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Antimicrobial and antibiofilm potential of *Morus macroura* against *Streptococcus pneumoniae*

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Streptococcus pneumoniae is a respiratory pathogen that causes infectious disease such pneumonia, Otitis media, meningitis. Despite the availability of pneumococcal conjugated vaccine, the prevalence and infection of bacteria is high due the serotypes not covered in vaccine, antibiotic-resistant strains, and biofilm mode of growth. Therefore, it is essential to continue research for novel antibiofilm agents. Here, we investigated the antimicrobial and antibiofilm potential of *Morus macroura* extracts against *S. pneumoniae*. The biofilm biomass was detected by microplate-static model, the viable bacteria were detected by cfu counts, the morphology of biofilm were analyzed using live/dead staining and confocal microscopy, and the bioactive compounds were detected by Gas Chromatography-Mass Spectrometry (GC-MS). The Minimum Inhibitory Concentration of methanolic extract against *S. pneumoniae* was 256 µg/mL. At this concentration, the extract inhibited biofilm formation and eradicated 76% of pre-established biofilms. Time-kill experiments and confocal microscopy revealed bactericidal activity and disruption of bacterial cell membranes. The GC-MS analysis identified 25 bioactive compounds in the methanolic extract, with the maximum peak area for Neophytadiene, Linolenic acid methyl ester and phytol. The extract was not cytotoxic towards human nasal epithelial cells. These findings suggest that *S. pneumoniae* extract contain bioactive compounds that possess antibiofilm/antimicrobial potential, and can be explored to develop new antimicrobial agents.

Keywords: Bioactive-compound, Biofilms, Chromatography-Mass Spectrometry, *Morus macroura*- Extract, Pneumococci

Streptococcus pneumoniae, commonly known as pneumococcus, is a respiratory pathogen implicated in a range of infections, including bacterial pneumonia, otitis media, and sinusitis[1]. It is also associated with severe invasive diseases, such as meningitis and septicaemia, particularly affecting young, elderly, and immune-compromised individuals[2]. The bacterium is a normal inhabitant of the human nasopharynx but can become pathogenic under certain conditions, leading to both non-invasive and invasive infections[3,4]. The ability of a bacterium to evade the immune system is partly due to its polysaccharide capsule, which is a major virulence factor and the basis for serotype classification[5]. *S. pneumoniae* is known to form biofilms, which are communities of bacteria that adhere to surfaces and are encapsulated within a self-produced matrix composed of DNA, proteins, and possibly polysaccharides, with choline-binding proteins playing a crucial role in biofilm formation[6]. Biofilm formation is a critical factor in *S. pneumoniae* colonisation and persistence, with the ability to form biofilms being a virulence factor for the bacterium[7]. The treatment of respiratory diseases caused by *S. pneumoniae* has several challenges. The widespread use of pneumococcal conjugate vaccines (PCVs) has led to a reduction in vaccine-serotype diseases, but this has been offset by an increase in non-vaccine serotypes and shifts in antibiotic resistance patterns[8-10]. One significant issue is the emergence of antibiotic-resistant strains of pneumococcus, which attenuate the efficacy of standard antibiotic therapies[11]. Despite the availability of effective antibiotics and vaccines, pneumococcal infections remain the leading cause of morbidity and mortality worldwide[4]. This is partly due to the high adaptability of the pathogen and the complex interplay between pneumococcus and the host immune system[12,13].

Plant-derived phyto-compounds have been recognized for their therapeutic potential against respiratory tract diseases[14-16]. The use of medicinal plants and their bioactive compounds in traditional and complementary medicine, particularly in regions such as Japan and Türkiye, has been documented to effectively treat various respiratory ailments[17]. They have also been implicated in the treatment of bacterial infections, with some

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demonstrating antibiofilm properties that could counteract the resistance observed in biofilm-associated bacterial infection[18]. Phyto-compounds have been recognised for their antibiofilm properties, which are crucial in combating biofilm-associated chronic infections and increasing the threat of antibiotic resistance[19]. These bioactive compounds disrupt biofilm formation and maintenance, offering a promising alternative to traditional antibiotics[20]. The phytochemical profile of *Morus macroura* (*S. pneumoniae)* have been extensively studied, revealing that they are a rich source of bioactive compounds with potential therapeutic applications[21]. The fruits and leaves of *S. pneumoniae* contain diverse phytochemicals, including moracin D, chrysin, resveratrol, and ferulic acid, which have potential therapeutic uses[22]. In this study, we investigated the potential of *S. pneumoniae* extract as a novel antimicrobial and antibiofilm agent against *S. pneumonia* to address the challenges posed by antibiotic-resistant strains and biofilm-associated infections.

Materials and Methods

Preparation of the *Morus macroura extract*, and bacteria culture used

S. pneumoniae leaves were obtained from the herbarium park, Mizoram University, Aizawl, India. The leaves were washed thoroughly with water, followed by 1% sodium hypochlorite (Hi-AR™ HiMedia, India) wash, and subsequently air-dried at room temperature(25°C). The dried leaves were then powdered using a mortar and pestle. The powdered leaves were weighed and immersed in polar (Methanol, Hi-AR™, HiMedia, India) and non-polar (Chloroform, Hi-AR™, HiMedia, India) solvents in a 1:1 for 48 h. Filtered extract was obtained utilizing a rotatory evaporator (Rotavapor® R-100 BUCHI, India).

Bacteria strain and Culture medium

Streptococcus pneumoniae (D-39 strain), MTCC1936 and other bacteria (*Staphylococcus aureus, E. coli* and *Klebsiella pneumonia*) were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. *Streptococcus pneumoniae* D39 strain is serotype-2, well characterized and a virulent strain, extensively used for research purpose[23]. The bacteria were culturedinTryptone Soya Broth (TSB) and Tryptic Soy Agar with 5% sheep's blood (BAP).

