Section A-Research Paper

Comparative Analysis of Phenolic Content and Antimicrobial Activity of Herbal Extracts from *Calotropis gigantea* and *Achyranthes aspera*.

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ABSTRACT

The medicinal applications of plant-based secondary metabolites have been extensively documented due to their perceived affordability, non-toxicity, and safety for human use. With the global concern over microbial resistance to antibiotics, there is a pressing need for the discovery of novel and potent antimicrobial agents. Therefore, this study aims to compare the antimicrobial activities of different solvent extracts from *Calotropis gigantean* and *Achyranthes aspera* and to investigate the effect of the total phenol content present in the extracts on their antimicrobial activity. The study utilized the agar disc diffusion method to assess the antimicrobial activity of various solvent extracts against *Escherichia coli* and *Bacillus subtilis*. Additionally, the minimal inhibitory concentration and minimum bactericidal concentrations were estimated using the liquid microdilution assay. The Folin-Ciocalteu method was used to determine the total phenol content. All of the extracts demonstrated significant antimicrobial activity against *E. coli* and *B. subtilis*. The methanol extracts from both plants showed the highest activity against both bacterial strains, and had the highest phenol concentration. The MBC/MIC ratio indicated that all of the extracts exhibited bactericidal activity. These findings suggest that the leaves of these plants, which contain significant antimicrobial properties, may be a valuable source of natural antimicrobials.

Key Words: *Achyranthes aspera*, *Calotropis gigantean*, Comparative, Antimicrobial activity, Bactericidal, Total phenol, Medicinal plants

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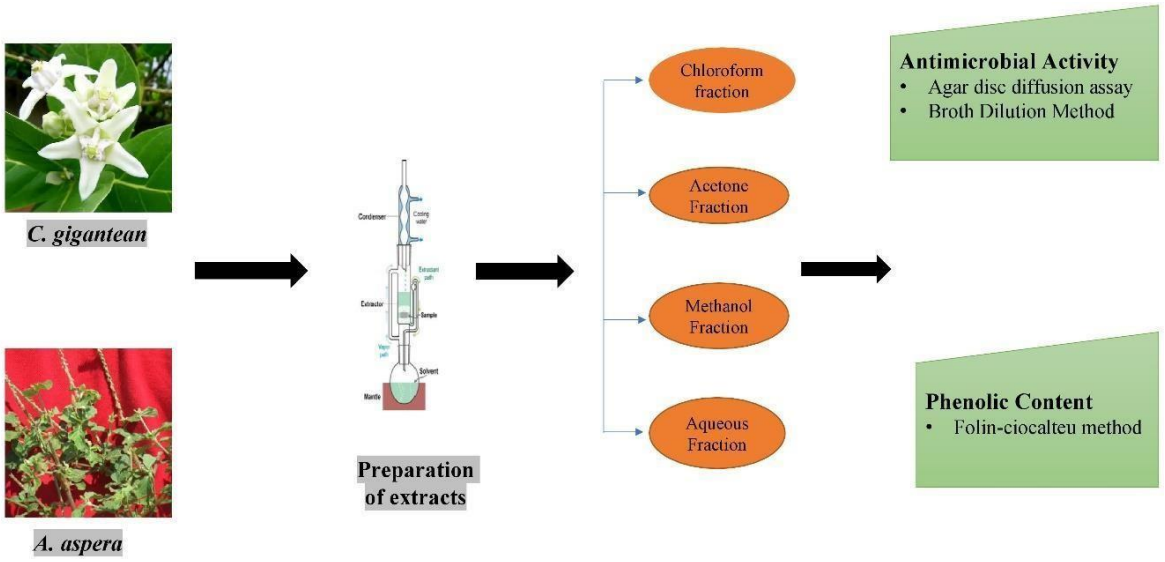


