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Investigating the Antiphage Activity of the Methanolic Extract of *Aegle marmelos* against Lactic Acid Bacteriophages

Research Article

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Abstract

Bacteriophages are common nuisance viruses that cause tremendous loss to the dairy industry by lysing lactic acid bacteria. Thus, an intervention using inclusion of ayurvedic herbs to prevent such lysis of potential lactic acid bacterial strains was set up. In this study, a thorough phytochemical analysis of methanolic extract of *Aegle marmelos* leaf powder undertaken showed the presence of major groups of secondary metabolitesin the extract while LCMS and GCMS identified various bioactive compounds in it. Antiphage assay using crude methanolic extractdemonstrated a significant reduction in P001 phage with a log reduction of 0.0846 ± 0.083 after 20 min of exposure and 0.708 ± 0.081 after 90 min of exposure for the extract without inhibiting the probiotic host culture. These findings offer valuable insights into the extract's potential for protecting the viability of the probiotic host culturein dairy applications.

Keywords: Antiphage Activity; Bael; *Lactococcus lactis*; Dairy Bacteriophages

Introduction

Aegle marmelos is a plant native to India belonging to the family *Rutaceae*. It is an important medicinal plant used in traditional Indian Ayurvedic medicine. It has various names in colloquial language such as Bengal quince, Indian quince, Holy fruit, Golden apple in English, "Bilva" in Sanskrit while in Hindi it is known as "Bael". According to Charaka (1500 BC), Bael is considered as an emblem of fertility and is a holy tree capable of healing and strengthening the body. Diverse ethnomedical properties of Bael have been reported in the pharmacological studies which include antimicrobial, antifungal, anti-inflammatory, antipyretic, hypoglycemic, antidyslipidemic, immunomodulatory, antiproliferative, wound-healing, insecticidal, anticancer, antidiabetic, and cardioprotective properties[1].This plant contains a diverse groups of bioactive compounds like alkaloids, saponins, tannins, cardiac glycosides, flavonoids, and phytosterols which provide medicinal properties to the plant [2]. The major phytochemicals reported in Bael include Skimmianine, Aegeline, Lupeol, Cineol, Citral, Citronella, Cuminaldehyde, Eugenol, Marmesinine, Marmelosin, Luvangetin, Aurapten, Psoralen, and Marmelide[3]. All the parts of the *Aegle marmelos* plant including fruits, stem, bark, and leaves exhibit medicinal properties but the most health benefits associated with Bael are attributed to the numerous phytocompounds present in its leaves [4].

Fermented foods have received attention worldwide for their varied health benefits. Lactic acid bacteria are widely used in food industry, particularly in dairy industry to manufacture a range of fermented foods [5]. However, their use in dairy industry is encountered with several challenges, most importantly being attacked by bacteriophages which lyse them causing fermentation disturbances [6]. Lytic phages are known to infect probiotic starter cultures in commercial manufacturing facilities leading to loss of productivity. Moreover, they are resistant to pasteurization

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temperatures and traditional phage control measures [7]. Thus, by conducting in-depth studies on these phages, targeted strategies to prevent their infections in commercial manufacturing plants can be devised. This study thus plans to evaluate the potential of methanolic extract of *Aegle marmelos* against *Lactococcus* P001 phage along with its thorough phytochemical profiling.

Materials and Methods

Procurement of Plant material

Complete plant of *Aegle marmelos* was procured from the Botanical Garden & Nursery within Bhavan's College Campus, Mumbai and confirmed of its authenticity by Botanists at Department of Botany, Bhavan's College. The leaves of the plant were dried and ground into fine powder using a grinder and stored in airtight containers until further use.

Preparation of plant extract and Phytochemical analysis

5 grams of the powdered material was dissolved in 50 ml methanol [Loba Chemie] and kept on a rotary shaker for 3 days. Every day, the shaker was switched on for 8 hours thus ensuring 24 hours of effective shaker treatment. Each extract was then filtered by whatmann paper No 1 [Himedia] to separate the powder. Preliminary qualitative testing for alkaloids, phenolic compounds, tannins, flavonoids, saponins along with quantitative testing for phenolic compounds, alkaloids and flavonoids,was conducted using standard protocols [8-11].