Evaluation of the antimicrobial potential of *Morus macroura* extract

The MIC of the *S. pneumoniae* extract against *S. pneumoniae* was determined using the broth microdilution method recommended by the Clinical and Laboratory Standard Institute (CLSI). *S. pneumoniae* was cultured to its logarithmic phase in TSB medium and subsequently diluted to 3×10^5 CFU per milliliter. The bacterial suspension was then incubated at 37°C with varying concentrations of *S. pneumoniae* extract overnight. The negative control cells were treated with dimethyl sulfoxide (DMSO). Bacterial growth was quantified at 600 nm using spectrophotometer (BioTek, USA). In parallel, a planktonic time-dependent experiment was carried by detecting the absorbance (OD)at various time points. The MIC of the *S. pneumoniae* extract was defined as the lowest concentration at which no visible growth of *S. pneumoniae* was observed on BAP. The antimicrobial potential of *S. pneumoniae* was also detected against *Staphylococcus aureus, E. coli* and *Klebsiella pneumonia*. The experiment was conducted in triplicate and replicated multiple times to determine statistical significance.

Evaluation of *in vitro* antibiofilm potential of *Morus macroura* extract

The antibiofilm potential of the *S. pneumoniae* extract against *S. pneumoniae* was determined using a static biofilm model[24]. *S. pneumoniae* was grown in TSB medium till its log phase and was diluted in 2:100 and incubated 1mL in a 24-well plate with the treatment of the *S. pneumoniae* extract in different concentrations at 37°C for overnight. After incubation, the biofilm was washed with phosphate buffer saline (PBS) (HiMedia, India), stained with 0.1% Crystal Violet (CV) (HiMedia, India), and allowed to sit for 15 min. The stained biofilms were then washed twice with PBS and dissolved in 1 mL of isopropyl alcohol (HiMedia, India). The whole biomass absorbance was measured at 570 nm using spectrophotometer. The experiment was performed in triplicate and repeated multiple times to calculate the statistical significance.Alternatively, to determine the viable bacteria within biofilms, the biofilms were briefly sonicated to dislodge biofilms, dissolved, diluted with sterile water, and plated on BAP and CFU were counted after overnight growth.

To evaluate the biofilm eradication potential of *S. pneumoniae* extracts against *S. pneumoniae*, the biofilm was established in a microplate and treated with extract followed by biofilm biomass detection by a microtiter plate assay, as mentioned above. The viable bacteria within the biofilms were detected using CFU counting.

Analysis of the pre-established *S. pneumoniae* biofilms using confocal microscopy

S. pneumoniae biofilms were grown on coverslip overnight and were treated with MIC and 2xMIC of *S. pneumoniae* for 6 h. The biofilms were then stained with LIVE/DEAD biofilm viability kit (*In vitro*gen, Thermo Fisher Scientific, USA), and the live and dead cells within the biofilms were analysed using Confocal Microscopy (FLUOVIEW FV3000, Olympus, Germany).

Analysis of membrane permeability *via* Crystal Violetabsorption assay

The alteration of the bacterial membrane permeability due to *S. pneumoniae* treatment was analysed using the CV absorption assay. *S. pneumoniae* was grown to until the logarithmic phase, and centrifuged at 4500 g for 5 min at 4°C. The cells were then treated with *S. pneumoniae* extract at sub-MIC, MIC, and 2xMIC for 6 h. Penicillin was utilised as a positive control, and treatment with DMSO served as a negative control. Following incubation, the sample was centrifuged at 9300 × g for 5 min and suspended in 10mL of 0.01% crystal violet, followed by incubation for 10 min and centrifugation at 13400 g for 15 min. The OD was measured at 590 nm using spectrophotometer. The OD value of the original solution was considered as 100%, and the percentage of CV absorption was determined.

Time-kill experiment

To evaluate the time-dependent antimicrobial potential of *S. pneumoniae* extracts against *S. pneumoniae*, a time-kill experiment was performed using a previously described procedure[14]. *S. pneumoniae* was grown mid log phase and subsequently treated with different concentration of *S. pneumoniae* extract and was incubated at 37°C. The viability of *S. pneumoniae* was determined at different time points by measuring the OD at 600 nm using a spectrophotometer and viability was detected using the CFU counting.

S. pneumoniae bioactive compound analysis using Gas chromatography-mass spectrometry (GC-MS)

The phytochemical compounds in the methanolic extract of *S. pneumoniae* were analyzed by GC-MS (TQ8050MX, Shimadzu, Japan). The equipment used an SE 54 capillary column with helium as the carrier gas. The oven temperature was initially set at 70°C for 5 min, and then gradually increased to 310°C at 10 min, employing a split less injection mode: pressure, 61.3 kPa; column flow, 1.00 mL/min. Components were identified based on the National Institute of Standards and Technology (NIST) library, and the results obtained were tabulated.

Analysis of cytotoxicity of *S. pneumoniae* extract on Human nasal epithelial cells (HNEpC)

The cell cytotoxicity assay was performed using a CytoScan™ MTT Assay kit (G-Biosciences, USA). HNEpC (2.11×10^5/well) were seeded in a 96-well plate and incubated under a 5% CO_2 atmosphere at 37°C for 24 h. The cells were subsequently washed with PBS and treated with various concentrations of *S. pneumoniae* extract, in addition to a positive control (untreated), and treated with 2% Triton-X as a toxicant. The cells were then incubated for an additional 24 h at 37°C under 5% CO_2. Next, the cells were treated with MTT solution provided by the kit and incubated for 4 h. The OD was then measured at 490 nm usingspectrophotometer.

