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Original Article

Comprehensive Phytochemical Profiling and Antiphage Activity of Methanolic Extract of *Withania somnifera* against Lactic Acid Bacteriophages

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ABSTRACT

Introduction: Bacteriophages present a significant challenge to commercial dairy manufacturing facilities impacting product quality and causing great economic losses to the dairy industry. Despite routine phage control measures, these phages exhibit resistance to conventional methods, including pasteurization. *Withania somnifera* (L.) Dunal is a popular medicinal plant traditionally used in Ayurvedic medicine and has various health benefits. **Objectives:** This study is aimed at conducting a comprehensive phytochemical analysis and evaluating its potential in preventing *Lactococcus* phage infections in the dairy industry. **Materials and Methods:** In this study, qualitative and quantitative phytochemical testing, HPTLC fingerprinting, LC-MS and GC-MS of *Withania somnifera* methanolic root extract were done. The MIC of the methanolic extract against the probiotic host was determined and antiphage activity of the methanolic extract was done against *Lactococcus lactis* P001 phage at a concentration that did not inhibit the probiotic host. **Results:** Qualitative tests revealed the presence of secondary metabolites like alkaloids, phenolic compounds, and tannins in the plant extract, while LCMS and GCMS identified various bioactive compounds. Antiphage assay using crude methanolic extracts demonstrated a significant reduction in the phage titre with a log reduction 0.713±0.08 after 20 min of exposure and 0.736±0.18 after 90 min of exposure without any observed inhibition of the host. **Conclusion:** These findings offer valuable insights into the extract's potential in preventing phage infection within lactic probiotic cultures.

Keywords: Ashwagandha, Antiphage, Bacteriophages, *Lactococcus lactis*.

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INTRODUCTION

India has a rich tradition of using herbal medicine for its therapeutic applications and drug development. Plant based formulations when used in the right proportions prove to be an economically viable and ecofriendly alternative with minimum side effects.

Withania somnifera (L.) Dunal, commonly known as Ashwagandha or the Indian ginseng is the most popular herb of Ayurveda and belongs to *Solanaceae* family. It is known to possess antioxidant, anxiolytic, adaptogen, memory enhancing, antiparkinsonian, antivenom, anti-inflammatory and antitumor properties.[1] Additionally, it is also effective against notable viruses like COVID-19, SARS-CoV-2, H1N1 Influenza, Herpes Simplex virus, Newcastle disease virus, Chikungunya virus etc.,[2] Major phytochemicals reported in leaves of ashwagandha are withanolides, condensed tannins, flavonoids, glycosides, free amino acids while roots encompass alkaloids, steroids, volatile oils, reducing sugars and amino acids.[3] However, most of the medicinal properties and health benefits of ashwagandha are found in the roots and are attributed to the numerous bioactive compounds like withaferin A, withanone and withanolides.[4,5]

While scientific literature extensively covers the different therapeutic properties of Ashwagandha, its efficiency against bacteriophages remains unexplored. Bacteriophages infecting probiotic starter cultures are a known threat in commercial manufacturing facilities. Severe phage infections are known to cause complete loss of starter culture resulting in dead vats filled with partly acidified milk, leading to product loss.[6] Moreover, these phages are resistant to routine phage control measures and pasteurization temperatures.[7] Hence it is important to conduct in-depth research to understand this issue better. By studying these phages further, specific strategies can be formulated to minimize their impact on commercial production processes and maintain the quality of commercial probiotic products. Thus, this study highlights the potential of methanolic extract of *Withania somnifera* against bacteriophages infecting *Lactococcus lactis*

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along with a thorough phytochemical profiling with an effort to identify the compounds responsible for this activity.