HPTLC fingerprinting of the extract

- HPTLC fingerprinting of the extract was carried out using mobile phase consisting of ethyl acetate: water: formic acid: acetic acid (100:26:11:11- v/v/v/v). The stationary phase consisted of TLC silica gel 60 F_{254} (Merck -100 X 100 mm). Specified volumes of sample was applied on to the TLC plates using Linomat 5 (CAMAG), semiautomatic sample dispenser. The TLC plates were scanned by CAMAG Scanner 4 which had a 6 X 0.45 mm micro-slit dimension and a scan speed of 20mm/sec and data resolution of 100µm/step. The developed chromatogram was visualised at 254 nm, 366 nm, and white light after derivatization with anisaldehyde sulphuric acid reagent and Natural Product A reagent and photodocumented.

Liquid Chromatography Mass Spectrometry (LC-MS) Analysis

- LCMS analysis of the extract was carried out using Q-TOF mass spectrophotometer with Dual AJS ESI (G6550A, Agilent Technologies, USA). The mobile phase consisted of 0.1 % formic acid (A) and methanol (B). The analysis followed a linear gradient program where in initial conditions were solvent A 95%:B 5%; 0-25 min, changed to solvent A 0%:B 100%; 25-30 min and back to solvent A 95%:B 5%; 31-35 min. The flow rate was maintained at 0.3 ml/min and injection volume was 5 µL and the column used was Infinity HPLC G1316C (Agilent, USA). The data generated was processed, analysed, and interpreted using Mass Hunter software (Agilent, USA).

Gas chromatography Mass Spectrometry (GC-MS) Analysis

GCMS analysis of the extract was carried out on 7890B GC system (Agilent, USA) connected to Jeol AccuTOF GCV with FID detector and head space injector. (Agilent, USA). The stationary phase column was HP-5 (30 mX0.32 mm, 0.25 µm) and helium was used as a carrier gas at a constant flow rate of 1 mL/min. The oven temperature was initially set at 60°C for 1 min and then was increased by 6°C per min and maintained at 200°C for 2 min. Final temperature was maintained at 280°C. The AccuTOF MS (Jeol) detector was used for eluting the molecules enabling their detection. The total run time was 28 min. The compounds eluted from the extract were identified and characterized by employing standard spectral libraries like NIST.

Preparation of plant extract for antiphage activity

50 g of the powdered plant was dissolved in 300 mL methanol [Loba Chemie, India] and kept on a rotary shaker for 3 days. Every day, the shaker was switched on for 8 hr thus ensuring 24 hr of effective shaker treatment. The extract was then filtered by whatmann paper No 1 [Himedia, India] to separate the powder and the methanol was allowed to evaporate. The residue was weighed and dissolved in DMSO [Loba Chemie, India] and DMSO dissolved extract was used for further experimentation.

Procurement and maintenance of LAB host and its bacteriophage

Lactococcus lactis [DSM-4366], a probiotic host culture used in the cheese fermentation and its virulent bacteriophage*Lactococcus phage P001* [DSM-4262] were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures, Germany. The cultures were grown and maintained on M17 medium [Himedia, India] at 30°C under aerobic conditions.

Determination of MIC against *Lactococcus lactis* host

MIC of the extract against the *Lactococcus lactis* probiotic host culture was performed by standard tube dilution technique to quantify the absence of extract activity on the host strain. DMSO dissolved extract was double diluted in M17 broth [Himedia, India] in two sets. The first set was labelled as 'test' and the second set was labelled as 'control'. Post dilution, 50 µL of the actively growing host was added to the 'test' set while 50 µL saline was added to the 'control' set. The tubes were incubated at 37°C for 24 hr. Post incubation, 10 µL extract from all the tubes of 'test' and 'control' sets were spotted on M17 agar plate (Himedia) and the plates were incubated at 37°C for 24 hr. Post incubation, growth of culture at the spot was considered as positive result. Appropriate controls were set up to eliminate false results and each set was run in triplicates and repeated three times to confirm the reproducibility of the result.