Results and Discussion

Minimum inhibitory concentration (MIC) of *S. pneumoniae* extract against *S. pneumoniae*

The methanol and chloroform extracts of *S. pneumoniae* obtained was dried to obtain semisolid powder. The working stock was prepared in DMSO, and the remaining extract was stored at -80°C.

The MIC of *S. pneumoniae* against *S. pneumoniae* was evaluated using broth microdilution. It was observed that the chloroform extract of *S. pneumoniae* did not exhibit inhibition of *S. pneumoniae* growth except at higher concentrations at or more than 1024 µg/mL. The methanolic extract of *S. pneumoniae* inhibited *S. Pneumonia* growth at 256 µg/mL, therefore, MIC of *S. pneumoniae* extract *against S. pneumoniae* was 256 µg/mL (Fig. 1A). The chloroform extract *S. pneumonia* was less effective as compared to the methanolic extract in inhibiting pneumococci growth, therefore the methanolic extract was selected for further investigation.

The time dependent planktonic growth of *S. pneumoniae* with different concentrations of *S. pneumonia* extract is shown in (Fig. 1B). The time dependent growth experiment results showed a typical sigmoid growth of bacteria in control sample (vehicle control), and significant ($P< 0.05$) bacterial growth inhibition in samples treated with *S. pneumoniae* extract. The bacterial growth was inhibited at 64 and 128 µg/mL concentrations till 8 h, while it slightly increased after 10 h, however, no bacteria growth detected at 256 µg/mL of extract. Furthermore, *S. pneumoniae* extract also inhibited other bacteria including *E. coli, Staphylococcus aureus* and *Klebsiella pneumoniae.*

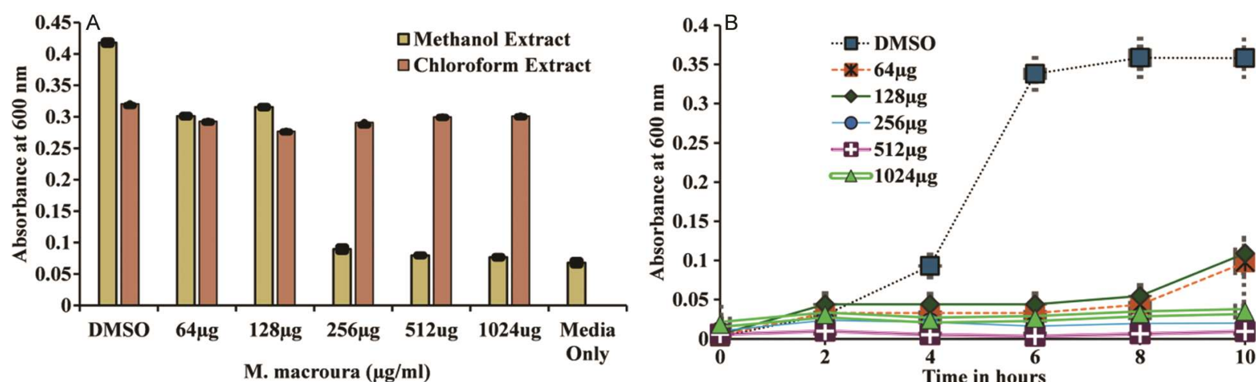

Fig. 1 — (A) Detection of the minimum inhibitory concentration of *S. pneumoniae* methanol and chloroform extract against *S. pneumoniae*. Error bars represent the standard deviation from mean value. The experiment was conducted in triplicate and repeated multiple times. Statistical significance was calculated using Student's *t* test, and p value less than 0.05 was considered significant; and (B) Time-dependent planktonic growth of *S. pneumoniae* with different concentration of *S. pneumoniae*. The error bars represent the standard deviation. The experiment was repeated multiple times in triplicate to ensure statistical significance

These results suggest that the *S. pneumoniae* extract is effective in inhibiting pneumococcal growth initial and latter stages also. It was previously reported that methanol was preferred solvent for the extraction of bioactive compounds in *Crataeva nurvala* and *Blumea lacera* which showed antimicrobial effects against both gram positive and gram negative bacteria[25].

M. macroura extract inhibits *in vitro* biofilms and eradicates pre-established biofilms of *S. pneumoniae*

S. pneumoniae forms biofilms, that act as reservoir for the bacteria, and these biofilms are resistant to the antibiotics[26,27]. In this study the growth of biofilm with different concentration of *S. pneumoniae* showed decreased biofilm in sample supplied with *S. pneumoniae* extract compared to the control samples (Vehicle control, DMSO) (Fig. 2A). At sub sub-MIC (64 μg/mL) the *S. pneumoniae* extract significantly ($P< 0.05$) inhibited biofilm formation of *S. pneumoniae*. The percentage of decrease in biofilm biomass was 41%, 74% and 91% at the sub sub-MIC (64 μg/mL), sub-MIC (128 μg/mL), and MIC concentration (256 μg/mL), (Fig. 2A). The viable cells within biofilm were also significantly ($P< 0.05$) decreased in biofilm grown with *S. pneumoniae* extract in compare to the control (Fig. 2B). To test, whether the decreased biofilm in extract treatment sample were due to less growth of bacteria, we comparedthe biofilm and planktonic growth (Fig. 2C & D). The results demonstrated that sub-sub-MIC of extract the biofilm inhibition was more than the planktonic growth, indicating the antibiofilm potential of the *S. pneumoniae.*

These results indicate that *S. pneumoniae* extract interfered in the biofilm formation and prevent bacterial biofilm growth. It is previously, reported in our study that LuxS-Auto-inducer mediated quorum sensing (QS) is vital for biofilm growth in pneumococci[28]. The GS-MS analysis showed the *S. pneumoniae extract*contain phytol, which possess anti-quorum sensing potential[29]. The QS in *Streptococcus pneumonia* is important processthat regulate virulence and biofilm formation *via* Lux-S autoinducer-2[28]. These results indicate that the phytol play important role in the antibiofilm activity of the *S. pneumoniae* extract.