1. INTRODUCTION

Plants have been revered for centuries as a rich source of therapeutic compounds, serving as a critical contributor to traditional and modern medicine, folk medicine, food supplements, nutraceuticals, pharmaceutical intermediates, and chemical entities for drug discovery [1]. They have been used as therapeutic agents since the dawn of human civilization due to their affordability, non-toxicity, and safety for human use. Bioactive principles derived from plants, such as secondary metabolites, have long been incorporated into conventional healthcare systems as natural defenses against microorganisms, insects, and higher predators [2, 3, 4]. However, with the emergence of antibiotic-resistant bacterial also called multi-drug resistant (MDR) bacteria, it has become increasingly important to explore natural alternatives that can combat this condition without causing harmful side effects. The potential of plant-based drugs as a promising solution lies in the antibacterial bioactive compounds they contain, which have been found to be highly effective with minimal or no side effects compared to synthetic antibiotics [5].

In the pursuit of discovering novel antimicrobials from nature, we studied two plants, *Achyranthes aspera* and *Calotropis gigantean. A. aspera* Linn., belonging to Amaranthaceae family, commonly known as “Devil's Horsewhip” is a medicinal herb widely distributed in tropical regions. It has been traditionally used for various purposes, including treating malaria symptoms, as well as possessing antiperiodic, diuretic, laxative, hepatoprotective, anti-allergic, and antiasthmatic properties [6, 7]. On the other hand, *C. gigantean* commonly known as “Giant Milkweed” from the Asclepiadaceae family is a laticiferous shrub found primarily in India, Bangladesh, Pakistan, Burma, and the sub-Himalayan tract [8]. It has been used in traditional medicine to treat a variety of diseases, including fevers, indigestion, rheumatism, colds, coughs, asthma, eczema, elephantiasis, nausea, vomiting, leprosy, and diarrhea [9]. Scientifically, *C. gigantean* has been reported for its cytotoxic, wound healing and antipyretic activities [10].

This study aims to evaluate the antibacterial activity *A. aspera* and *C. gigantean* leaf extracts against clinically isolated as well as standard microbial cultures of *E. coli* and *B. subtilis*. The efficacy of the extracts was compared with that of the commercially available antibiotic gentamycin. To determine the underlying mechanism of action for the antibacterial properties of the extracts, the total phenol content, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) were determined. These parameters are important indicators of potential antibacterial activity and can aid in the development of alternative or complementary antimicrobial agents.

2. METHODS

2.1 Chemicals

Methanol, Acetone, Chloroform were obtained from Rankem Pharmaceutical Company, Haryana; Beef extract, Peptone, Sodium Chloride, Tween 80, Folin-Ciocalteu reagent, Sodium Carbonate, Gallic acid were procured from Central Drug House Pvt. Ltd., New Delhi, India; Agar was obtained from Fischer Scientific and Mueller Hinton agar medium was purchased from HIMEDIA laboratories Pvt. Ltd., Mumbai, India. All the chemicals used in this study were of analytical grade.

2.2 Plant Material collection

Leaves of *A. aspera* (AA) and *C. gigantean* (CG) were collected from the local area of Modinagar, India. The identification and authentication of plant materials was done by Dr. D. K. Awasthi, Professor of Botany at Multanimal Modi College, Modinagar. A specimen of plant material has been preserved in the Botany Department of M. M. College, Modinagar, Meerut, India.

2.3 Preparation of extracts

Leaves were washed with double distilled water, shade dried and grounded to a coarse powder. The 10 grams of powder were extracted separately with 250 ml chloroform, acetone, methanol, and water in Soxhlet for 24 hours. Solvents were evaporated under vacuum by Buchi type rotary evaporator at 40^0C. Concentrated extracts were kept in the desiccator until constant weight was obtained. Extracts were stocked at 4ºC for further use [11]. The % yield of extracts was calculated [12]. Tween 80 and distlled water in 1:9 ratio were used to prepare specific concentration (w/v) of extracts for biological experiments.

2.4 Bacterial strains

Lyophilized samples of *E. coli* and *B. subtilis* were collected from MTCC (Microbial Type Culture Collection and Gene Bank), Chandigarh, India and were maintained in nutrient broth (NB) at 40°C. *E. coli* was precultured in NB for 12-18 hours in a shaker incubator at 37°C, and *B. subtilis* at 30°C. Growth of both cultures was monitored spectrophotometrically until absorbance at 600nm reached about 0.600 [13].