Determination of antiphage activity [12]

450 µL of the plant extract (at the dilution that did not inhibit the probiotic host) was mixed with 50 µL of the *Lactococcus* phage P001 and incubated at 37°C for different time intervals viz 20 min and 90 min. For control, 450 µL of St. saline was mixed with 50 µL of the Lactococcus phage P001 and incubated at room temperature for same time intervals. 100 µL of the test and control aliquots were then mixed

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with 300 µL of actively growing *Lactococcus lactis* host culture and subjected to plaque assay by standard double agar overlay method. Post incubation, the plaques were counted the log reduction values for two periods of exposure was calculated. Each set of exposure times was run in triplicates and repeated three times to confirm the reproducibility of the result.

Statistical Analysis

Statistical analysis was done using Prism Version 9.0 (Graph Pad Software, Inc USA). Absorbance values of standards for estimation of alkaloids, flavonoids, and phenolic compounds were entered into the software yielding a calibration curve equation. This equation was then used to interpolate absorbance values of the sample, and the concentrations determined were expressed as mg/ml. The log reduction values were presented as mean ± SD. To access the statistical significance of the findings, a statistical level of $P<0.05$ was employed.

Results and Discussion

The present study comprehensively investigates the phytochemical composition of the methanolic extract of *Aegle marmelos* leaves and its potential to prevent phage infection of *Lactococcus lactis* P001. Qualitative phytochemical analysis confirmed the presence of key secondary metabolites such as alkaloids, phenolic compounds, reducing sugars, flavonoids and saponins. Tannins were not detected, and the absence of tannins is particularly interesting as phenolic compounds typically co-occur with tannins in many plant extracts. The presence of these metabolites is significant as they have been reported for anti-inflammatory, antiviral and antibacterial properties [13,14]. Alkaloid content was quantified using a standard curve plotted with atropine, resulting in an equation Y=0.3457*X+0.02381(Figure 1). The concentration of alkaloids was found to be 0.104 ± 0.04 mg of atropine/mg. Phenolic content was determined using a calibration curve with gallic acid, yielding the equation Y=0.01429*X+0.002857 (Figure 1). The phenolic concentration was 19.75 ± 0.49 mg of gallic acid/mg. Flavonoid content was quantified using catechin as a standard, with a calibration curve equation Y=0.009914*X–0.02571(Figure 1). The flavonoid concentration was 54.03 ± 1.42 mg of catechin/mg. These high levels of phenolic compounds highlight the strong antioxidant potential of the extract [15].

HPTLC fingerprint of the methanolic extract of *Aegle marmelos* was developed using the mobile phase composed of ethyl acetate: water: formic acid: acetic acid (100:26:11:11 v/v). After development, the TLC plate was derivatized with universal derivatizing agents anisaldehyde sulphuric acid and natural product A reagent revealing seven distinct bands. Each band corresponded to the separation of a different compound with a unique R_f value as shown in Table 1. Specific identities of these compounds could not be definitively determined without comparison to known standards. (Figure 2A) represents the HPTLC fingerprint documented under 366nm light following derivatization with natural product A reagent while (Figure 2B) shows the fingerprint under white light after treatment with anisaldehyde sulphuric acid reagent.

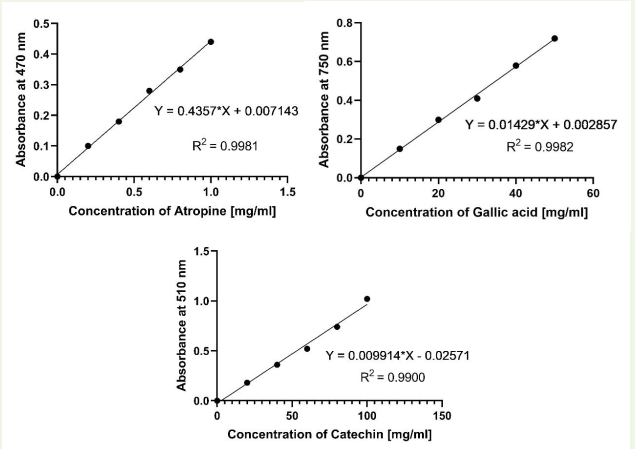

Figure 1: Calibration curve for estimation of alkaloids using atropine standard and phenolic compounds using gallic acid standard & flavonoids using catechin standard.