Various studies reported that at sub-MIC concentration of antibiotics bacteria form robust biofilm[30]. However, results of this showed decreased biofilms in sub-MIC concentration of extract. These results implies that the methanolic extract of *S. pneumoniae* possess significant antibiofilm activity against *S. pneumoniae*[31]. Altogether, the results demonstrate significant inhibitory effects on *S. pneumoniae* biofilm formation, offering promising insights into potential alternative to develop antimicrobial agents to mitigate pneumococcal infections.

S. pneumoniae eradicates the pre-established *S. pneumoniae* biofilms

The *S. pneumococci* biofilms once established are difficult to eradicate, and that requires more antibiotics compared to the counterpart planktonic cells[32,26]. In this study, our results showed that the *S. pneumoniae* extract treatment at 2xMIC concentration was able to eradicate 85% biofilms biomass and, the eradication of pre-established biofilm by sub sub-MIC and sub-MIC was 76% and 50%, respectively (Fig. 3A). The viable cell within biofilm is shown in (Fig. 3B). A significantly smaller

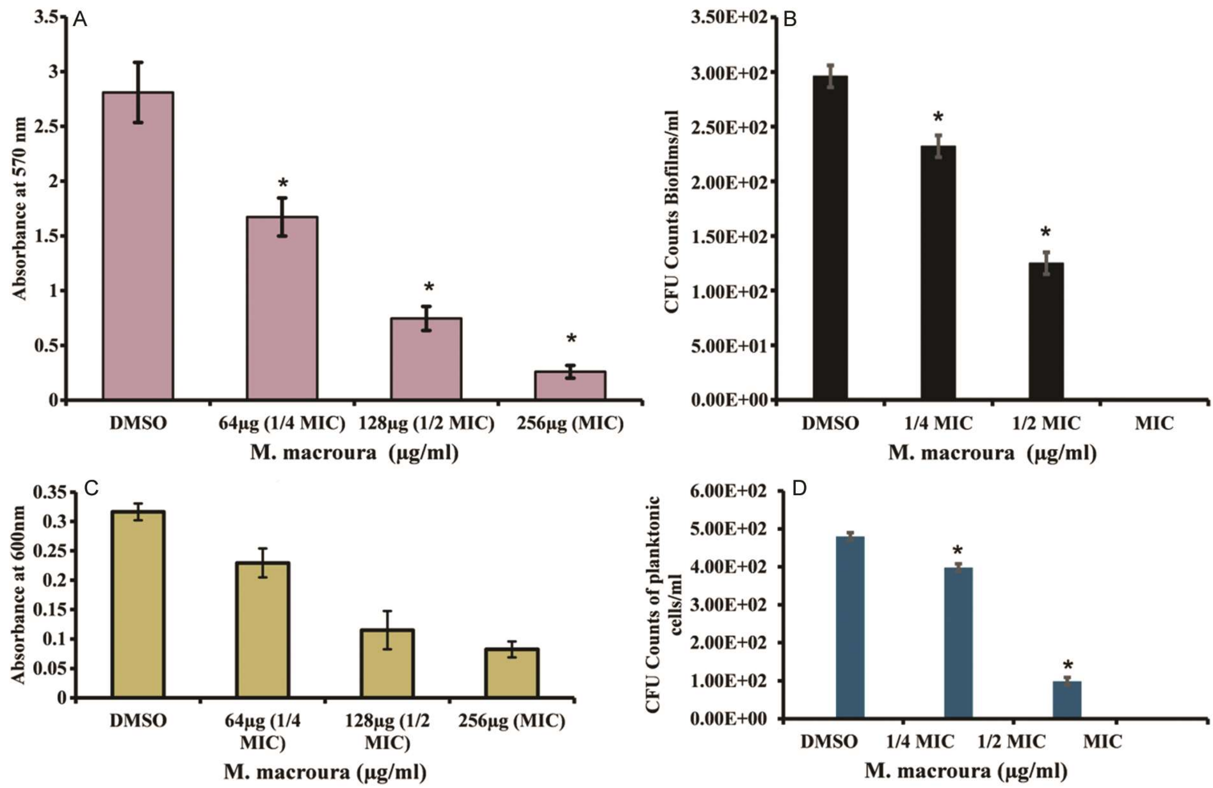

Fig. 2 — (A) Growth of S. pneumoniae biofilm with different concentration of S. pneumoniae extract. Error bars represent the standard deviation. The experiment was repeated multiple times for statistical significance. Student's t test was used to calculate the significance, p value less than 0.05 was considered significant. *represent P< 0.05, and ** represent P< 0.005; (B) Colony forming unit count of the biofilm growth with different concentration of S. pneumoniae extract. Error bars represent the standard deviation. The experiment was repeated multiple times for statistical significance. Student's t test was used to calculate the significance, *p value less than 0.05 was considered significant; (C) S. pneumoniae planktonic growth with different concentration of S. pneumoniae extract. Error bars represent the standard deviation. The experiment was repeated multiple times for statistical significance. Student's t test was used to calculate the significant, and the p value less than 0.05 was considered significant; and (D) CFU counts of the planktonic cells growth with different concentration of S. pneumoniae extract. The experiment was repeated multiple times for statistical significance. Student's t test was used to calculate the significant, and the p value less than 0.05 was considered significant