2.5 Antimicrobial assay

The agar disk diffusion technique was employed to conduct antimicrobial testing of the plant extracts [14, 15]. Various concentrations of the extracts, including 1mg/ml, 5mg/ml, 10mg/ml, 15mg/ml, and 20 mg/ml, were tested. In sterile Petri plates, 20 ml of molten NAM (Nutrient Agar Media) was seeded, and after solidification, selected strains were swabbed onto the media. Sterile filter paper discs of 5.0 mm diameter were soaked with 15 µl of plant

extract of varying concentrations and placed on the agar plates swabbed with bacteria. The inoculated plates were incubated for two days at 37°C for *E. coli* and 30°C for *B. subtilis*, after which the zone of inhibition (ZOI) was measured. Gentamycin was utilized as the control.

2.6 Minimum Inhibitory Concentration (MIC)

The micro dilution method was utilized to measure the minimum inhibitory concentration (MIC) by using serial dilutions of the plant extracts [16]. The four solvent extracts from both plants were diluted serially in test tubes to obtain concentrations ranging from 0.5 mg/ml to 10 mg/ml. Fresh nutrient broth was used as a diluent. A sterile suspension of 50 µl of standard culture inoculum was added to the broth dilutions, and they were incubated at 37°C for 18 hours. The MIC was determined as the lowest concentration of extracts that did not exhibit any visible growth [17].

2.7 Determination of Minimum Bacterial Concentration (MBC)

MBC values of samples were determined by subculturing the samples that exhibited no visible bacterial growth in MIC assay. For this purpose, the test tube samples which did not show any bacterial growth were streak-seeded on the surface of the Muller-Hinton agar (MHA). The Petri dishes were incubated at 35 ± 2 °C for 18-24 hours and examined for bacterial growth. The lowest concentration of plant extracts, which did not show any bacterial growth on the subculture, was recorded as the MBC [18].

2.8 Determination of total phenol content (TPC) in plant extract

The spectrophotometric method was employed to determine the total phenol content in various solvent extracts [3]. 0.1ml plant extracts (1 mg/ml) were diluted to 1 ml with double distilled water (DDW) and subsequently mixed with0.5 ml of FC reagent (1:1) and 1.5 ml sodium carbonate (0.2 g/ml). The mixture was then allowed to stand at 25^0Cfor 2 hours. After the incubation period, 7.0 ml DDW was added to each sample, and the absorbance was recorded at 765nm.To determine the amount of phenol in each extract, a standard curve was constructed using gallic acid, and the overall amount of phenol present was expressed as mgGAE/g PM (Gallic Acid Equivalent/g Plant Material).

2.9 Statistical analysis

The results presented in this study are reported as the mean ± standard deviation of three replicates. The data were subjected to analysis using one-way ANOVA, and p-values < 0.05 were considered statistically significant. Graphical presentation was created using MS Office.

3. RESULTS

3.1 Percent yield of different plant extracts

Different solvents with varying polarity indexes, namely water (aqueous), methanol, acetone, and chloroform, were employed to extract and isolate a diverse range of phytochemicals from the plant samples. Results in Table 1 shows the highest extraction yield was obtained with water, which had the highest polarity index (27.03% for AA and 36.70% for CG), followed by methanol (11.3% for AA and 22.87% for CG), chloroform (4.2% for AA and 9.83% for CG), and acetone (3.2% for AA and 8.16% for CG). These findings suggest that the extraction efficiency of the plant extracts is influenced by the polarity index of the solvents used.