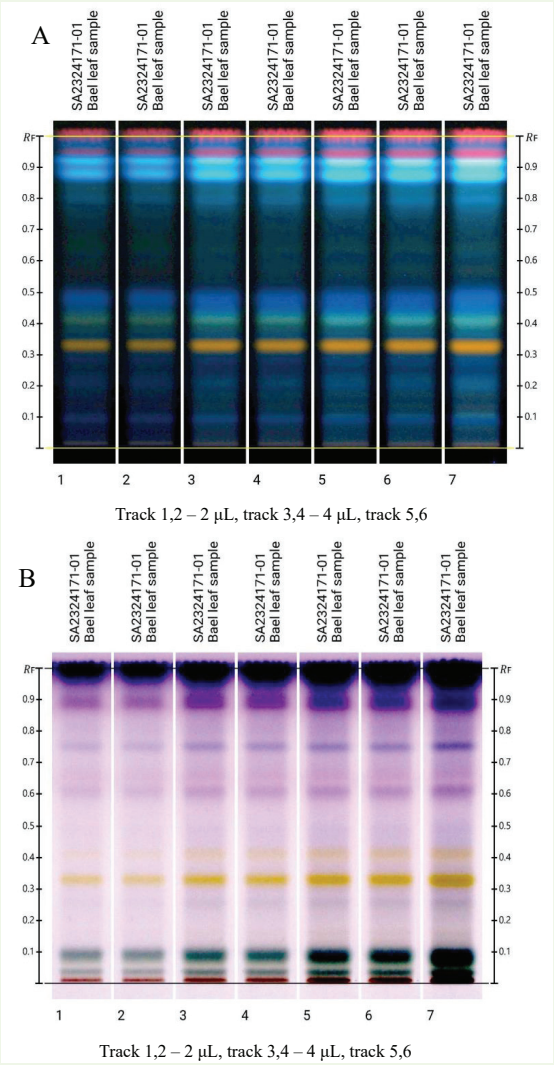

Figure 2: A:Image under 366nm after derivatization with Natural Product A reagent; B: Image under white light after derivatization with anisaldehyde sulphuric acid

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Table 1: Rf value of compounds separated by HPTLC analysis.

Image under 366 nm after derivitization with Natural Product A reagent			Image under white light after derivitization with Anisaldehyde sulphuric acid reagent	
Compound No	**Rf Value**	**Color**	**Rf Value**	**Color**
1	0.10	Blue	0.10	Green
2	0.33	Yellow	0.26	Purple
3	0.41	Green	0.33	Yellow
4	0.49	Blue	0.42	Yellow
5	0.87	Blue	0.61	Purple
6	0.92	Blue	0.76	Purple
7	0.95	Red	0.89	Purple

Table 2: Compounds identified in the extract by LCMS analysis.

RT	Compound	Molecular Formula	Compound nature	Score
5.151	Carnegine	$C_{13}H_{19}NO_2$	Alkaloid	85.83
6.471	Solanocapsine	$C_{27}H_{46}N_2O_2$	Alkaloid	91.9
10.618	Myrtenylformate	$C_{11}H_{16}O_2$	Terpenoid	96.76
12.973	Piperine	$C_{17}H_{19}NO_3$	Alkaloid	98.6
15.239	α, β onoceradienedione	C30 H46 O_2	Triterpenoid	95.96
16.51	5-alpha-cholesta-7, 24-dien-3-beta-ol	$C_{27}H_{44}O$	Sterols	65.47
17.835	Manglupenone	$C_{30}H_{44}O_2$	Triterpenoid	96.06
19.095	Oleanolic acid	$C_{30}H_{48}O_3$	Triterpenoid	95.42

