

In-vitro Evaluation of Antioxidative, Antibacterial, and Hepatoprotective effect of *Smilax zeylanica* L. and *Berberis aristata* DC

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Abstract

Background: The research examined the antioxidant, antibacterial, and hepatoprotective properties of hydroalcoholic extracts derived from *Smilax zeylanica* L. and *Berberis aristata* DC. Alkaloids, tannins, flavonoids, and glycosides were the major phytoconstituents, with a significant quantity of alkaloids and flavanones.

Objective: The hydroalcoholic extract of *Smilax zeylanica* exhibited an IC₅₀ value of 6.29 µg/ml, while *Berberis aristata* demonstrated an IC₅₀ value of 9.26 µg/ml in the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay for antioxidant activity.

Methods: The occurrence of biologically active compounds in the hydroalcoholic extract was identified through column chromatography and GC-MS analysis. The modified extract showed significant antibacterial activity and *Enterococcus faecalis* exhibited significant susceptibility to the extract by using the disc diffusion method.

Results: The hepatoprotective activity of the extracts was assessed against HepG2 cells, and the extracts showed varying levels of hepatoprotection and cytotoxicity. *Berberis aristata* showed the highest hepatoprotective activity (IC₅₀ < 47.09) among all the extracts.

Conclusion: From the above, it is suggested that natural antioxidants, antibacterials, and hepatoprotective properties of plant extracts of *Smilax zeylanica* and *Berberis aristata* could be promising for further research and can be used to develop effective antibacterial agents against microbial infections.

Keywords- Hepatoprotective, Antioxidant activity, Antibacterial activity, Cytotoxicity, Cell line.

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Introduction

In many living things, oxidation is essential because it produces the energy needed to keep biological processes going. However, unchecked production of free radicals originating from oxygen can harm cellular structures and functions and perhaps start a chain reaction. Thus, more free radicals are formed as a result of these reactions.¹ Atoms or chemical groups with at least one unpaired electron, which makes them unstable and extremely reactive, are known as free radicals. Free radicals are present in the environment for humans because of things like pollution and radiation.²

Free radicals, which would otherwise have harmful effects on living things, are neutralized by antioxidants in biological cells. Superoxide dismutase (SOD) is a particularly important enzyme in reducing the effects of oxidative stress brought on by free radicals. This metalloenzyme is an important modulator of oxidative reactions in cells, distinguished by its sub unitary structural arrangement. It counteracts oxidative damage by facilitating the recombination of

oxygen radicals. By blocking the production of hydrogen peroxide and singlet oxygen, therapeutic uses of SOD have demonstrated efficacy in treating a variety of clinical diseases and delaying their onset. Enzymes like superoxide dismutase and catalase, together with dietary antioxidants like ascorbic acid and tocopherols, protect almost all organisms against free radicals.³

The world is beginning to acknowledge Ayurveda, the age-old Indian medical tradition, as a successful treatment practice. Antioxidants are plentiful in many Ayurvedic medicines, many of which are derived from medicinal plants. If consumed as dietary supplements, these organic substances may significantly lower the prevalence of illnesses linked to oxidative stress.⁴ Infectious diseases remain a critical concern for health organizations, pharmaceutical firms, and governments globally, resulting in over 50,000 deaths daily. This issue is exacerbated by the rising trend of multidrug resistance among both emerging and re-emerging bacterial pathogens to contemporary antibiotics.⁵ The

extensive use of antibiotics in clinical practice, agriculture, and veterinary medicine has contributed to the development of resistance among infectious microorganisms, creating significant challenges in managing pathogenic infections. The proliferation of drug-resistant strains and the resurgence of infectious diseases present substantial obstacles to public health systems.⁶ Notably, the emergence of antibiotic resistance has impeded the progress of developing and deploying new antibiotics to the public.⁷

The rise of antibiotic-resistant bacteria has increasingly undermined the effectiveness of treating infectious diseases. Recent reports indicate that in the United States alone, 23,000 deaths annually are attributed to bacterial infections that cannot be managed due to resistance, while the European Union experiences approximately 25,000 such fatalities each year.⁸ Antibiotic resistance has led to rising rates of morbidity and mortality from infectious illnesses, according to epidemiological data. The need for new antibacterial drugs to fight resistant bacterial strains is highlighted by the low efficacy of current antibiotics. Natural products have long been used in pharmaceutical research to produce molecules that are physiologically active. As a result, a lot of effort has gone into looking for antibacterial compounds in natural sources, which have shown promise as potential medication candidates.⁹ Plant extracts and essential oils have attracted attention as natural product sources throughout history. The possibility of these chemicals as alternative therapies for different infectious illnesses has been assessed. Aromatic and medicinal plants are a significant source of naturally occurring chemical compounds and are widely used in therapeutic applications. These plants serve as safer and more affordable substitutes for antibacterial agents.¹⁰

Depending on the degree of cellular damage, acute hepatitis may progress to chronic hepatitis, which, if left untreated, may eventually result in cirrhosis or malignant tumours.¹¹ Liver function abnormalities can change the liver's subcellular composition or chemical makeup. Portal hypertension, ascites, jaundice, increased bleeding, and other metabolic disorders impacting other organs can all be caused by slight alterations in the structure and function of the liver. Established hepatitis viruses are typically the source of acute viral hepatitis, which is characterized by extensive liver inflammation and frequently coexists with clinical and biochemical abnormalities.¹² Liver cirrhosis is the primary cause of death in Western nations due to alcoholic liver disease (ALD), a serious consequence of long-term alcohol usage. Excessive and prolonged consumption of ethanol is linked to liver cancer, cirrhosis, fibrosis, and cellular proliferation.¹³