Table 1. %Yield of different extracts of plants

Solvent extracts	Percent yield (%)	
	Achyranthes aspera	*Calotropis gigantean*
Acetone	3.2±0.010	8.16±0.001
Chloroform	4.2±0.055	7.83±0.007
Methanol	11.3±0.050	22.87±0.094
Aqueous	27.03±0.080	36.7±0.065

Note: Data given are mean of three replicates ±SE, P< 0.005

3.2 Antimicrobial activity of plant extracts

Table 2 demonstrates that all extracts from both A. aspera and C. gigantean plants exhibited significant antimicrobial activity against both bacterial strains. A concentration-dependent response was observed in all extracts tested. The methanol extract of both plants demonstrated the highest activity against *E. coli* and *B. subtilis.*

Table 2. Antibacterial activity of solvents extracts of *A. aspera* and *C. gigantean* against different bacterial strains

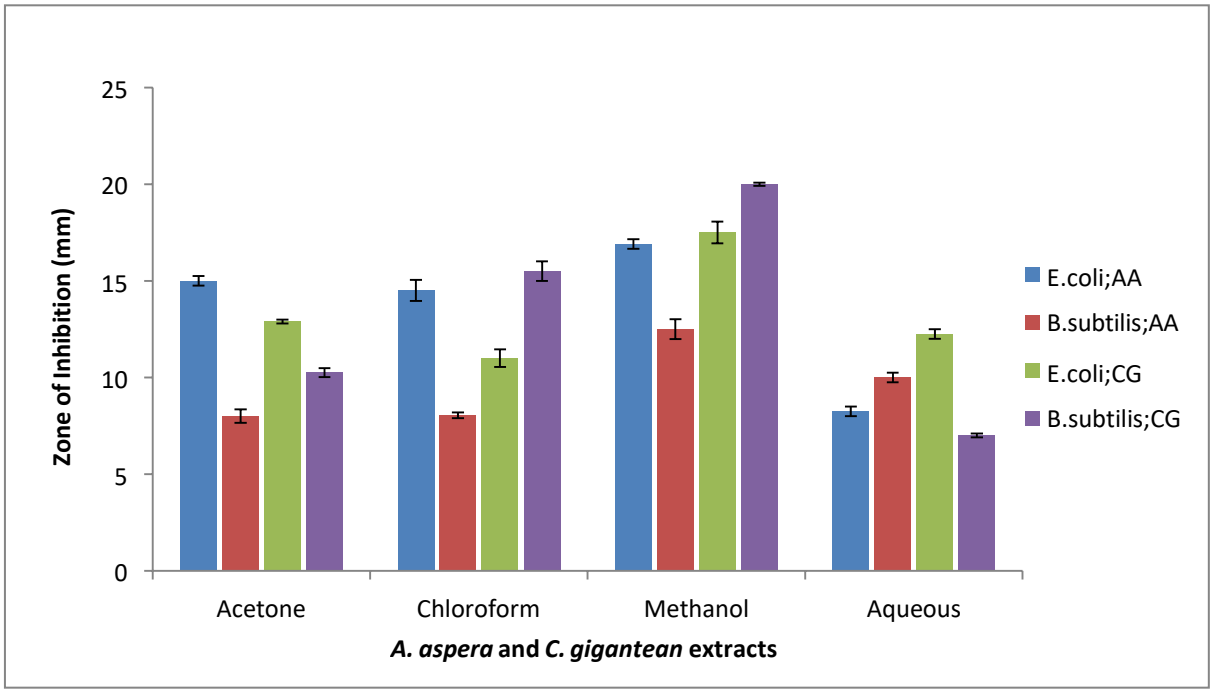
Plant extracts	Concentration	*E. coli* Zone of Inhibition (in mm) ± SD				
		Acetone	Chloroform	Methanol	Aqueous	Gentamycin
A. aspera	20 mg/ml	15.00±0.251	14.50±0.542	16.90±0.250	08.25±0.251	23.00±0.001
	15 mg/ml	10.75±0.190	10.00±0.001	13.00±0.109	08.00±0.052	18.50±0.250
	10 mg/ml	08.75±0.255	07.50±0.502	10.50±0.502	07.25±0.205	10.50±0.500
	5 mg/ml	07.25±0.236	06.10±0.100	08.00±0.055	05.75±0.321	09.00±0.001
	1 mg/ml	00.54±0.961	00.10±0.850	00.75±0.255	00.07±0.502	08.05±0.050
C. gigantean	20 mg/ml	12.90±0.100	11.00±0.461	17.50±0.560	12.25±0.250	23.00±0.001
	15 mg/ml	09.50±0.502	07.50±0.510	10.50±0.050	08.50±0.050	18.50±0.250
	10 mg/ml	07.25±0.021	06.50±0.530	08.50±0.520	06.00±0.001	10.50±0.500
	5 mg/ml	04.25±0.250	06.00±0.001	06.50±0.500	06.00±0.001	09.00±0.001
	1 mg/ml	02.25±0.250	04.50±0.250	05.50±0.601	03.50±0.570	08.05±0.050
		B. subtilis Zone of Inhibition (in mm) ± SD				
A. aspera	20 mg/ml	08.00±0.350	08.05±0.150	12.50±0.521	10.00±0.251	22.00±0.001
	15 mg/ml	07.00±0.257	07.25±0.252	10.00±0.001	08.00±0.352	18.00±0.001
	10 mg/ml	06.00±0.001	06.50±0.723	08.10±0.100	05.00±0.505	09.00±0.050
	5 mg/ml	04.00±0.500	04.50±0.500	07.00±0.001	04.00±0.431	08.00±0.001
	1 mg/ml	00.06±0.500	00.07±0.502	05.00±0.001	00.05±0.810	07.00±0.020
C. gigantean	20 mg/ml	10.25±0.230	15.50±0.503	20.00±0.081	07.00±0.100	22.00±0.001
	15 mg/ml	08.00±0.050	08.10±0.050	10.00±0.001	06.00±0.250	18.00±0.001
	10 mg/ml	06.00±0.150	06.90±0.001	08.25±0.250	02.00±0.300	09.00±0.050
	5 mg/ml	03.75±0.250	04.25±0.250	06.00±0.001	00.50±0.200	08.00±0.001
	1 mg/ml	00.50±0.500	00.55±0.500	01.00±0.881	00.10±0.500	07.00±0.020

