scholaris*(L.) bark extraction by soxhlet apparatus

UV-visible spectroscopy[14]

10 mg Bark extract was dissolved in 100 ml water. The plant extract have been scanned for the wave length ranging at 190nm to 400 nm using schematzu Spectrophotometer.

200nm wavelength was selected for extract. Absorbance was checked and took calibration curve by plotting the absorbance against the concentration of extract and conc. of extract was calculated by calibration curve. In that characteristic peaks were detected. The peak values at the UV-VIS were recorded.

FT-IR Analysis[14]

The characteristic peaks and functional groups were used to check by FT-IR Spectroscopy in ranging from 400- 4000 cm .The peak values of the FTIR were recorded and were as each and every analysis was repeated twice for the spectrum confirmation.

HPTLC Study of *Alstonia scholaris linn*. Bark Extract Containing Stigmasterol [15, 16]

High-Performance Thin-Layer Chromatography (HPTLC) is an advanced analytical technique used for the qualitative and quantitative analysis of bioactive compounds in plant extracts. It is widely used for fingerprinting medicinal plants and identifying phytoconstituents such as stigmasterol in *Alstonia scholaris* bark extract.

Materials and Methods for HPTLC of Alstonia scholaris bark extract

Chromatographic Conditions [17]

HPTLC analysis of *Alstonia scholaris* bark extract was carried out using precoated silica gel plates as the stationary phase. Square silica gel 60 F_{254} plates with dimensions of 100 mm × 100 mm were employed for the study. The thickness of the silica gel layer was uniform and suitable for densitometric scanning.

Sample application was performed using an HPTLC applicator (Aetron, Model: SPRAYLIN AE-05) operated through IDS 5.0 software**,** ensuring precise and reproducible band application. Chromatographic scanning was conducted using a TLC scanner (Aetron, Model: PROLITE DX AE-22) equipped with Just TLC software for data acquisition and processing. The average chromatographic run time for each analysis was approximately 30 minutes**.**

Detection was carried out using a UV detector**,** and nitrogen gas was employed during sample application to ensure uniform solvent evaporation and prevent band diffusion.

Preparation of Extracts and Standards

The bark extract of *Alstonia scholaris* was prepared using a suitable solvent system and filtered prior to analysis. Standard solutions of reference compounds were prepared at appropriate concentrations using analytical-grade solvents. Both sample extracts and standards were freshly prepared and stored under controlled conditions until analysis.

Plate Activation and Sample Application

Prior to sample application, the silica gel plates were pre-washed with methanol to remove impurities and activated by drying in a hot air oven at **60 °C for 30 minutes.** After cooling to room temperature, standard solutions and sample extracts were applied as discrete bands on the activated plates using the HPTLC applicator under controlled conditions.

Chromatographic development was performed in a twin-trough development chamber. To ensure reproducibility of retention factor (Rf) values, the chamber was pre-saturated with the mobile phase for **30 minutes** using saturation pads. Chamber saturation was essential, as higher Rf values were observed in unsaturated conditions.

Following saturation, the plates were developed up to a predetermined distance. After development, the plates

were removed from the chamber and dried at room temperature to eliminate residual solvents.

Densitometry Analysis

Quantification is performed by scanning the TLC plate at **366 nm**. The peak areas of the sample extracts are compared with standard compounds to determine concentration.

Brine Shrimp Lethality Assay for Toxicity Profiling and Dose Selection of bark extract[18]:

Collection of seawater: sea-water was collected for brimp shrimp activity from Goa Sea

Hatching of brine shrimps: Here the test organisms of Artemia salina leach (brine shrimp eggs) were collected from pet shops. Seawater was occupied in the small tank and shrimp eggs remained added to one side of the tank and then this side was enclosed. As nauplii it took two days to hatch the shrimp to be matured. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were involved to the lamp through the perforated dam and they were occupied for experiment. Living shrimps were used having 5 ml of seawater to each of the test tubes with the help of pipette.