The potential of herbal treatments to improve the liver's natural healing mechanisms and manage a variety of liver ailments is becoming more widely acknowledged. Because they have few adverse effects and are generally low in toxicity, plants have been used historically to cure a variety of illnesses.¹⁴ The effectiveness, perceived minimal side

effects, and affordability of herbal medications make them widely recommended, especially in cases when the precise biologically active ingredients are not well understood.¹⁵ Furthermore, antioxidants are becoming more and more popular, especially those that come from natural sources, as a means of reducing the possible negative effects of free radicals and halting the deterioration of lipids and other food ingredients. Because natural antioxidants are thought to be safer than synthetic ones, they are recommended. In the framework of contemporary medical research, several plants have been evaluated for their antioxidant and hepatoprotective qualities.¹⁶

Material & Methods

Drugs

Silymarin, *Smilax zeylanica* DC, *Berberis aristata* L.

Chemicals

Hydrochloric acid, Mayer's reagent, Sodium hydroxide, Ammonia, Ferric chloride, Acetic acid, Sulfuric acid, Ciprofloxacin, Dimethyl sulfoxide, Chloroform, Ethyl acetate, Methanol, Ethanol, Silica gel, Folin's reagent, DPPH, Distilled water, Bovine serum albumin.

Collection of plant extracts

The extract of *Smilax zeylanica* L. and *Berberis aristata* DC was procured from Ambe NS Agro Products Pvt. Ltd. located in Swasthya Vihar (Near Preet Vihar Metro Station), New Delhi-110092, India.

Phytochemical Screening analysis of plant extracts

The following procedures were used to conduct a chemical test on *Berberis aristata* L. and *Smilax zeylanica* DC extracts to identify different phytochemicals.¹⁷

DPPH Method for Determination of Antioxidant Activity

DPPH i.e., 2,2-diphenyl-1-picryl-hydrazine-hydrate was a persistent free radical that reacts with methanol to produce a violet-colored solution; however, when antioxidant molecules are added, it changes to a colorless solution.¹⁸

Procedure

Ascorbic acid was used as standard with different concentrations of 10 to 100 µg/ml in methanol. The same concentrations of plant extract samples were prepared. Prepare 0.1 mM DPPH in methanol and add 2.4 ml of this solution to each dilution of standard and extracts. Mix this reaction mixture well using a vortex and keep it aside for thirty minutes. A blank solution was prepared using a 0.1 mM concentration of DPPH dissolved in methanol. After 30 minutes, measure absorbance at 517 nanometers using a UV spectrophotometer. All procedure was performed in triplicate. Percentage inhibition of standard and extracts was calculated by the given equation:

$I\%: (Ac - At) * 100 / Ac$

I% = % Inhibition

Ac = Control absorbance,

At = Standard absorbance / Plant extract absorbance.^{19, 20, 21}

GC-MS Analysis

For GC-MS Analysis, an Agilent gas chromatograph and mass spectrophotometer were utilized. The equipment was equipped with an HP-5MS fused silica column, which had dimensions of 30.0 meters in length, 250 meters in diameter, and a film thickness of 25.25 meters. The column was connected to a 5675C Inert MSD with Triplet-axis detector.^{22, 23}

Helium gas was used as the carrier gas at a flow rate of 1.0 milliliter per minute. The ion source temperature was set to 250 °C, while the interface temperature was maintained at 300.00 °C. The pressure was set at 16.2 psi, and the outlet was set to 1.8 millimeters. A 1 litter injector was used in split mode with a split ratio of 1:50, and the injector temperature was set to 300 °C.^{24, 25}

Anti-microbial Activity Assay

The Zone of Inhibition Method, occasionally referred to as the Kirby-Bauer approach, was utilized to evaluate the antibacterial activity. Mueller-Hinton Agar (MHA) plates were uniformly coated with a 100 µl solution of *Enterococcus faecalis*, which had been adjusted to a cell density of 0.5 McFarland Unit (about 1.5×10^8 CFU/mL). The plates were then covered with discs that had been impregnated with 10 µl of different doses ranging from 0 to 100 mg/mL. A positive control was represented by a Ciprofloxacin disc (10 µg) and a negative control was created using discs that contained just the solvent. The *Enterococcus faecalis* inoculated plates were incubated for 24 hours at 37 °C in an incubator made by Basil Scientific Corp. India. We next measured and recorded the zones of inhibition around the discs.^{26, 27, 28}

In-vitro Cytotoxicity Evaluation

Using the MTT test, the sample's cytotoxicity was examined on the HepG2 cell line (obtained from NCCS Pune). For a duration of 24 hours, 10,000 HepG2 cells per well were cultivated in 96-well plates using Dulbecco's Modified Eagle Medium (DMEM-AT149-1L), which was enhanced with 10% Fetal Bovine Serum (FBS-HIMEDIA-RM 10432) and 1% antibiotic solution. The plates were then incubated at 37°C with 5% CO₂. The next day, untreated cells were used as controls and cells were subjected to different concentrations of the sample (as specified in the Excel sheet). Following a 24-hour incubation period, the cell monolayer was dissolved in 100 µl of Dimethyl Sulfoxide (DMSO-SRL-Cat no.-67685) and the culture supernatant was disposed of. At 540 and 660 nm, absorbance was measured with an ELISA plate reader (iMark, Biorad, USA).^{29, 30}