MATERIALS AND METHODS

Procurement of Plant Material

Complete plant of *Withania somnifera* (L.) Dunal (WSR) was procured from the Botanical Garden and Nursery within Bhavan's College Campus, Mumbai and confirmed of its authenticity by Botanists at the Department of Botany, Bhavan's College. The roots 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 g of the powdered plant material was added in 50 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. Preliminary qualitative and quantitative testing was conducted using standard protocols.[8,9]

HPTLC fingerprinting of the extract

HPTLC fingerprinting of the extract was carried out using mobile phase consisting of chloroform: ethyl acetate: methanol: distilled water (3:8:4.4:1.8-v/v/v/v). The stationary phase consisted of TLC silica gel 60 F_{254} (Merck, USA -100x100 mm). The development chamber consisted of saturated twin trough Chamber (CAMAG) with Whatmann filter paper no 1 with the development distance of 70 mm from the application point. Specified volume of sample was applied on to the TLC plates using Linomat 5 (CAMAG), semiautomatic sample dispenser. The TLC plates were scanned by CAMAG Scanner 3 and the developed chromatogram was visualized at 254 nm, 366 nm and white light before and after derivatization with anisaldehyde sulphuric acid reagent and photodocumented.

Liquid Chromatography Mass Spectrometry (LC-MS) Analysis

LCMS analysis of the extract was carried out using Q-TOF mass spectrophotometer (G6550A, Agilent, USA) with Dual AJS ESI (Agilent, 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 (Aligent, USA). The data generated was processed, analyzed and interpreted using MassHunter 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 MBC of methanolic extract

MBC 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]

Briefly, 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 with 300 µL of actively growing *Lactococcus lactis* host culture and subjected to plaque assay by standard double agar overlay method.[13] 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 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 are presented as mean±SD. To access the statistical significance of the findings, a statistical threshold of $p<0.05$ was employed.

RESULTS

Phytochemical analysis

Qualitative phytochemical analysis of methanolic extracts of Ashwagandha root sample detected the presence of secondary metabolites, namely alkaloids, phenols, and tannins. Alkaloid content in the extract was calculated using the standard curve plotted with atropine as a standard in the concentration range of 0.2 to 1.0 mg/mL as shown in Figure 1. According to the standard curve, the equation obtained was Y=0.4357*X+0.007143. The alkaloid content was calculated to be 0.328±0.03 mg equivalent of atropine/mg. Concentration of phenolic compounds in the extract was determined from the calibration curve plotted using gallic acid as a standard in the concentration range of 10 to 50 mg/mL as shown in Figure 1. The equation obtained from the curve was Y=0.01429*X+0.002857. The concentration of phenolic compounds was found to be 10.79±0.49 mg of gallic acid/mg.

HPTLC fingerprint of the extracts

The chromatogram for HPLTC fingerprinting of the methanolic extract of *Withania somnifera* was developed using the mobile phase chloroform: ethyl acetate: methanol: distilled water (3:8:4.4:1.8-v/v/v/v). Post development, there were no bands seen at 254 nm and white light (data not shown) indicating lack of compounds with a significant absorbance at 254 nm. However, presence of distinct bands at 366 nm indicated the presence of compounds fluorescing at this specific wavelength. Furthermore, derivatization of TLC plates with universal derivatizing agent anisaldehyde sulphuric acid showed 13 distinct bands indicating separation of 13 different compounds with different R_f values at white light as shown in Figure 2. However, the detailed class of compound analysis was not done, hence the identity of the bands cannot be deciphered based on this fingerprint data. The 3D chromatogram revealed nine distinct peaks across seven tracks highlighting the complexity of the compounds present in the root sample. Figure 2A shows HPTLC fingerprint photodocumented under white light after derivatization with anisaldehyde sulphuric acid reagent while Figure 2B and Figure 2C exhibit HPTLC fingerprint photodocumented under 366 nm after derivatization with anisaldehyde sulphuric acid reagent and 3D chromatogram respectively. Table 1 shows R_f values and corresponding spot colors different compounds obtained after derivatization with anisaldehyde sulphuric acid reagent.