Advanced analytical techniques, specifically LC-MS and GC-MS, were employed to gain deeper insights into the bioactive compounds present in the extract. LC-MS analysis identified several significant compounds, including alkaloids like piperine, which is known for its potential antiviral properties against Dengue and Ebola viruses as suggested by computational studies [16]. The presence of high molecular weight compounds, such as triterpenoids and sterols, is particularly relevant as these compounds are often associated with enhanced bioactivity due to their structural complexity, which may facilitate stronger interactions with biological targets, including viral particles and phages. The detailed list of compounds identified by LC-MS is provided in (Table 3), and the chromatogram obtained from LC-MS analysis is shown in (Figure 3) and structures identified are shown in (Figure 4).

GCMS analysis revealed the presence of several compounds, each identified based on its specific retention time, analysing their mass spectra and corresponding m/z values using NIST spectral databases. The chromatogram obtained from GC-MS analysis is shown in Figure 5, with the detailed list of identified compounds provided in (Table 3).

The determination of the MIC ensured that the plant extract did not inhibit the probiotic host culture, *Lactococcus lactis*. The MIC value was determined to be 80 mg/ml, with the highest concentration tolerated by the host being 70 mg/ml. This concentration was then used in subsequent experiments to evaluate the extract's antiphage activity, ensuring that the host culture remained viable and unaffected by the extract.

The antiphage activity was further evaluated by exposing the phage

Table 3: Compounds identified in the extract by GCMS analysis.

RT [min]	Component	Molecular Formula
8.62	Nonanal	$C_9H_{18}O$
12.27	n-Hexadecylsuccinic anhydride	$C_{20}H_{36}O_3$
14.57	2-Dodecenal, (E)	$C_{12}H_{22}O$

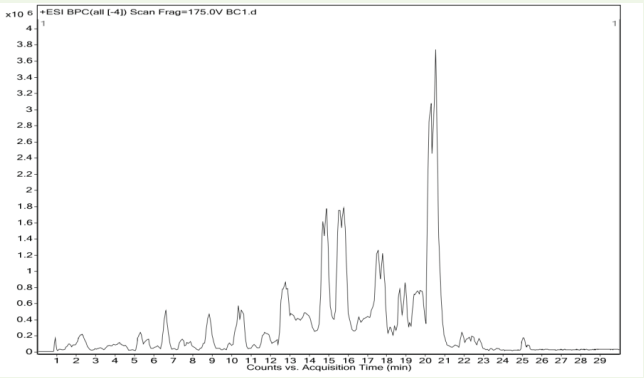

Figure 3: The chromatogram obtained from LC-MS analysis of the methanolic extract.