Statistical Analysis

All the results are presented as the mean ± standard deviation (SD), and the statistical analysis was performed using SPSS

(version 15.0). The analysis involved two-way ANOVA with subsequent Bonferroni post hoc correction. The significance levels for this investigation were p values less than 0.05.³¹

Results

Information about of plant extraction: Here hydroalcoholic extracts of *Smilax zeylanica* L. and *Berberis aristata* DC. plants used for in vitro investigation of Antioxidative, Antibacterial, and Hepatoprotective Properties.

Main Phytochemical results

The phytochemical examination of hydroalcoholic extracts from *Smilax zeylanica* L. and *Berberis aristata* DC. showed presence of alkaloids tannins, glycoside and flavonoids.

Antioxidant analysis by DPPH method

The antioxidant properties and IC₅₀ value are determined through DPPH radical inhibition, as illustrated in Table 1. Ascorbic acid serves as the standard reference drug for comparison. To measure antioxidant activity, the IC₅₀ value is derived using the DPPH radical inhibition technique.

Analysis of Column Chromatography

Table 2 summarizes the mobile phases, fraction details, and retention factor (R. f.) values obtained during the column chromatography of *Smilax zeylanica* L. Moreover, the mobile phases, fraction details, and retention factor (R.f.) values observed during the column chromatography analysis of *Berberis aristata* DC. are reported in table 3.

Fraction 7 to 11 for hydroalcoholic extracts of *Smilax zeylanica* L. and Fraction 6 to 10 for hydroalcoholic extracts of *Berberis aristata* DC were eluted and used for further investigation by GC-MS analysis. Selection of the fractions

Table 1: The ability of *Smilax zeylanica* and *Berberis aristata* to eliminate DPPH radicals can be described as their capacity for DPPH radical scavenging.

Percentage Inhibition of DPPH Radical			
Concentration (µg/ml)	Ascorbic acid	<i>Smilax zeylanica</i> L. Extract	<i>Berberis aristata</i> DC. Extract
10	31.33	21	5
20	39.42	28.5	11.3
30	44.41	34.04	19.2
40	51.72	39.9	24.2
50	54.86	43.47	27.1
60	60.5	48.4	31.6
70	65.65	54.43	38.1
80	70.64	59.5	43.33
90	75.044	60.9	48.8
100	78.7	69.7	53.73
IC ₅₀ (µg/ml)	4.104183567	6.288040812	9.257371533

were done the TLC results. Only that fraction will be selected, which exhibited the highest number of spots on the TLC plate. Therefore, it was chosen for additional studies such as GC-MS analysis at the Central Laboratory of Patanjali Food and Herbal Park Pvt. Ltd. located in Haridwar, specifically on Laksar Road in Padartha.

GCMS study of selected fraction of both plant

Figure 1 for GC-MS Study of Selected Fraction of Hydroalcoholic Extracts of *Smilax zeylanica* L. Figure 2 for GC-MS Study of Hydroalcoholic Extracts of *Berberis aristata* DC The chromatograms display the retention times and intensity of the detected compounds in the selected fractions of both extracts. The GC-MS analysis confirms the presence of compounds contributing to antioxidant properties.

Table 2: Hydroalcoholic extracts of *Smilax zeylanica* L. for Column Chromatography.

S. No.	Mobile Phase	% ratio	No. of fraction collected	No. of spots	R. f. value
1	Methanol	100	F1 to F2	1	0.12
2	Methanol: Ethanol	50:50	F3 to F6 (SZ-1)	3	0.80, 0.81
3	Ethyl acetate: Ethanol	50: 50	F7 to F11 (SZ-2)	3	0.22, 0.71, 0.83

In vitro analysis for Anti-bacterial

This section covers the organization of an in vitro antibacterial study using the disc diffusion method for assessing the efficacy of selected samples against *E. faecalis*. Figure 3 representation of antibacterial zones of inhibition for S1, S2, S3, and the positive control.

Determination of Antibacterial activity

Based on the results obtained from the study, when test organism was treated with different amount of sample on agar plate, it was found that Sample S1, S2 and S3 was found not active against the test organism *Enterococcus faecalis* as compared to positive control (Max zone of Inhibition 31.5 mm at 50 µg dose). The zone of inhibition is an area around a disk on an agar plate where no bacterial growth is observed due to the presence of an antimicrobial agent. It is used to

Table 3: Hydroalcoholic extract of *Berberis aristata* DC Column Chromatography.