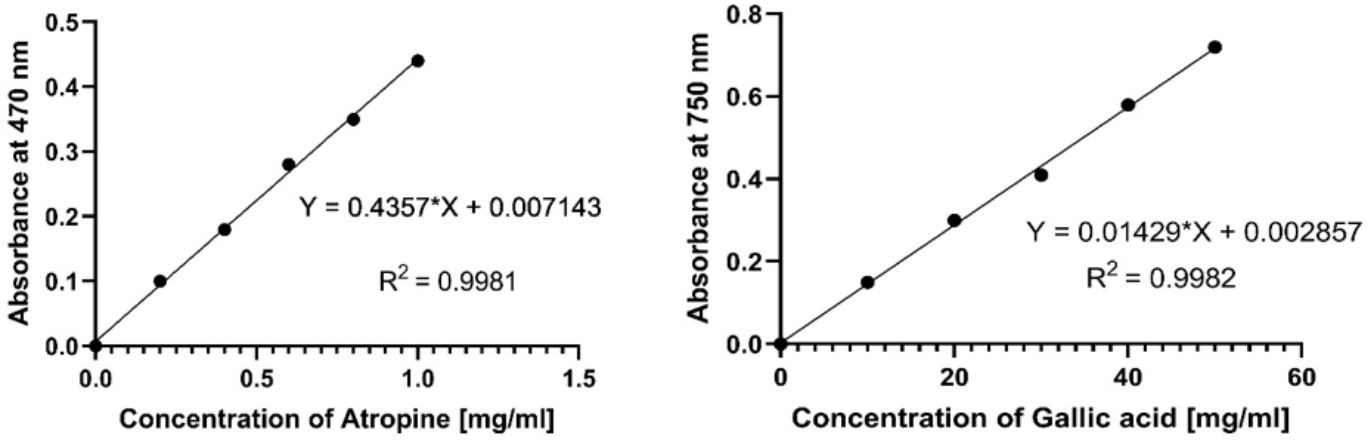

Figure 1: Calibration curve for estimation of alkaloids using atropine standard and phenolic compounds using gallic acid standard.

LC-MS analysis of the extract

The analysis of the chromatogram obtained from LC-MS data revealed the presence of several compounds. Each compound was identified based on its characteristic retention time, analyzing their mass spectra and corresponding m/z values. Identified compounds belonged to diverse array of secondary metabolites like alkaloids, phenolics and terpenoids. The chromatogram obtained is shown in Figure 3 and the detailed list of compounds identified is given in Table 2.

Table 1: R_f **value table for compounds separated by HPTLC of Ashwagandha Root Sample.**

Image under white light after derivitization with anisaldehyde sulphuric acid reagent		
Compound No	**R_f Value**	**Color**
1	0.09	Green
2	0.14	Green
3	0.30	Blue
4	0.38	Blue
5	0.44	Blue
6	0.52	Blue
7	0.54	Blue
8	0.58	Blue
9	0.63	Purple
10	0.69	Purple
11	0.74	Purple
12	0.79	Purple
13	0.88	Purple

GC-MS analysis of the extract

GC-MS analysis revealed the presence of several compounds, each identified based on its specific retention time, analyzing their mass spectra and corresponding m/z values using NIST spectral databases. Compounds like heptanal, nonanal, 1-octene, 3,7 dimethyl suggest the presence of volatile compounds contributing to its potential biological activity and aroma and flavor. The chromatogram obtained is shown in Figure 4 and the detailed list of compounds identified by GC-MS is shown in Table 3.

Determination of MBC of the methanolic extract against LAB host

For determination of MBC of the plant extract against the host, the host exhibited growth on M17 agar [Himedia] plate at concentrations up to 90 mg/mL for the extract. This indicated that the highest concentration of the extract tolerated by the host was 90 mg/mL. This concentration was further used for antiphage activity. However, the host did not show growth at extract concentration of 100 mg/mL indicating this concentration to be the minimum inhibitory concentration. The results of MBC are shown in Table 4.

Determination of antiphage activity

To determine the efficacy of the extract against P001 phage, the highest concentration tolerated by the host i.e. 90 mg/mL was considered. Plant extract exposed to the P001 phage for two exposure times of 20 min and 90 min showed the mean log reduction of P001 phage of 0.713±0.08 after 20 min of exposure and 0.736±0.18 after 90 min of exposure. No statistically significant difference (p>0.05) in the antiphage activity of the plant extract was reported between 20 min and 90 min of exposure times. It suggests that the duration of exposure does not impact the efficiency of the plant extract against P001 phage.

Table 2: Compounds identified in the methanolic extract by LC-MS.