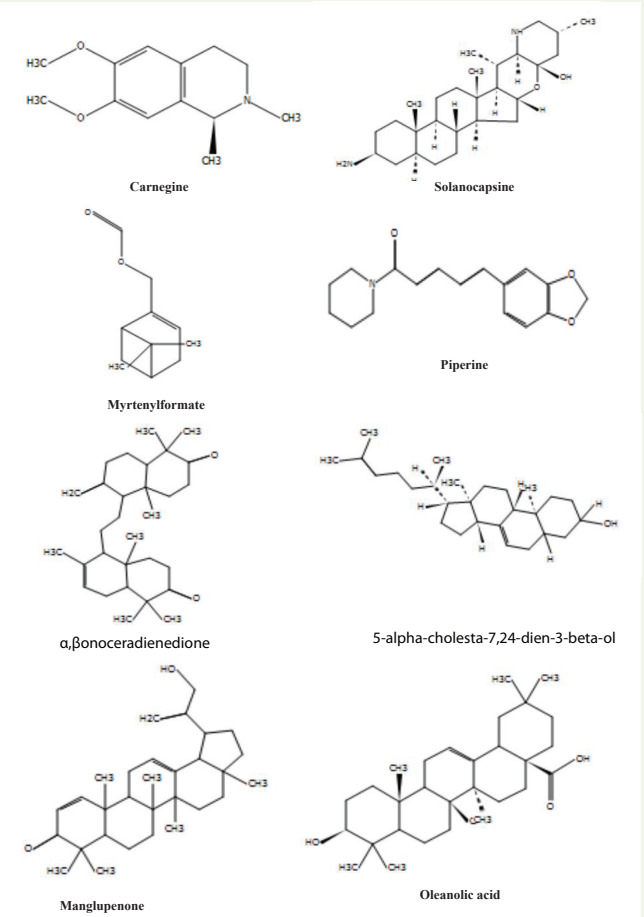

Figure 4: Structures of compounds identified by LCMS.

Citation: Mulay R, Bhathena Z. Investigating the Antiphage Activity of the Methanolic Extract of *Aegle marmelos* against Lactic Acid Bacteriophages. J Plant Sci Res. 2025;12(1): 272

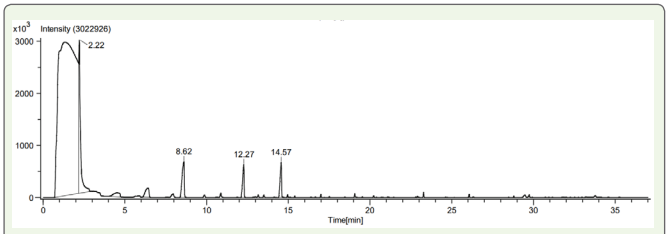

Figure 5: The chromatogram obtained from GC-MS analysis.

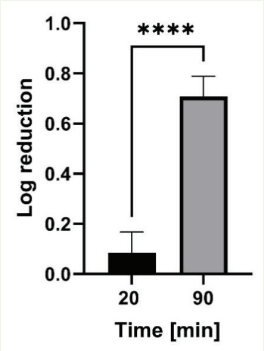

Figure 6: Effect of methanolic extract of *Aegle marmelos* on *Lactococcus* P001 phage (**** = p <0.0001).

to the extract at concentration of 70 mg/ml for two different durations, 20 minutes and 90 minutes. The results showed a significant time-dependent increase in antiphage activity, with a mean log reduction of 0.0846 ± 0.083 observed after 20 minutes of exposure, which increased to 0.708 ± 0.081 after 90 minutes of exposure (Figure6). The statistically significant difference in antiphage activity between the two exposure times ($p < 0.0001$) suggests that the bioactive compounds in the extract may interfere more effectively with different stages of phage replication over extended exposure times. This finding implies that prolonged contact with the extract enhances its capacity to inhibit phage activity, potentially by disrupting key processes in the phage life cycle.

These findings align with previous studies, including those involving other plant extracts like *Withania somnifera*, where a log reduction of 0.713 ± 0.08 after 20 minutes and 0.736 ± 0.18 after 90 minutes of exposure to the phage was observed, showing that different plant extracts might share common mechanisms of action against phages [17]. Other plant extracts, such as those from *Phoenix dactylifera* [18] and *Plantago major* [19], have also been reported to exhibit significant antiphage activity, further supporting the potential of plant-derived compounds in phage control strategies.

The current study provides valuable insights into the potential applications of *Aegle marmelos* leaf extract in the dairy industry, particularly in mitigating phage infections that can disrupt fermentation processes. The extract's ability to inhibit *Lactococcus* P001 phage without adversely affecting the probiotic host highlights its potential as a natural and effective solution to phage-related challenges. This study opens avenues for the development of new, medicinal plant-based strategies for phage control in industrial applications.

Conclusion

Studying the phytochemical profile of *Aegle marmelos* leaves extract highlighted the rich composition of varied secondary metabolites including alkaloids, phenols, flavonoids, and saponins. This study also demonstrated the effectiveness of the methanolic extract for reducing the phage infection of P001 phage without impacting the viability of the probiotic host culture suggesting its application in preventing phage infection. Presently, our focus lies on purifying the plant extract to separate the bioactive compounds, elucidating their antiphage effects and understanding their precise mechanisms.

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Conflict of Interest: The authors declare no conflict of interest in this study.

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