S. No.	Mobile Phase	% ratio	No. of fraction collected	No. of spots	R. f. value
1	Methanol	100	F1 to F2	1	0.82
2	Ethyl acetate	100	F3 to F5 (BA-1)	2	0.80, 0.81
3	Methanol: Ethanol	100	F6 to F10 (BA-2)	3	0.22, 0.71, 0.83

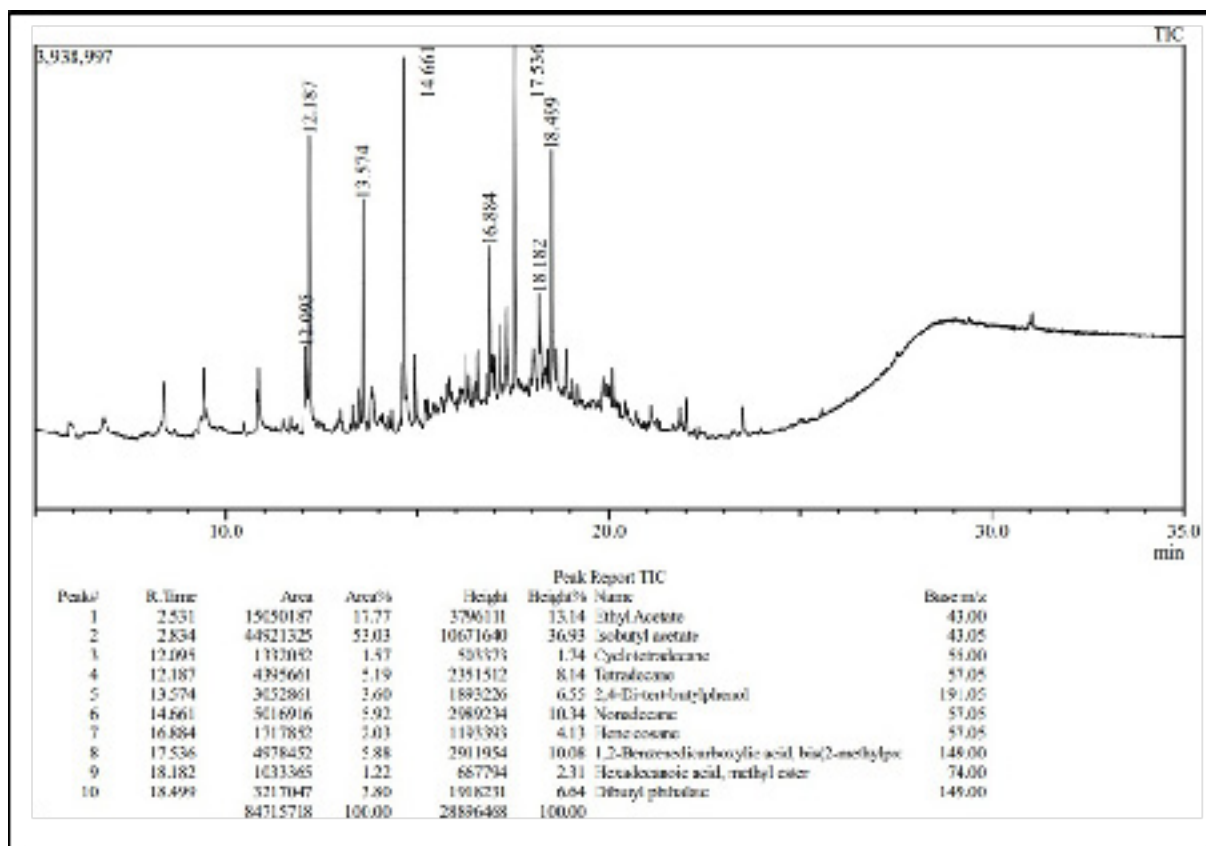


Figure 1: GCMS chromatogram presentation of hydroalcoholic extracts of *Smilax zeylanica* L. fraction.