Note: Data given are mean of three replicates ±SE, P< 0.005

Specifically, at a concentration of 20 mg/ml, the methanol extract of AA exhibited a ZOI of 16.90±0.250 mm against *E. coli*, while the acetone, chloroform, and aqueous extracts had ZOIs of 15.00±0.251, 14.50±0.542, and 08.25±0.251 mm, respectively. For CG, the methanol extract displayed a ZOI of 17.50±0.560 mm, followed by acetone, aqueous, and chloroform extracts with ZOIs of 12.90±0.100, 12.25±0.250, and 11.00±0.46 mm, respectively.

Against *B. subtilis* at the concentration of 20 mg/ml, the methanol extract of AA showed a ZOI of 12.50±0.521 mm, while the aqueous, chloroform, and acetone extracts exhibited ZOIs of 10.00±0.251, 8.05±0.150, and 8.00±0.350 mm, respectively. The methanol, chloroform, acetone, and aqueous extracts of CG showed ZOIs of 20.00±0.081, 15.50±0.503, 10.25±0.230, and 7.00±0.100 mm, respectively.

Figure 1 presents a comparison of the antibacterial activity of both plants, indicating that CG exhibited stronger antimicrobial activity against both bacterial strains than AA. These results were compared with standard gentamycin, which displayed a ZOI of 23.00±0.001 and 22.00±0.001 mm against *E. coli* and *B. subtilis*, respectively. Correlation and regression analyses revealed that the antimicrobial activity of the plant extracts was concentration dependent.