Preparation of the test sample: The cytotoxic potential of the *Alstonia scholaris* linn. Bark extract was evaluated using the Brine Shrimp Lethality Assay. Brine shrimp (Artemia salina) eggs were hatched in sea water. The solution was aerated continuously for 48 hours under illumination to obtain active nauplii (larvae).

The hydroalcoholic bark extract of *Alstonia scholaris* (L.) was dissolved in distilled water and diluted with sea water to obtain different test concentrations of 100, 200, 400, 600, 800, and 1000 µg/ml. Nauplii were transferred into each test tube containing 5 ml of the respective concentrations. A control group was maintained containing only Distilled water and sea water.

All test tubes were kept under illumination at room temperature (25–28°C) for 24 hours. After the incubation period the number of survivors was counted, and the percentage mortality was calculated using the formula:

Percent Death= (Number of Dead Nauplii)/ (Total Number of Nauplii) ×100

PHARMACOLOGICAL SCREENING STUDY OF OPTIMIZED FORMULATION:

Preparation of Extract:

Extracts dissolved in DMSO (100 %)for make 10 mg/ml master stock. The stock solution
was syringe filter sterilized (o.22 µM) size with PVDF membrane made by Millipore and
further diluted with culture medium (MEM) to make different concentrations without antibiotics and 2% FBS.

Cell culture and Media:

Vero CCL-81 cells were used for Antiviral experiment which was maintained in Minimal
Essential Medium (MEM) made by HiMedia with 10% FBS (US Origin) in T- 25 cm 2 and T-
75 cm 2 flasks (TPP make).

Vero CCL-81 cells were seeded in flat bottom 96 well plate (make-TPP) for Cell Cytotoxicity assay (MTT assay) and for further antiviral studies cells were seeded in 24 well plate.

Determination MTT assay[19, 20,21]:

Vero CCL-81 cells were seeded in 96-well plates at a density of 20,000 cells per well for the 120-h assay. Blank control wells containing only culture medium and cell control wells containing untreated cells were also included. The plates were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 to allow cell attachment. After incubation, the culture medium was removed and 100 µL of the extract at the desired concentrations was added to each well. The plates were then incubated at 37 °C with 5% CO_2 for the virus incubation period (120 h). Following incubation, 10 µL of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and the plates were incubated for 3 h in the dark to allow formation of formazan crystals. The supernatant was carefully discarded, and 100 µL of DMSO or acidified solubilization solution (5% 0.1 M HCl in isopropanol) was added to dissolve the formazan crystals, followed by incubation for 1 h at 37 °C in the dark. Absorbance was measured using a microplate reader (Lux Varioscan) at 570 nm with a reference wavelength of 690 nm. The percentage of cell viability and cytotoxicity was calculated based on the absorbance values in comparison with the cell control.

Percent toxicity calculated by following Formula:

O.D. of cell control – O.D. Compounds / O.D. of cell control x 100

Percent cell Viability = 100 - Percent Toxicity

In vitro antiviral assays: [22-29]

Screening of Extract:

Vero CCL-81 cells were seeded in 24 well plates (50000 cells per well for DENV-2 antiviral Study) incubated at 37 ° C for 24 hrs with 5% CO2. The Extract concentrations were selected by MTT assay while choosing the highest non cytotoxic conc. (Cell viability of compound should be more than 90 % for the particular concentration.). The single concentration (highest non cyto toxic concentration) extract and treated with cells and Virus with different concentrations of extract as Pre-treatment, Co treatment and post treatment in triplicates.

1. Pre Treatment: Extract was treated with cells for 24 hrs with selected Concentration. After incubation the compound was removed and cells infected with DENV virus we used 0.1 MOI after infection cells were washed with PBS twice to remove unbound virus particles and MEM without antibiotics was added with 2% FBS (maintenance medium). Plates were incubated at 37ºC for 5 days/120 hrs as incubation period of DENV.

Plates were freezed at -80ºC post incubation and proceed for further assays.

2.. Post Treatment: Cells infected first with Virus and then extract and same procedure followed as pre-treatment.

3. Co Treatment: Extract and virus treated and incubated at 37ºC for 1 hr & thenadded to cells. Cells then incubated at37ºC for 1 hr. Cells then washed with PBS twice and maintenance medium added to plate.