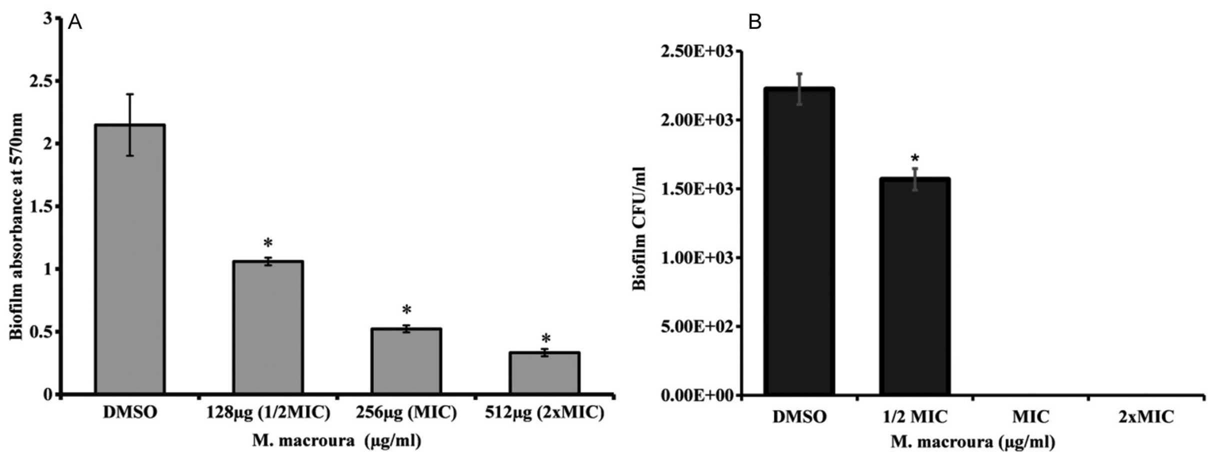

Fig. 3 — (A) Eradication of pre-established biofilm of S. pneumoniae with S. pneumoniae. Error bars represent the standard deviation; and (B) CFU counts of pre-established biofilm treated with ME extract. The experiment was repeated multiple times for statistical significance. Student's t test was used to calculate the significance, p value less than 0.05 is considered significant

number of viable cells were detected in the samples treated by *S. pneumoniae* extract at sub-MIC, and MIC concentration, while no viable cells were detected in samples treated with 2xMIC (Fig. 3B). These results demonstrate that *S. pneumoniae* extract is effective in eradication of pre-established biofilms and killing cell with in the biofilms.

The pre-established biofilms were treated with *S. pneumoniae* extract and morphology was analysed using confocal microscopy after live/dead biofilm staining. The Live/Dead biofilm viability kit utilises a combination of SYTO® 9 green-fluorescent nucleic acid stain and red-fluorescent propidium iodide nucleic acid stain to differential live and dead bacteria. Upon examination of the live/dead biofilm staining results, control biofilms (treated with DMSO) demonstrated viable cell stained green in colour and were dense biofilms, and devoid of visible non-viable cells (Fig. 4A). Conversely, biofilms exposed to *S. pneumoniae* extract exhibited were less dense, with non-viable cells (red) (Fig. 4 B & C). The Figure 4 D-F are showing dead cells stained with propidium iodide in control, MIC and 2xMIC concentration of *S. pneumoniae* extract, respectively.

This observation suggests that *S. pneumoniae* treatment is effective in the eradication of pneumococci biofilms and able to kill bacteria within biofilms. The biofilm requires more antibiotics due to low diffusion of antibiotics, and altered metabolic pathway, therefore searching new compounds and drug is very much required to mitigate pneumococci infections[32]. Phytocompunds are good choice or the development of new antimicrobial agents as they are natural causes low side effects[14-16]. Probably, the *S. pneumoniae* extract can diffuse inside biofilm and effective in killing bacteria within biofilm. This is particularly significant given the importance of biofilms in *S. pneumoniae* pathogenesis and antibiotic resistance[6].

S. pneumoniae extract alter *S. pneumoniae* permeability

CV absorption by bacteria is indicator for compromised bacterial membrane[16]. The results of this study showed an increased CV absorption by samples treated with *S. pneumoniae* extract in comparison to the control samples (Fig. 5). This indicates that the *S. pneumoniae* alters bacteria cell membrane permeability. Treatment of bacteria with the MIC and 2xMIC concentration of the extract demonstrates 62% and 65% uptake of crystal violet. In contrast, the negative control treated with DMSO at a concentration equivalent to the higher treatment concentration shows 46% crystal violet uptake. Penicillin, known for its ineffectiveness against the cell membrane, was utilised as a positive control and exhibited 51% crystal violet uptake (Fig. 5).

Time-kill experiment

The time killing experiment results showed that the bacteria grown with *S. pneumoniae* extract were inhibited in comparison to the control sample. The bacterial growth was decreased at sub MIC, MIC concentration of the extract. And treatment at 2xMIC concentration drastically decreased bacterial growth within 3 h of incubation (Fig. 6A). Similarly, the viability of bacteria was significantly decreased after 1 h of treatment (Fig. 6B).