RT	Compound	Molecular Formula	Compound nature	Score
3.232	Ammothamnine	$C_{15}H_{24}N_2O_2$	Alkaloid	99.78
4.049	Matrine	$C_{15}H_{24}N_2O$	Alkaloid	99.47
4.271	Geijerone	$C_{12}H_{18}O$	Terpenoid	78.07
6.022	Petasinine	$C_{13}H_{21}NO_3$	Terpenoid	85.94
6.481	Agroclavine	$C_{16}H_{18}N_2$	Alkaloid	85.2
8.19	N-Feruloyltyramine	$C_{18}H_{19}NO_4$	Phenolic compound	99.19
11.303	Withaferin A	$C_{28}H_{38}O_6$	Alkaloid	97.55
14.313	Euglobal VII	$C_{28}H_{38}O_5$	Terpenoid	98.84
11.467	Withasomnine	$C_{12}H_{12}N_2$	Alkaloid	99.24
11.465	Boviquinone 4	$C_{26}H_{36}O_4$	Terpenoid	66.39
11.656	Alnustone	$C_{19}H_{18}O$	Terpenoid	85.46

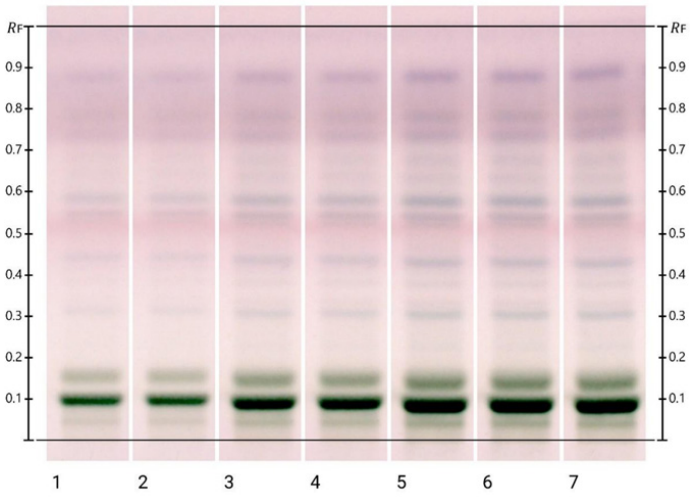

Figure 2A: Image under white light after derivitization with anisaldehyde sulphuric acid reagent.

Track 1,2 – 2 µL, track 3,4 – 4 µL, track 5,6 – 6 µL, track 7 – 8 µL.

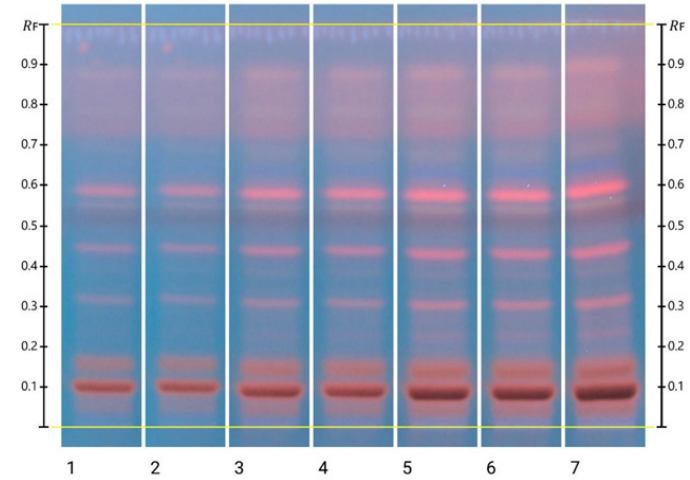

Figure 2B: Image under 366 nm after derivitization with anisaldehyde sulphuric acid reagent.

Track 1,2 – 2 µL, track 3,4 – 4 µL, track 5,6 – 6 µL, track 7 – 8 µL.

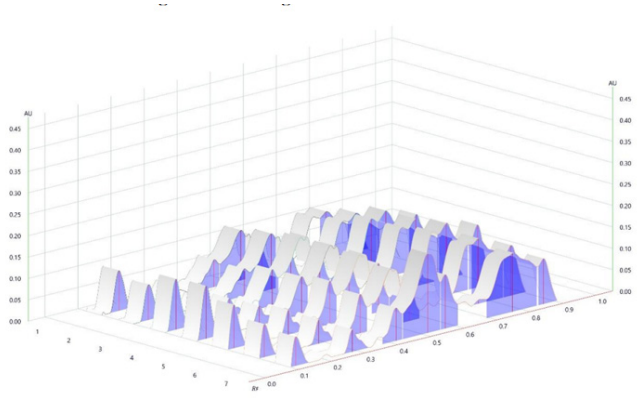

Figure 2C: 3D Chromatogram of *Ashwagandha* root sample.