The plates were incubated as per the incubation period of the virus.

Plates freeze –thawed and proceed for further assays. The supernatant of the above plates were diluted 10 fold and added on cells seeded plate to perform FFU assay.

(Note: Each experiment included cell control and virus control as per type of treatment in triplicates.)

FFU assay [30-36]**:**

Titration Procedure:

1. Vero CCL-81 cells seeded in 96 well plate (20000 cells per well for DENV) in MEM with antibiotics and 10% FBS and incubated at 37 ° C for 24 hrs with 5% CO2.Test samples were thawed and serially diluted in 96 well dilution plates in triplicate in chilled condition and added on cells seeded plate with respective wells.Plate then incubated at 37 ° C for 1 hr, after incubation medium removed and overlay medium (2xMEM + 2% CMC + 2% FBS in 1:1 ratio)added to plate and again incubated at 37 ° C for 120 hrs for DENVwith 5% CO 2.

FFU assay Protocol:

The focus-forming unit (FFU) assay was performed to quantify viral infectivity following treatment. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline containing Tween-20 (PBST) and fixed using a chilled acetone–methanol mixture (1:1, v/v) for 20 min in a freezer. Following fixation, the cells were blocked with blocking reagent consisting of 1% bovine serum albumin (BSA) in PBS, with 100 µL added to each well, and the plate was incubated at 37 °C for 45 min to minimize non-specific binding. The cells were then washed twice with PBST, and the primary antibody (in-house developed monoclonal anti-dengue virus antibody) was added at a volume of 50 µL per well, followed by incubation at 37 °C for 45 min. After washing twice with PBST, 50 µL of secondary antibody (anti-mouse IgG HRP conjugate; Sigma) was added to each well and the plate was incubated at 37 °C for an additional 45 min. Subsequently, the cells were washed twice with PBST and 50 µL of chromogenic substrate (True Blue substrate; KPL) was added to each well. The plate was incubated in the dark at room temperature for 10–25 min until the appearance of a blue coloration in the wells and distinct foci at the bottom. The substrate was then removed, the plate was air-dried, and the wells were analyzed using an ELISpot reader for higher resolution. The foci-forming units were counted and compared with the virus control, and the viral titre for each compound was calculated accordingly.

Result and Discussion

Phytochemical Screening Tests

The presence of these bioactive compounds was determined using standard qualitative phytochemical tests, as summarized in Table 1 and representative images of the performed tests are presented in Fig. 2**.**

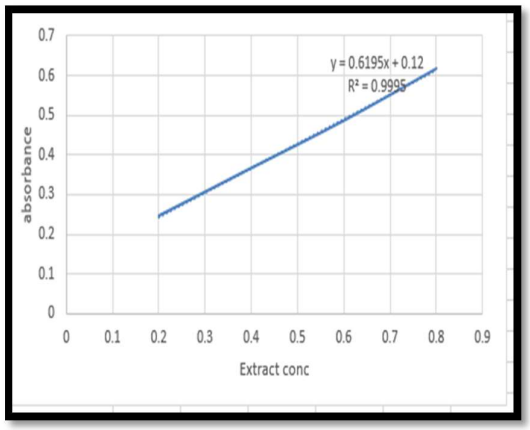
Table 1: Phytochemical test of *Alstonia Schoalsris k*

Chemical Test	**Result**
1.Alkaloid test: Dragendroff test	Present
Mayer's test	Present
Hager's test	Present
Wagner's test	Present
2.Glycosides (killer killani test)	Present
3. Flavonoid (shinoda test)	Present
4. Steroid (salkovaski test)	Present

Fig.2: Phytochemical test of bark extract of *Alstonia*

UV-VIS analysis

The UV-VIS analysis preformed for identification of phytoconstituents present in hydro alcoholic bark extract of *Alstonia scholaris Linn*. The qualitative UV-VIS profile of extract was taken at the wavelength of 190 nm to 400 nm. Serial dilute con. of bark extract were taken 0.2 µg to 10 µg per 10 ml and absorbance were shown as per below graph. The calibration curve of bark extract shown in fig. 3.