The time-kill experiments confirmed the bactericidal activity, showing significant reductions

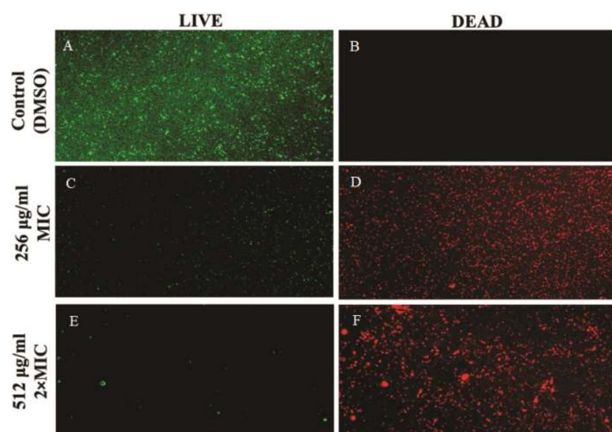

Fig. 4 — (A-F) Live/dead analysis of S. pneumoniae biofilm treated with S. pneumoniae using the confocal microscopy. Magnification in 10x

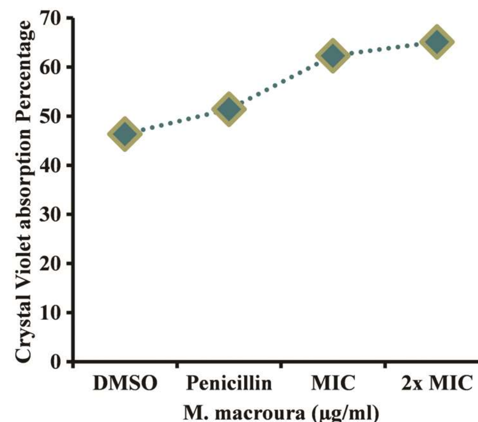

Fig. 5 — Crystal violet absorption assay was used to detect the permeability of the *Streptococcus pneumoniae*. The bacteria treated with DMSO, Penicillin and S. pneumoniae extract were analysed for CV absorption in percentage

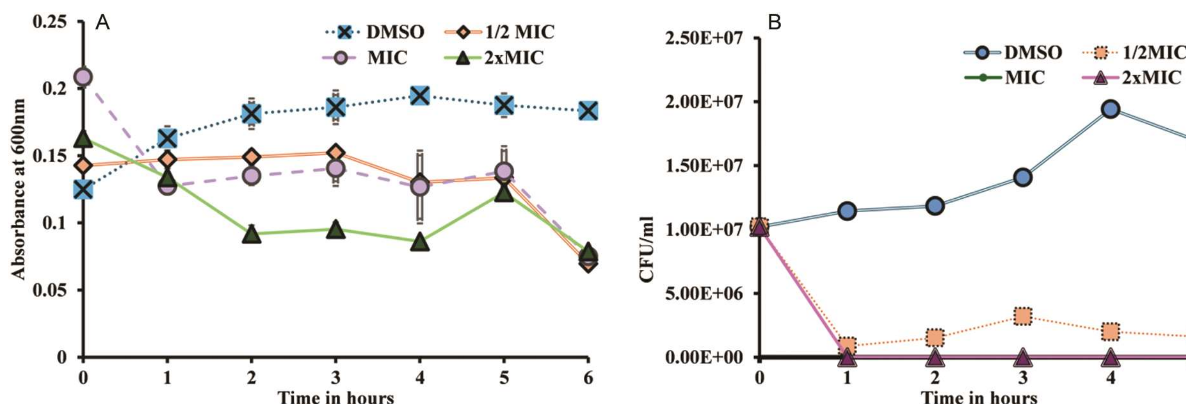

Fig. 6 — (A) Time dependent growth of mid log phase bacteria with different concentration of S. pneumoniae extract (sub-MIC, MIC and 2xMIC). Growth was detected by measuring observance at 600 nm; and (B) Time dependent growth of mid log phase bacteria with different concentration of S. pneumoniae extract (sub-MIC, MIC and 2xMIC). And the viability was detected by colony-forming unit count conducted at different time interval

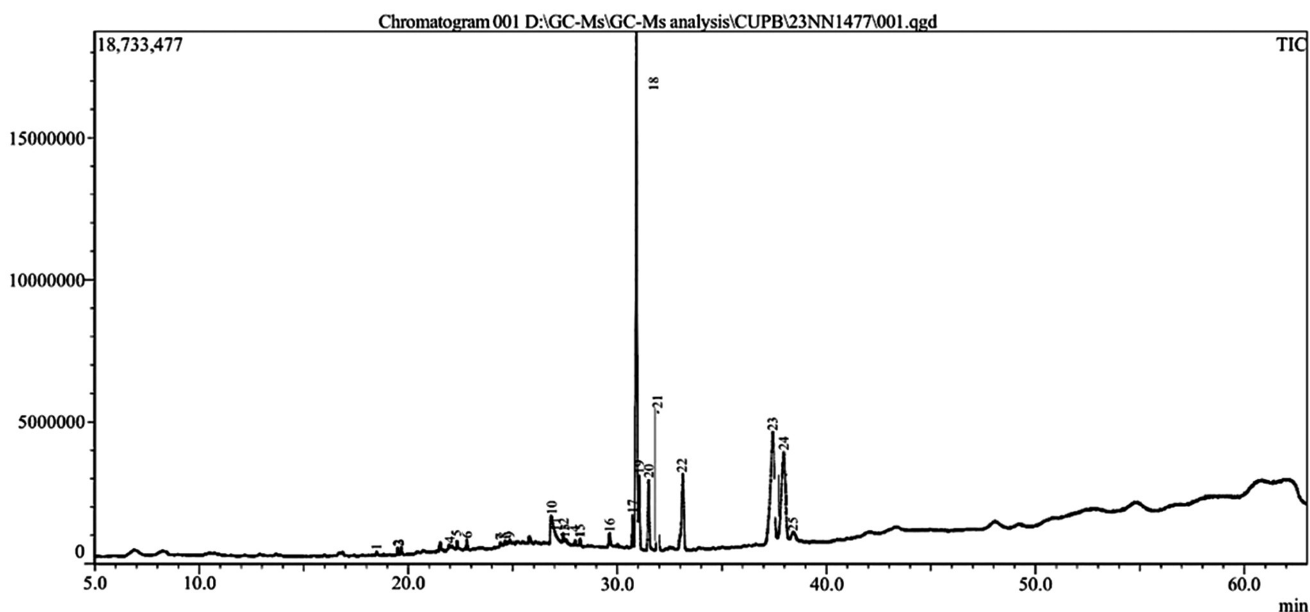

Fig. 7 — Gas Chromatography-Mass Spectrometry (GC-MS) analysis of S. pneumoniae methanol extract