Note: AA; *Achyranthes aspera*, CG; *Calotropis gigantean*, Extract conc. 20mg/ml

Figure 1: Antibacterial activity of different solvent extracts of *A. asper*, *C. gigantean*, and gentamycin against *E. coli* and *B. subtilis.*

3.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of plant extracts

To evaluate the antimicrobial efficacy of various plant extracts, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined against each bacterium. The MIC is the lowest concentration of plant extract that inhibits the growth of the tested microbe after overnight incubation, while the MBC is the lowest concentration of antibacterial substance required to produce a sterile culture [19]. After

determining the MICs, the MBC was evaluated, and the MBC/MIC ratio was calculated to describe the antibacterial activity of plant extracts as bacteriostatic or bactericidal. A ratio ≤ 4 is considered bactericidal, while a ratio > 4 is considered bacteriostatic [20]. All results were compared to the standard antibiotic gentamicin.

The MIC and MBC values of various plant extracts against *E. coli* and *B. subtilis* are presented in Table 3 and Table 4. The results showed that the methanol extracts had the lowest MIC values, consistent with the ZOI results. Specifically, the methanol extract *A aspera* had a MIC value of 2.00 mg/ml against *E. coli*, while the methanol extract of *C. gigantean* had a MIC value of 0.50 mg/ml. For *B. subtilis*, the methanol extracts of *A. aspera* and *C. gigantean* had MIC values of 0.75 mg/ml and 2.00 mg/ml, respectively. The MBC/MIC ratio for all solvent extracts from both plants showed a bactericidal effect against both bacterial strains, indicating their effectiveness in killing the bacteria. These findings suggest that *A. aspera* and *C. gigantean* could potentially be used as natural antibacterial agents.

Table 3. MIC and MBC values of *A. aspera* extracts against *E. coli* and *B. subtilis*

Bacterial strain	Extracts	*A. aspera*			
		MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	Effect
E. coli	Acetone	3.00±0.451	5.00±0.543	1.6	Bactericidal
	Chloroform	3.00±0.620	5.00±0.221	1.6	Bactericidal
	Methanol	2.00±0.534	3.00±0.532	1.5	Bactericidal
	Aqueous	4.00±0.287	7.00±0.756	1.75	Bactericidal
	Gentamycin	0.50±0.411	1.00±0.501	2	Bactericidal
B. subtilis	Acetone	5.00±0.543	7.00±0.271	1.4	Bactericidal
	Chloroform	4.00±0.750	6.00±0.432	1.5	Bactericidal
	Methanol	0.75±0.386	2.00±0.366	2.6	Bactericidal
	Aqueous	5.00±0.350	10.00±0.624	2	Bactericidal
	Gentamycin	0.50±0.502	1.00±0.521	2	Bactericidal

Note: Data given are mean of three replicates ±SE, P< 0.005

Table 4. MIC and MBC values of *C. gigantean* extracts against *E. coli* and *B. subtilis*

Bacterial strain	Extracts	*C. gigantean*			
		MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	Effect
E. coli	Acetone	2.00±0.510	6.00±0.850	3	Bactericidal
	Chloroform	0.75±0.102	2.00±0.420	2.6	Bactericidal
	Methanol	0.50±0.220	1.00±0.565	2	Bactericidal
	Aqueous	1.00±0.345	4.00±0.323	4	Bactericidal
	Gentamycin	0.50±0.411	1.00±0.501	2	Bactericidal
	Acetone	5.00±0.723	8.00±0.030	1.6	Bactericidal

B. subtilis	Chloroform	4.00±0.278	7.00±0.048	1.75	Bactericidal
	Methanol	2.00±0.623	5.00±0.320	2.5	Bactericidal
	Aqueous	10.00±0.742	14.00±0.046	1.4	Bactericidal
	Gentamycin	0.50±0.502	1.00±0.521	2	Bactericidal

Note: Data given are mean of three replicates ±SE, P< 0.005

3.4 Total phenolic content

The results presented in Figure 2 indicate the phenolic content in all extracts under investigation. Methanol extracts of both plants exhibited the highest concentration of phenols, followed by chloroform, acetone, and water (aqueous) extracts. The trend of phenol concentration in different solvent extracts was nearly identical in both plants. Additionally, the data showed that the concentration of phenols in all solvent extracts of *C. gigantea* was greater than that in *A. aspera*.