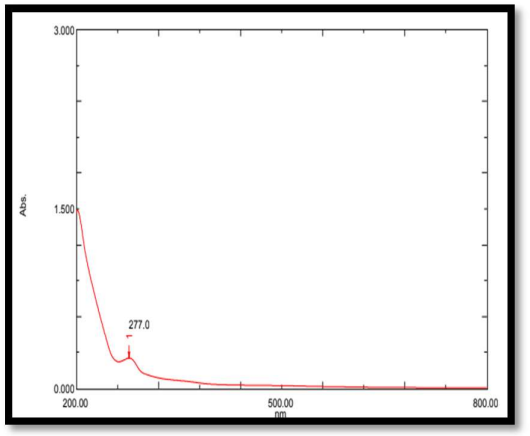


Fig. 3: Calibration curve and λ max of bark extract *Alstonia scholaris L.*

FTIR Study of bark extract of Alstonia scholaris:

FTIR study has shown various functional groups which are present in bark extract of *Alstonia Scholaris* which deplicated in fig.4.

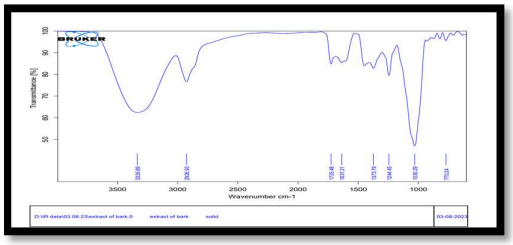


Fig. 4: FTIR study of bark extract *Alstonia Scholaris*

Interpretation of FTIR study of bark extract Alstonia Scholaris:

FTIR Peak values functional groups of Ethanolic bark extract of *Alstonia scholaris linn.* are showed in table 2

Table 2: FTIR Peak values functional groups of Ethanolic bark extract of *Alstonia scholaris linn.*

S. NO	Peak Value	Functional group	Vibrations
1.	770.24	=C–H	bending
2.	1030.29	C-O-	Stretching
3.	1244.45	C-O(ester)	Stretching
4.	1373.79	C-H(Alkyl)	Bending
5.	1637.21	C=C(alkene)	Stretching
6.	1725.48	C=O(carbonyl)	Stretching
7.	2926.93	C-H (Alkyl)	Stretching
8.	3335.69	O-H(Hydroxyl)	Stretching

HPTLC Analysis of *Alstonia scholaris* Bark Extract: [17]

The Extract of Alstonia *scholaris* were analysed by HPTLC method .

Stationary Phase: - Silica Gel 60 F_{254}

Mobile Phase: - Toluene: Ethyl acetate: Formic acid (5:4:1)

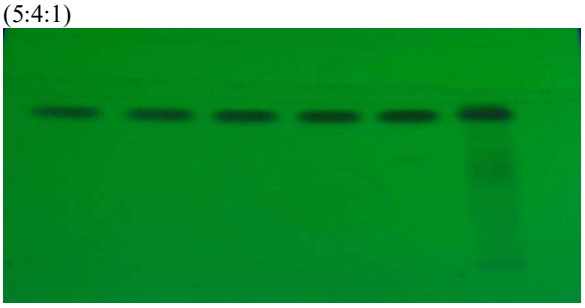


Fig.5: TLC plate of extract

Table 3: Lane Profile Graphs

ID	Width	Bands	Volume
1	309	1	107009481
2	317	1	131264945
3	303	1	140931057
4	296	1	181792248
5	280	1	207509680
6	299	4	264213443

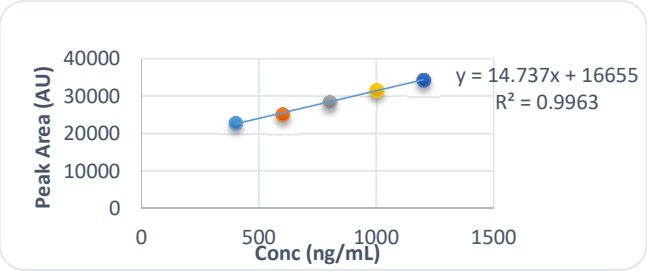
Table 4: Bands of extract compared with standard biomarker