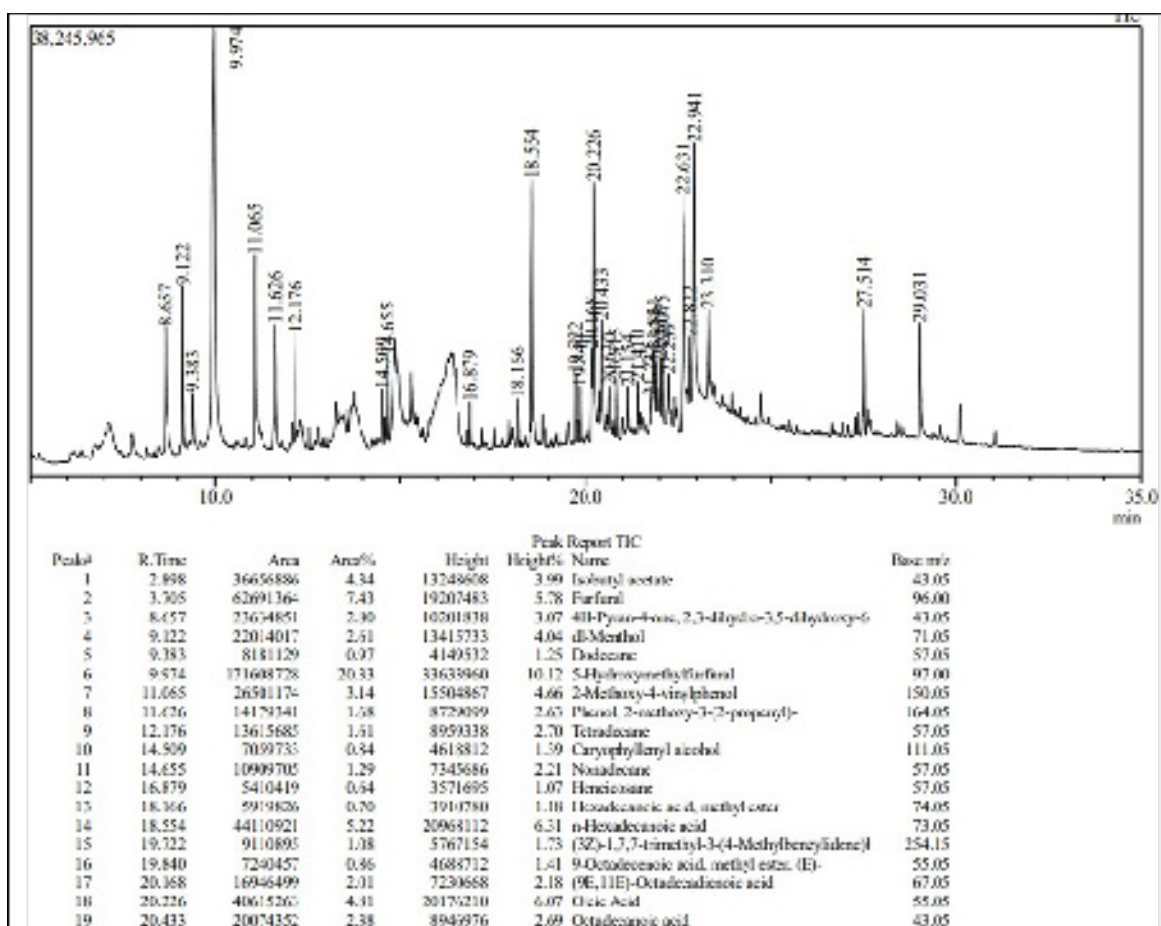


Figure 2: GCMS chromatogram presentation of Hydroalcoholic extracts of *Berberis aristata* DC. Based on the clarification provided by the GC-MS analysis, the hydroalcoholic extract derived from the plant were proven that these have antioxidant properties.

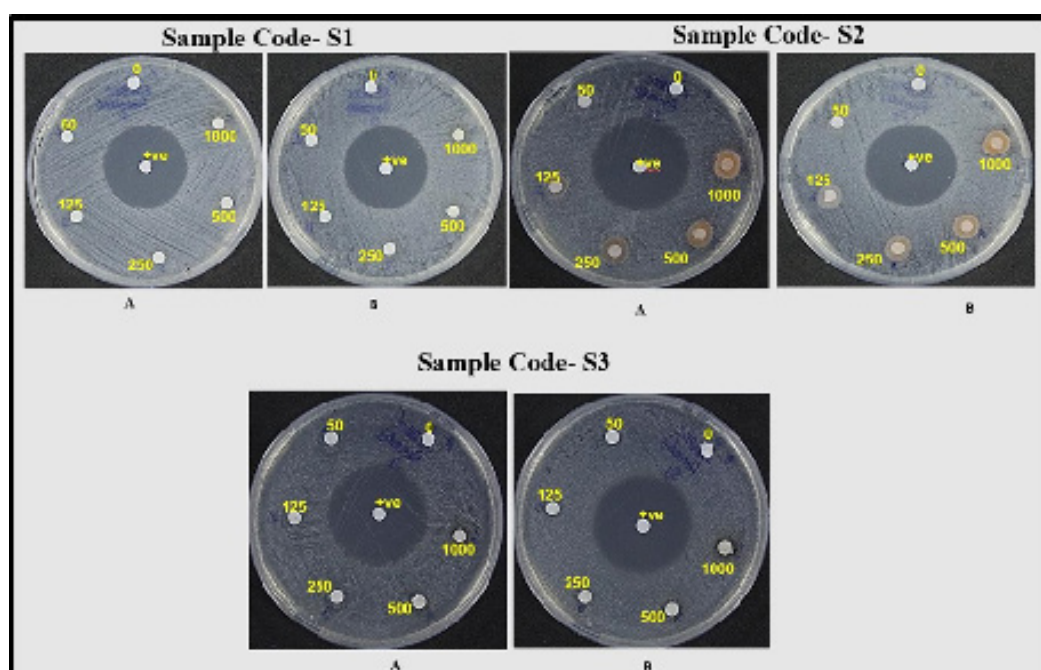


Figure 3: Antibacterial Zone Inhibition Test. Disc Diffusion Method. Test organism- *E. faecalis*. Amount present per disc in µg. Dispensed Volume- 10 µL. Positive Control- 10 µg. Sample code S1, S2 and S3 (Silymarin, Berberis and Smilax).