Table 3: Compounds identified in the methanolic extract by GC-MS analysis.

RT [min]	Component	Molecular Formula
4.45	Heptanal	$C_7H_{14}O$
6.35	Octanal	$C_8H_{16}O$
8.70	Nonanal	$C_9H_{18}O$
9.93	1-Octene, 3,7-dimethyl	$C_{10}H_{20}$
10.19	2.4-O-Methylphorbol 12,13-didecanoate	$C_{41}H_{66}O_8$
10.91	(5β)Pregnane-3,20β-diol, 14α,18α-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate	$C_{28}H_{43}NO_6$
12.43	2-Decenal, (E)-	$C_{10}H_{18}O$
12.54	Nonanal dimethyl acetal	$C_{11}H_{24}O_2$
14.71	2-Undecenal, E-	$C_{11}H_{20}O$

Table 4: Results of MBC of methanolic extract against *L. lactis* host.

Tube No	Concentration of extract [mg/mL]	*Withania somnifera* root extract	
		Test Set	Control Set
1	40	+	-
2	50	+	-
3	60	+	-
4	70	+	-
5	80	+	-
6	90	+	-
7	100	-	-
8	PC	+	+
9	NC	-	-
10	MC	-	-

Each set was performed in triplicates and repeated three times. Key-+: growth in the form of spot seen on M17 agar, -: no growth in the form of spot seen on M17 agar.

DISCUSSION

Medicinal plants have gained significant attention in recent times due to their numerous potential health benefits. This study was aimed at investigating the phytochemical profile of Ashwagandha root extract and studying its potential to prevent phage infection of *Lactococcus lactis* P001 phage.

Qualitative phytochemical tests detected the presence of alkaloids, phenols, tannins, amino acids, carbohydrates, starch, flavonoids and reducing sugars while saponins were not detected in the extract. The presence of alkaloids is particularly important as they are known for their diverse pharmacological properties. Also, the presence of phenols and tannins is noteworthy as these compounds are known for their antioxidant properties. Alkaloids and phenolic compounds were quantified in the extract providing valuable insights into the chemical composition of the extract and understanding its potential pharmacological properties. HPTLC fingerprint showed distinct peak patterns at 366 nm after derivatization reflecting the complex interplay between the compounds. However, identification of each separated compound can be achieved definitively and accurately by using standards.

Furthermore, advanced analytical techniques like LC-MS and GC-MS facilitated the identification of specific compounds within the extract. The detected compounds predominantly belonged to classes of alkaloids, terpenoids and phenolic compounds. Compounds identified through LCMS analysis exhibited a diverse range of molecular structures and compositions spanning carbon numbers from C12 to C28 incorporating nitrogen and oxygen atoms indicating various functional groups like amines, amides and ethers. Higher carbon number compounds suggest greater molecular complexity and potentially lower volatility. GCMS analysis, on the other hand, mainly identified compounds with lower carbon numbers C7 to C10 which are anticipated to be more volatile in nature and the presence of oxygen and nitrogen in some compounds ($C_{28}H_{43}NO_6$) may influence their volatility and chemical properties. Matrine, an alkaloid and withaferin A which is a steroidal lactone have been previously reported for their antiviral activities against various animal viruses.[14] The presence of these compounds suggests the potential of the extract to contribute to its antiphage activity.

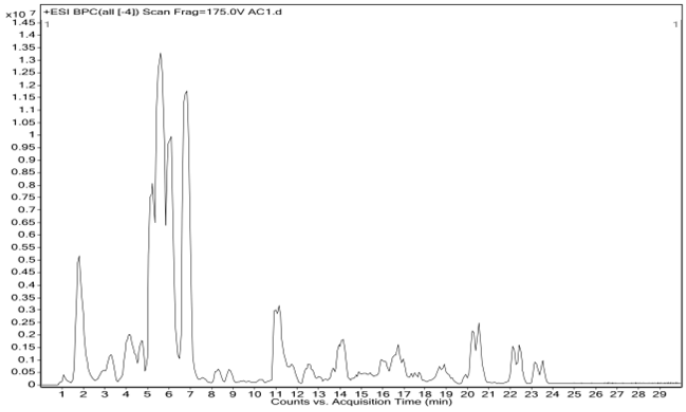

Figure 3: The chromatogram obtained from LC-MS analysis of extract of *Ashwagandha* root sample.