Lane ID	Band ID	Rf	Area
1	1	0.85	22866
2	1	0.849	25043
3	1	0.85	28422
4	1	0.849	31528
5	1	0.848	34360
6	1	0.849	34684
6	2	0.596	17342
6	3	0.511	31993
6	4	0.296	22425

Table 5. Quantification bio constituent of stigmasterol in *Alstonia scholaris* bark extract

Conc (mg/mL)	Rf	Peak Area	Notes
400	0.85	22866	
600	0.849	25043	
800	0.85	28422	
1000	0.849	31528	
1200	0.848	34360	
Extract (600)	0.849	34684	0.2038 mg/ 100 mg Extract
	0.596	17342	
	0.511	31993	
	0.296	22425	

Fig. 6. Quantification bio constituent of stigmasterol



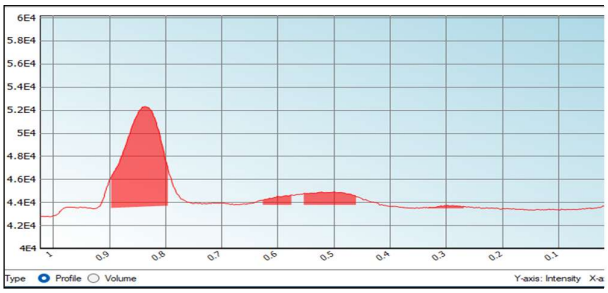


Fig.7. Lane profile of Extract

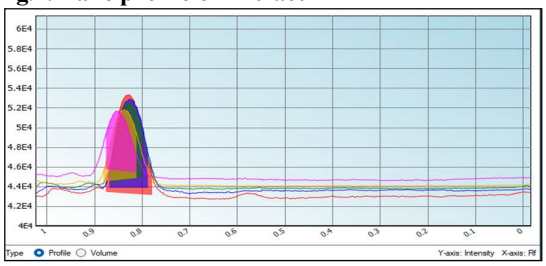


Fig. 8: Std. Overlay of extract with standard biomarker stigmasterol

The hydroalcoholic bark extract of *Alstonia scholaris* was analyzed using High-Performance Thin-Layer Chromatography (HPTLC) for phytochemical profiling and quantification of the biomarker compound. Chromatographic separation was achieved on silica gel 60 F_{254} plates using toluene: ethyl acetate: formic acid (5:4:1, v/v/v) as the mobile phase. The developed plates were visualized under UV light band volume, which represents concentration of the detected component, increases from Lane 1 to Lane 6 shown in table 3.

The TLC plate of the extract exhibited well-resolved and distinct bands, indicating the presence of multiple phytoconstituents which shown in fig.5. The lane profile graphs obtained from densitometric scanning showed sharp and symmetrical peaks with minimal baseline noise, suggesting the presence of multiple constituents in the bark extract. Rf value (~0.85) between the extract bands and the standard biomarker indicate the target compound in all tested samples present shown in table 4.The amount of stigmasterol present in the Alstonia scholaris bark extract was found to be 0.2038 mg per 100 mg of extract shown in table 5.It indicating a significant presence of this bioactive phytosterol which shown in Fig.8.

Brine Shrimp Lethality Assay for Toxicity Profiling and Dose Selection:

The 100 µg/mL, only 7.1% mortality was observed, indicating negligible toxicity at lower concentration. However, a marked increase in mortality was observed with increasing concentration of the extract as shown in fig. 9.

The results suggesting that the extract exhibits moderate to high cytotoxic potential at elevated doses shown in table 6**.** The sharp rise in mortality beyond 200 µg/mL indicates that the predicted LC50 value lies near 200µg/mL, classifying the extract as biologically active according to standard brine shrimp toxicity criteria as shown in fig. 10.