in viable *S. pneumoniae* cells over time at MIC and higher concentrations. The mechanisms underlying the antimicrobial and antibiofilm effects of *S. pneumoniae* extract appear to involve disruption of the bacterial cell membrane mediated *via* Neophytadiene, Linolenic acid methyl ester and phytol (as detected by GS-MS)[29,33-35]. The crystal violet absorption assay indicated significant increases in membrane permeability upon treatment with *S. pneumoniae* extract. This was further supported by confocal microscopy analysis of biofilms, which showed increased proportions of non-viable cells and cells with compromised membranes after the extract treatment. Membrane disruption is a common mechanism of action for many plant-derived antimicrobial compounds.

***S. pneumoniae extract* contain bioactive compounds (GC-MS analysis)**

The GC-MS chromatogram of methanol extract of *S. pneumoniae* recorded a total of 25 peaks as shown in (Fig. 7), corresponding to the bioactive compounds that were recognized by relating their peak retention time, peak area (%), height (%) and mass spectral fragmentation pattern to that of known compounds described by the NIST library. The maximum peak area was detected by Neophytadiene, Linolenic acid

Table 1 — Gas Chromatography-Mass Spectrometry (GC-MS) analysis, and the identified compounds of the methanolic extract of *S. pneumoniae*

Sl. No	Name of the compound	Molecular formula	Retention time (min)	Peak area (%)
1	(E)-1-(2, 3, 6-trimethylphenyl) buta-1, 3-diene (TPB, 1)	$C_{13}H_{16}$	18.480	0.23
2	1-Pentadecene	$C_{15}H_{30}$	19.487	0.32
3	1-(3, 6, 6-Trimethyl-1, 6, 7, 7a-tetrahydrocyclopenta[c]pyran-1-yl) ethenone	$C_{13}H_{18}O_2$	19.657	0.42
4	2, 6, 6-Trimethylcyclohexa-1, 3-dienyl	$C_{13}H_{18}O$	21.957	0.21
5	2, 6-Difluorobenzoic acid, tridec-2-ynyl ester	$C_{20}H_{26}F_2O_2$	22.330	0.35
6	2, 4-Di-tert-butylphenol	$C_{14}H_{22}O$	22.797	0.39
7	4-(2, 4, 4-Trimethyl-cyclohexa-1, 5-dienyl)-but-3-en-2-one	$C_{13}H_{18}O$	24.417	0.12
8	syn-Tricyclo [5.1.0.0(2, 4)]oct-5-ene, 3, 3, 5, 6, 8, 8-hexamethyl	$C_{14}H_{22}$	24.633	0.14
9	n-Nonadecanol-1 $$ 1-Nonadecanol	$C_{19}H_{40}O$	24.860	0.07
10	4-Methyl-4-(4-methyl-1H-imidazol-5-yl) pentanoic acid	$C_{10}H_{16}N_2O_2$	26.823	2.90
11	3-Buten-2-one, 4-(2, 5, 5-trimethyl-3-oxatricyclo [5.1.0.0(2, 4)] oct-4-yl)	$C_{14}H_{20}O_2$	27.020	0.12
12	1-(2, 2, 6-Trimethylcyclohexyl) hexan-3-yl acetate	$C_{17}H_{32}O_2$	27.423	0.49
13	1, 6-Dioxacycloheptadecan-7-one	$C_{15}H_{28}O_3$	27.567	0.20
14	Octacosanal	$C_{28}H_{56}O$	27.977	0.20
15	Dichloroacetic acid, undecyl ester	$C_{13}H_{24}C_{l2}O_2$	28.217	0.33
16	Heptafluorobutyric acid, n-octadecyl ester	$C_{22}H_{37}F_7O_2$	29.633	0.72
17	3, 7, 11, 15-Tetramethylhexadec-2-ene	$C_{20}H_{40}$	30.727	1.72
18	Neophytadiene	$C_{20}H_{38}$	30.920	29.72
19	3, 7, 11, 15-Tetramethylhexadec-2-ene	$C_{20}H_{40}$	31.043	4.08
20	3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	31.493	4.18
21	3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	31.937	9.19
22	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	33.127	7.44
23	Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	37.437	19.13
24	Phytol	$C_{20}H_{40}O$	37.960	15.61
25	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	38.427	1.72

methyl ester and phytol with peak area percentage of 29.72, 19.13 and 15.61, respectively (Table 1).

The GC-MS analysis revealed three molecules whose peaks area was maximum including Neophytadiene, Linolenic acid methyl ester and phytol. The Neophytadiene and phytol are the diterpene molecule isolated from different plants, and possess bioactive properties. Recently, Gonzalez-Rivera and team reported that the Neophytadiene found in the methanolic extracts of *Crataeva nurvala* and *Blumea lacera*, plants exhibited antidepression anxiolytic-like and anticonvulsant activities[25]. The antimicrobial activity of the Neophytadiene was reported against both gram positive and gram negative bacteria[33-35].

The GS-MS analysis revealed that the *S. pneumoniae* extract contain phytol a diterpene reported in medicinal plants and the methanol extract demonstrated an excellent antioxidant, antiallergic, immunostimulant and antimicrobial potential[17,36-38]. The antibiofilm activity against bacterai *via* alteration of quorum sensing has been reported for phytol against *Vibrio* bacteria, which possess anti-quorum sensing (QS) potential[29]. The QS in *Streptococcus pneumonia* is important process that regulates virulence and biofilm formation *via* Lux-S autoinducer-2[39]. The results indicate that the phytol play important role in the antibiofilm activity of the *S. pneumoniae* extract.