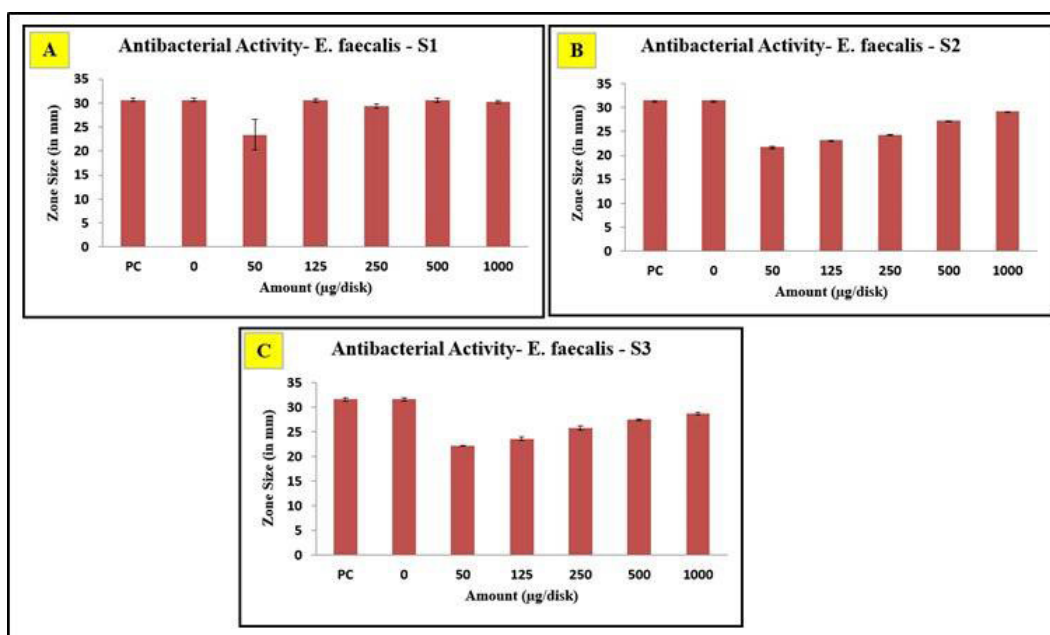


Figure 4: According to the study's findings, when the test organism was exposed to varying amounts of sample on an agar plate, it was discovered that Sample S1 (Silymarin), S2 (Berberis), and S3 (Smilax) exhibited activity toward the evaluate organism *Enterococcus faecalis*. These samples showed greater activity compared to the positive control, with a maximum zone of inhibition of 31.5 mm at a dose of 50 µg. The area of inhibition refers to the region surrounding the disk on a plate of agar where there is no growth of bacteria. This lack of growth is attributed to the fact that of an antimicrobial substance. This method is employed to ascertain the susceptibility of a certain test organism to the effects of a specific antimicrobial drug.

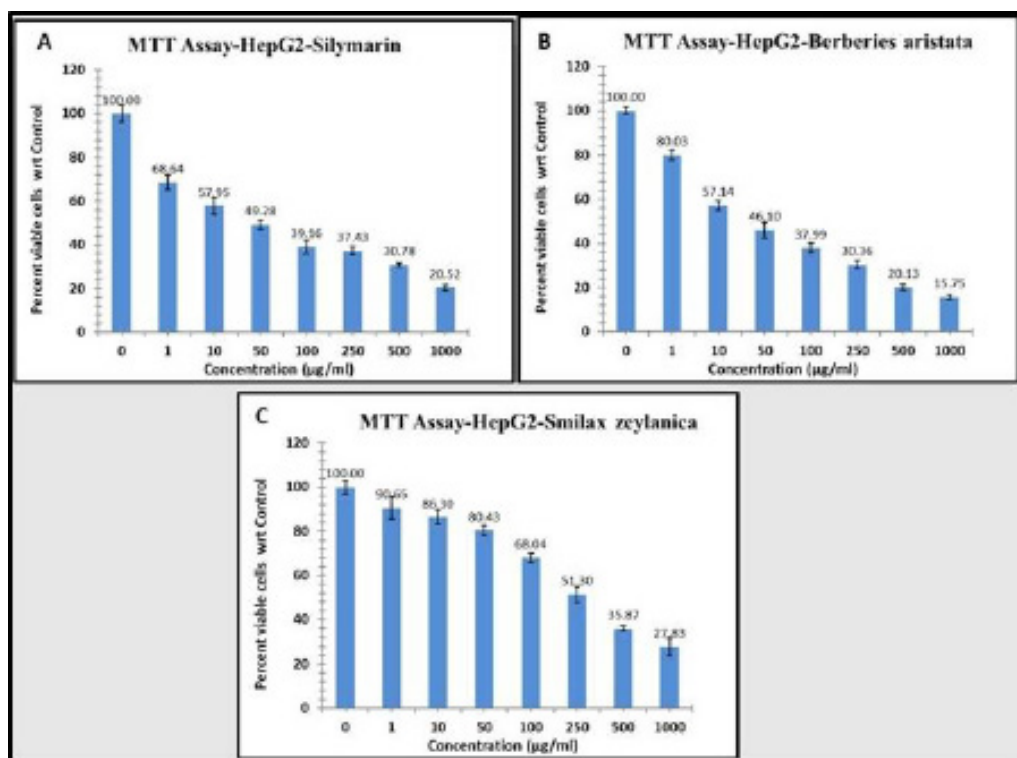


Figure 5: The MTT assay results revealed that the HepG2 cell line exhibited cytotoxic effects in response to various concentrations of the sample. Specifically, the cytotoxicity was observed for Silymarin ($IC_{50} = 64.61 \pm 0.21$ µg/ml), Barberis aristata ($IC_{50} = 47.09 \pm 0.17$ µg/ml), and Smilax zeylanica ($IC_{50} = 259.1 \pm 0.06$ µg/ml), with Barberis aristata demonstrating the highest efficacy among the samples tested. The IC_{50} value represents the concentration at which the number of viable cells is reduced by 50%.