The plateau observed between 400–600 µg/mL suggests a saturation effect, while the steep increase at 800–1000 µg/mL indicates enhanced toxicity due to higher bioavailability or accumulation of active constituents.The selected dose range **(**100–200 µg/mL) is suitable for subsequent experimental investigations shown in.

Numerical Calculation of LC50 (Brine Shrimp Lethality Assay)

The LC 50 (lethal concentration causing 50% mortality) was calculated using linear interpolation, as 50% mortality lies between 100 µg/mL (7.1%) and 200 µg/mL (53%)**.**

$$LC_{50} = C_1 + \left(\frac{50 - M_1}{M_2 - M_1}\right) \times (C_2 - C_1)$$

Where:

- $C_1 = 100$ µg/mL
- $C_2 = 200$ µg/mL
- $M_1 = 7.1\%$
- $M_2 = 53\%$

$$LC_{50} = 100 + \left(\frac{50 - 7.1}{53 - 7.1}\right) \times 100$$

$$LC_{50} = 100 + \left(\frac{42.9}{45.9}\right) \times 100$$

$$LC_{50} = 100 + 93.46$$

$$\boxed{LC_{50} \approx 193.5\ \mu g/mL}$$

Fig. 9: Living shrimps were used having 5 ml of seawater to each of the test tubes and image of dead shrimps under microscope

Table 6: Percentage of mortality by Brine shrimps activity

Sr. No.	Conc. of Control and extract (µg/ml)	No. of Brine shrimps per test sample	Average number of survivors	Average number of deaths	Percentage of mortality
	Control	11	11	0	0
	100	14	13	1	7.1
	200	15	7	8	53
	400	7	3	4	57
	600	11	5	6	54
	800	9	2	7	77
	1000	12	1	11	91

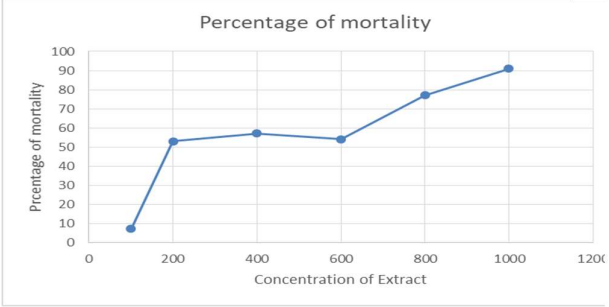


Fig .10: Determination of Percentage of mortality by Brine shrimps activity

MTT Study of Bark extract after 120 hrs.

The cytotoxicity of Bark extract was evaluated using the MTT assay at 120 hrs as shown in Table 7. The results demonstrated a dose-dependent reduction in cell viability with increasing concentrations for all tested samples as shown in fig. 11. Upon extending the exposure period to 120 h, decline in cell viability was observed, with CC_{50} values of Bark extract 105.5 µg/ml .These findings suggest a time-dependent increase in cytotoxicity.

Fig.11: MTT assay study of extract

MTT Assay for 120 days	OD values				% Cyto toxicity			% Cell viability		
Conc. µg/ml	**Bark Extract**				**Bark Extract**			**Bark Extract**		
200	0.21774	0.214545	0.24889	0.59246666667	63.300600	63.795454	57.989892	36.669400	36.204046	42.0108
100	0.29981	0.299900	0.32177	0.59246666667	49.684849	49.533330	45.701616	50.315151	50.467070	54.2984
50	0.33932	0.359858	0.43441	0.59246666667	42.747478	39.270808	26.730101	57.252522	60.729292	73.2699
25	0.39334	0.413737	0.46229	0.59246666667	33.599696	30.173333	21.869900	66.400404	69.826767	78.1310
12.5	0.44266	0.455353	0.53777	0.59246666667	25.295454	23.151818	9.2438	74.704646	76.848482	90.7562
6.25	0.47932	0.511717	0.59330	0.59246666667	19.117878	13.632323	-0.009000	80.882222	86.367677	100.09000

3.125	0.4796	0.49944	0.56005	0.59246666667	19.05083	15.70833	5.395555	80.94497	84.29177	94.6045
1.56	0.5583	0.56399	0.60064	0.59246666667	5.766969	4.821616	-2.35177	94.23311	95.17784	102.3517
0.78	0.5282	0.55576	0.65007	0.59246666667	10.84733	5.888500	-9.82900	89.15275	94.11501	109.8290
CC	0.5431	0.55770	0.67773	0.59246666667	8.333244	5.986863	-14.31887	91.66767	94.01377	114.3187

Table 7 MTT Study bark extract after 120 hrs.