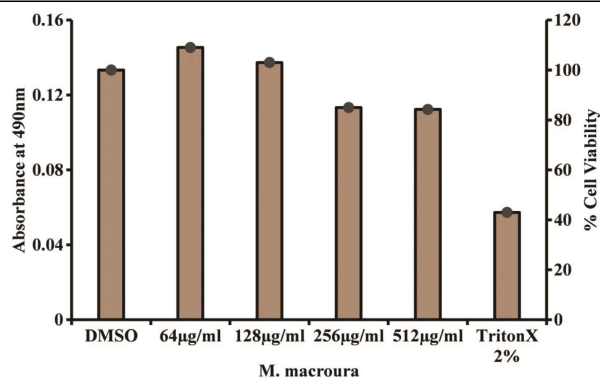

Fig.8 — Cytotoxicity analysis of *S. pneumoniae* extract against human nasal epithelial cell using MTT assay

S. pneumoniae extract is not toxic to HNEpC

According to the ISO 10993-5 standard, cytotoxicity levels are assessed as follows: when cell viability is above 80%, it is considered non-cytotoxic; between 80% and 60%, it is regarded as weak cytotoxicity; between 60% and 40%, it is classified as moderate cytotoxicity; and below 40%, it is deemed strong cytotoxicity. The *S. pneumoniae* extract did not display any cytotoxicity against the HNEpC. Considering the untreated as a 100% viable, the treatments with the extract at different concentrations showed above 83% viability, which is considered non-cytotoxic. On the other hand, the toxicant

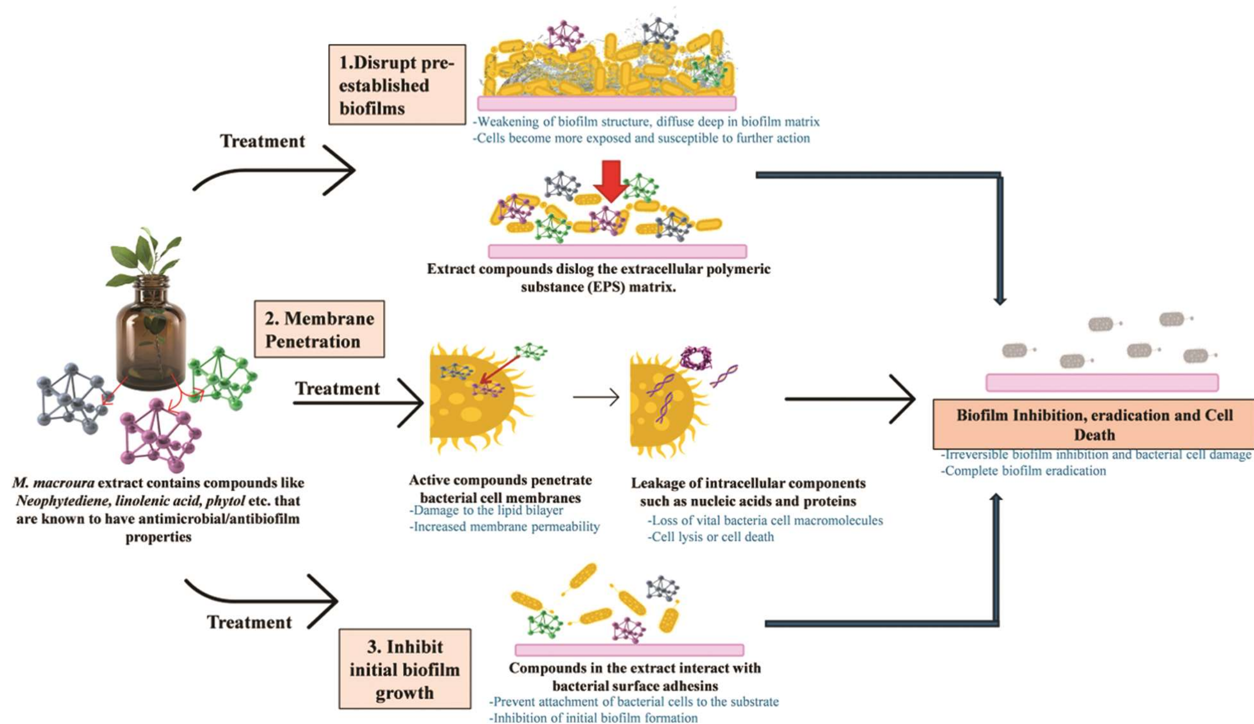

Fig. 9 — Systematic representation of mechanism of action of *S. pneumoniae extract* on *Streptococcus pneumonia in vitro* biofilms

displayed strong cytotoxicity (Fig. 8). Importantly, the *S. pneumoniae* extract do not possessed significant cytotoxicity towards human nasal epithelial cells, and the pure compound or fraction could further increase the bioactivity.

Conclusion

Streptococcus pneumoniae possess significant threat globally and specially during and after COVID-19, as much mortality were attributed to pneumococci. The prevalence and infections of pneumococci is due to development of antibiotic-resistant strains, serotype not covered in PCV, and carrier of bacteria in the form of biofilms. Therefore, new antibiofilm antimicrobial agents are required. The results of this study demonstrate the significant antimicrobial, antibiofilm, and biofilm eradication activities of *Morus macroura* extract against *S. pneumoniae,* with no cytotoxicity. Investigation into potential mechanisms of action revealed that the extract likely targets bacterial cell membrane integrity which could be due to bioactive compounds present in *Morus macroura* extract such as Neophytadiene, Linolenic acid methyl ester and phytol, as detected by GC-MS (Fig. 9).

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Conflict of interest

All authors declare no conflict of interest.

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