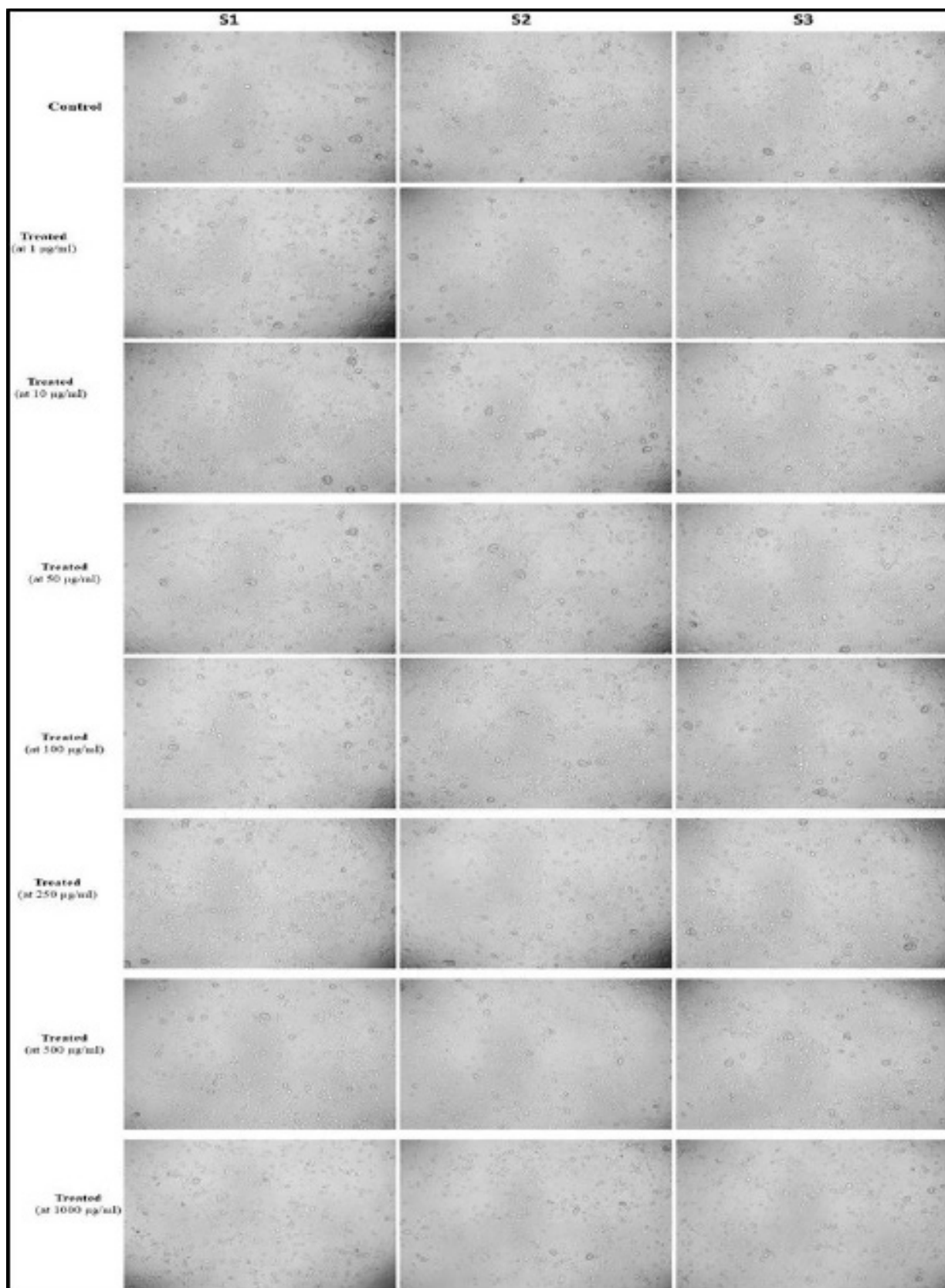


Figure 6: The following figure shows the microscopic images of the test results indicating how various concentrations of the test compounds affect the cell morphology. The images are organized into three columns: S1 would show the results of Silymarin treatment, S2 would reflect the impacts of Berberis, whereas S3 would exhibit the effects of Smilax. The rows correspond to the following conditions:

determine whether a particular test organism is susceptible to the action of a particular antimicrobial agent or not.

Determination of in vitro Hepatoprotective Effect of Compounds on HepG2 cell line

Based on the results obtained from the MTT assay, it was observed that when the cell line was exposed to different concentrations of the sample, cytotoxic activity was observed in samples. *Berberis aristata* was found to be most effective than other samples. The IC_{50} is the concentration of an inhibitor/sample/ formulation at which the viable cells reduced by half.

Microscopic images in Figure 6 showing the effects of various concentrations of test compounds (Silymarin, Berberis, and Smilax) on cell morphology. The images highlight the changes in cellular response and potential cytotoxicity at higher concentrations.

Control: Untreated cells.

- *Treated at 1 µg/ml*

Cells exposed on this case to 1 µg/ml of the test compound.

- *Treated at 10 µg/ml*

Cells receiving the highest concentrations of the test sample, 10 micrograms/ml.