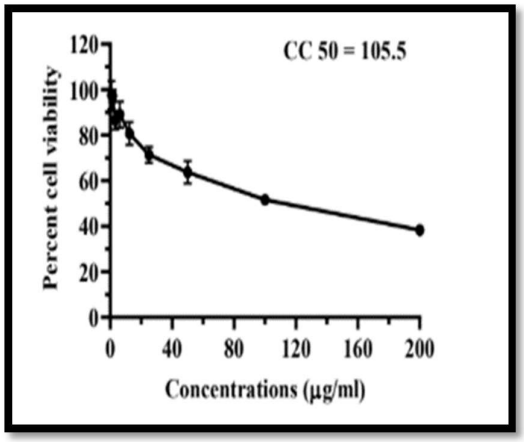


Fig. 12: MTT Study of bark extract after 120 hrs.

At 120 h of exposure, the bark extract exhibited a CC_{50} value of 105.5 **µ**g/mL, indicating moderate cytotoxicity. Compared to shorter exposure durations, a noticeable reduction in cell viability was observed upon prolonged treatment, suggesting a time-dependent cytotoxic effect as shown in fig.12. Although the bark extract remained relatively safe at lower concentrations, extended exposure led to decreased cell viability, highlighting the need for cautious dose optimization during long-term applications.

Antiviral study of Bark extract on DENV-2:

The single concentration 1.56 µM (highest non cyto toxic concentration) extract and treated with cells and Virus with different concentrations of extract as Pre-treatment, Co treatment and post treatment in triplicates shown in Table 8.

The study evaluates antiviral efficacy of control and bark extract against Dengue virus serotype-2 (DENV-2) **through** three treatment strategies as depicted in fig. 13.

Pretreatment – cells treated before viral infection (prophylactic activity)

Cotreatment – cells treated simultaneously with virus and compound (entry inhibition)

Posttreatment – cells treated after viral infection (replication inhibition)

This effective study of Bark extract on DENV-2 shown in Table 9 . The hydroalcoholic bark extract of *Alstonia scholaris Linn,* enriched with stigmasterol, exhibits promising antiviral efficacy against DENV-2

Table 8: Antiviral study of extract on DENV2

Antiviral study on Dengue	**Comp.**	**Conc.**	**Dilution**	**FFU no.**	**Titer**	**Copy no.**	**Log 10 value**	**Mean Log 10 value**	**Log Diff.**
Pre-treatment	**Bark Ext**	1.56 µM	10*-4	11, 21, 13	1.1 X 10*6	1100000	6.041392685		
					2.1 X 10*6	2100000	6.322192295		
					1.3 X 10*6	1300000	6.113943352	6.159185111	0.243987
	VC		10*-4	25, 27, 24	2.5 X 10*6	2500000	6.397940009		
					2.7 X 10*6	2700000	6.431363764		
					2.4 X 10*6	2400000	6.380211242	6.403171672	

Co-treatment	**Bark Ext**	1.56 µM	10*-4	11,5,13	1.1X10*6	1100000	6.04139 2685		
					0.5X10*6	5000000	5.69897 0004		
					1.3X10*6	1300000	6.11394 3352	5.951435347	0.2656577
	VC		10*-4	16,14,20	1.6X10*6	1600000	6.20411 9983		
					1.4X10*6	1400000	6.14612 8036		
					2.0X10*6	2000000	6.30102 9996	6.217092671	
Post-treatment	**Bark Ext**	1.56 µM	10*-4	49,54,53	4.9X10*6	4900000	6.69019 6088		
					5.4X10*6	5400000	6.73239 3766		
					5.3X10*6	5300000	6.72427 587	6.715621903	-0.011778
	VC		10*-4	75,54,42	7X10*6	7000000	55874856		
					5.4X10*6	5400000	6.73239 3766		
					4.2X10*6	4200000	6.62324 9929	6.703839302	

The y-axis represents log10 FFU/ml (focus-forming units per mL), indicating viral titer. Lower log values correspond to greater viral inhibition**.**VC shows the highest viral titer (~6 log10 FFU/mL) as shown in fig.13.