However, before exposing the extract to the phage, it was important to determine the concentration of the root extract that does not inhibit the probiotic host culture. Thus, the MBC study of methanolic extracts provided a basis for understanding the probiotic host culture's sensitivity towards the extract and ensured that the concentration of extract used for antiphage activity determination did not exert any adverse effects on its viability and growth. The study demonstrated the MIC value of 100 mg/mL for the extract, indicating that the highest concentration of the extract tolerated by the probiotic host was 90 mg/mL. To ensure the compatibility of the extract with the probiotic host, 90 mg/ml concentration was chosen for subsequent determination of antiphage activity. Results of antiphage activity revealed that the methanolic root extract successfully prevented the infection of *Lactococcus* phage P001, showing a log reduction of 0.713±0.08 after 20 min of exposure and 0.736±0.18 after 90 min of exposure. The observed lack of statistically significant difference (p>0.05) in the antiphage activity between the exposure times of 20 min and 90 min indicates that the ashwagandha root extract's inhibitory effect remains constant regardless of the duration of exposure, suggesting that its effect on phage infection might be exerted at an early stage of phage replication cycle, possibly during the

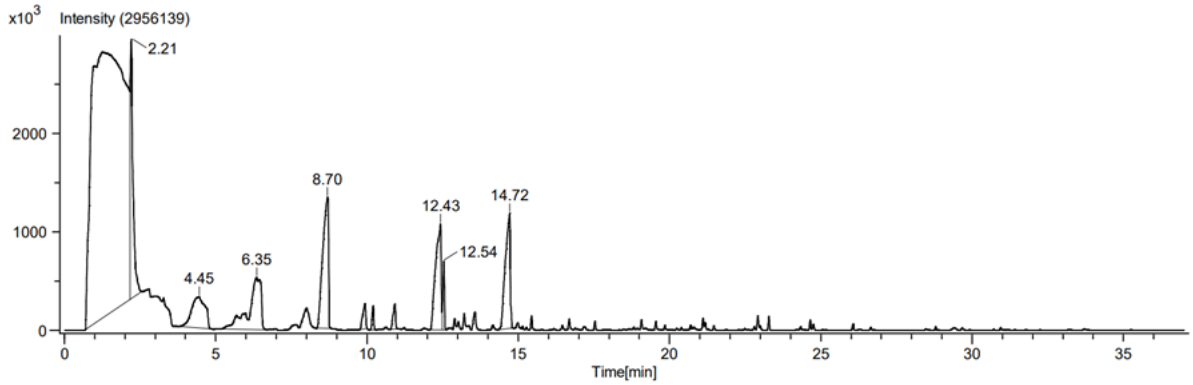

Figure 4: The chromatogram obtained from GC-MS analysis of *Ashwagandha* Root sample.

eclipse phase. These results are consistent with similar studies in the literature, where different plant extracts have demonstrated significant effects against bacteriophages. For instance, ethyl acetate fraction of *Phoenix dactylifera* has shown 96.48% reduction in coliphage activity[15] while *Plantago major* leaf extract exhibited a strong antiphage activity against coliphages with a log reduction of 0.927.[16] These collective findings highlight the potential of plant extracts in mitigating phage infections, with our study contributing valuable insights to the activity of *Withania somnifera* root extract against *Lactococcus* P001 phage without adversely affecting the probiotic host which is a potential starter culture in dairy fermentation industry. This opens the potential of *Withania somnifera* as a solution to address phage related challenges in the dairy industry.

CONCLUSION

Investigation of the phytochemical profile of *Withania somnifera* root extract highlighted the rich composition of diverse secondary metabolites including alkaloids, phenols, tannins, etc. 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 potential application in the dairy fermentation industry. Currently, we are actively focusing 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 that there is no conflict of interest.

ABBREVIATIONS

MBC: Minimum Bactericidal Concentration; **MIC:** Minimum Inhibitory Concentration.

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