- *Treated at 50 µg/ml*

Cells treated with 50 µg/ml with the test compound.

- *Treated at 100 µg/ml*

Cells exposed to 100 µg/ml of the test compound.

- *Treated at 250 µg/ml*

Based on the used concentration of the test compound 250 µg/ml cells were treated as mentioned above.

- *Treated at 500 µg/ml*

Cells exposed to 500 µg/ml of the test compound.

- *Treated at 1000 µg/ml*

This includes Cells treated with 1000 µg/ml of the test compound.

This format allows seeing the change in the cellular response to the increasing concentrations of the test compounds and comparing their possible cytotoxicity.

Discussion

Ethanol and water of *Smilax zeylanica* L. and *Berberis aristata* DC. were employed to study an in-vitro evaluation of their antioxidant, antibiotics, and liver-protecting effect. These plants were chosen basing on their uses in traditional medicine and the possibilities of having bioactive components. This extraction process wanted to enrich the plants with these phytochemicals that lead to the desired therapeutic effects.³²

Phytochemical screening of the hydro alcoholic of *Smilax zeylanica* L. and *Berberis aristata* DC. Some other standard chemical tests were also performed on the sample and the findings are enlisted in table 1. A study was carried out to determine the occurrence of alkaloids, saponin, tannins, flavonoids, and glycosides.³³

Through the data analysis it was evident that both extracts contained alkaloids and flavonoids with high flavonoids detected in *Smilax zeylanica* L. *Berberis aristata* DC. also revealed reasonable levels of alkaloids and tannins besides the triterpenoids and steroids.³⁴

In this view, column chromatography was used to partition and characterize hydroalcoholic extracts of *Smilax zeylanica* L. and *Berberis aristata* DC. This was done employing varying compositions of mobile phase, to separate various fractions, which were then evaluated for their phytochemical content.^{35, 36}

The objective of the present research was to assess the effectiveness of three samples; Silymarin (S1), Berberis (S2), and Smilax (S3) against *E. faecalis* through the Disc Diffusion Method and the results were presented in Figure 4. The findings of this research pointed to the fact that all the prepared samples possessed excellent antibacterial efficacy and potency compared with positive control at 10 µg, which has manifested a maximum zone of inhibition of 31mm.^{37, 38, 39}

The zone of inhibition is a crucial indicator of an antimicrobial agent's effectiveness, representing the area around a disc on an agar plate where bacterial growth is inhibited due to the presence of the agent. In this study, the observed zones of inhibition for S1, S2, and S3 suggest that these samples possess antimicrobial properties against *E. faecalis*. The effectiveness of the samples varied, with specific concentrations likely influencing the size of the inhibition zones observed. It may be concluded that Silymarin, Berberis, as well as Smilax could be used as potential natural antimicrobials. It is crucial to comprehend their effectiveness against *E. faecalis* due to the constantly growing problem of antibiotic resistance. The stringer research could investigate the best concentrations and preparations to improve their efficacy and extend the usage in medical and treat therapeutic processes. Hepatoprotective potentials of the Silymarin, Berberis aristata, and Smilax zeylanica were examined on HepG2 cell line employing MTT assay and the results were presented in Figure 5. The results showed varying degrees of cytotoxic activity at different concentrations, Silymarin: IC_{50} was 64.61 ± 0.21 µg/ml, *Berberis aristata*: Determined IC_{50} = 47.09 ± 0.17 µg/ml (most effective), *Smilax zeylanica*: IC_{50} = 259.1 ± 0.06 µg/ml.^{40, 41, 42}

The IC_{50} values show the concentration of the tested samples at which the living cell proportion was cut in half. Hepatoprotective activity increases with the decreasing values of IC_{50} . Among all the samples, *Berberis aristata* had the highest hepatoprotective activity and therefore it is regarded as the most prospective for further investigations related to the liver and disease prevention. Microscopic

part offered documentation of the impact of concentration differentiation of S1 (Silymarin), S2 (Berberis), and S3 (Smilax) on HepG2 cell structure. The images represented the morphological and optical density alterations of the cell structure in the not treated group and the groups treat with different concentrations of the subjected samples of up to 1000 µg/ml.^{43, 44, 45}

Conclusion

The results from both the experiment discussions underscore the potential of Silymarin, *Berberis aristata*, and *Smilax zeylanica* to be effective against *E. faecalis* as antimicrobial as well as cytoprotective against the cytotoxic effect of HepG2 cells. They also require more detailed studies about their action mechanisms, therapeutic scores, and possible uses in the management of bacterial diseases and liver disorders. It is for these reasons that these natural compounds are seen to offer solutions to modern-day diseases such as antibiotic resistance and liver illnesses through their many activities.

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Author contributions

Monika Singh was responsible for conceptualization, methodology, investigation, and the initial draft, which included software and data analysis. Manish Kumar Kaushik and Sadish Kumar Shanmugam managed the resources and oversaw the investigation. Nitin Kumar and Moumita Barman conducted the manuscript's review and editing. All the authors collectively assumed responsibility for all aspects of the project, including addressing any inquiries regarding the precision and honesty of every element of the effort and ensuring their thorough examination and resolution.

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Competing Interests

The authors have no relevant financial or nonfinancial interests to disclose.

Data availability statement

Manuscript has no associated Data.

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