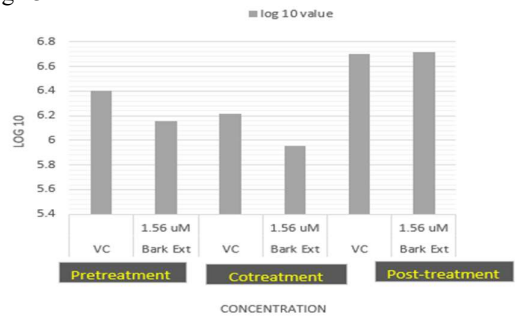


Fig. 13.Pretreatment, cotreatment and Post-treatment of bark extract on DENV-2

Table 9: Pretreatment, cotreatment and Post-treatment of bark extract on DENV-2

Virus	Treatment	Bark Extract	Remark
DENV-2	Pretreatment	**Moderate**	High significance
	Cotreatment	**Moderate**	Entry inhibition
	Posttreatment	**Moderate**	Late-stage inhibition

Discussion

Alstonia scholaris is a medicinally important plant and a natural source of steroid secondary metabolite content. By phytochemical study alkaloid, glycosides, flavonoids, tannins, terpenoids, and phenolics, sterols were present .UV-Visible spectroscopy shown the absorbance at various concentration. The IR study also shown the functional group which present in chemical constituent of bark extract of *Alstonia scholaris Linn*. In general analysis, the bark extract of *Alstonia scholaris* Linn showed the presence of steroids (stigmasterol). The IR spectrum of Alstonia scholaris bark extract confirms the presence of stigmasterol

through characteristic absorption peaks corresponding to hydroxyl, alkene, and aliphatic hydrocarbon groups. These functional groups align with the known structure of stigmasterol, supporting its presence in the extract. The HPTLC analyses for stigmasterol have revealed the existence of these constituents in sonicated bark extracts. The amount of stigmasterol was exhibited in hydroalcoholic bark extract.

By MTT assay study Bark extract have Moderate safety profile and acceptable at short exposure but needs caution for longer durations. Bark extract shows time-dependent cytotoxicity, indicating potential cumulative effects. Bark extract (1.56 µg/mL) showed moderate reduction in viral titter against DENV-2.Viral Control shows the highest viral titer (~6 $\log_{10}$ FFU/mL)

Conclusion: The present study demonstrates that the hydroalcoholic bark extract of Alstonia scholaris Linn. Possesses a significant phytochemical and antiviral potential. Phytochemical screening confirmed the presence of alkaloids, glycosides, flavonoids, steroids, and phenolic compounds. Spectroscopic characterization using UV–VIS and FTIR analyses revealed the presence of functional groups corresponding to stigmasterol, while HPTLC profiling validated stigmasterol as a principal bioactive marker within the extract.

The cytotoxicity assessment through MTT assay indicated that the bark extract exhibits moderate cytotoxicity, with CC_{50} values of 105.5 µg/mL /120 h. These results suggest that the extract is relatively safe at shorter exposure durations. Furthermore, antiviral screening against dengue virus serotype-2 (DENV-2) showed moderate inhibitory activity during pre-, co-, and post-treatment phases, suggesting interference with both viral entry and replication stages. The hydroalcoholic bark extract of *Alstonia scholaris Linn,* enriched with stigmasterol, exhibits promising antiviral efficacy against DENV-2 and could serve as a potential natural candidate for developing plant-derived antiviral therapeutics. Future studies should focus on the purification of active constituents, elucidation of the underlying mechanisms of action, and validation of antiviral efficacy through in vivo experiments.

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Declaration of competing interests

The authors declare no conflicts of interest.

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