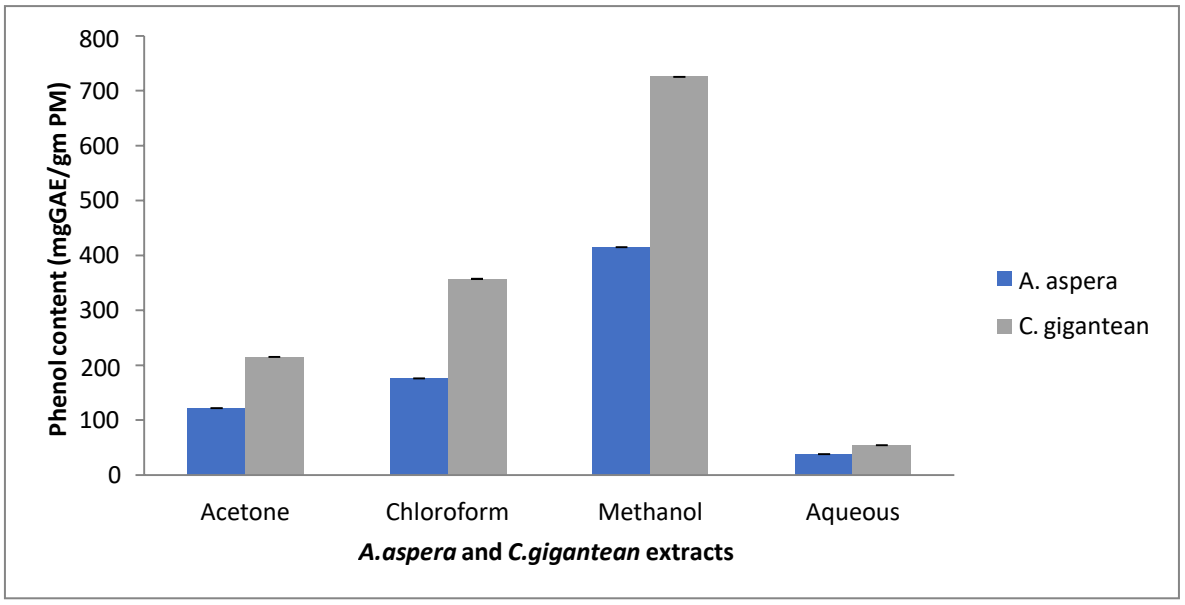


Figure 2: Total phenolic content in various extracts of *A. aspera* and *C. gigantean*

3.5 Correlation between total phenolic content and antimicrobial activity

The study evaluated the total polyphenol content of various solvent extracts from both plants, as many phenolic compounds have been shown to possess significant antibacterial activity in addition to their established antioxidant activity. As shown in Figure 2, our findings indicate that the total phenolic content in *C. gigantean* leaves was higher compared to *A. aspera* leaves in all the solvent extracts. Interestingly, a correlation was observed between the polyphenol content and the antibacterial activity, as evidenced by the zone of inhibition (Table 5). The methanol extract of *C. gigantean* displayed the highest concentration of polyphenols (725±0.002 mg GAE/gm PM), which resulted in a greater ZOI against both *B. Subtilis* (20.00±0.081 mm) and *E. coli* (17.50±0.560 mm). The methanol extract of *A. aspera* also exhibited a similar trend, but the ZOI values against *B. Subtilis* (12.50±0.521 mm) and *E. coli* (16.90±0.250 mm) were lower due to the lower amount of polyphenols (415±0.93 mgGAE/gm PM) compared to *C. gigantean*. This finding suggests that polyphenols could play a significant role in the antibacterial properties of these plant extracts.

Table 5. Correlation between total phenolic content and Zone of Inhibition.

Plants	Solvent Extracts	Phenol Content (mgGAE/gmPE)	Zone of Inhibition (20mg/ml)	
			E. coli	*B. subtilis*
A. aspera	Acetone	122±0.005	15.00±0.251	8.00±0.350
	Chloroform	176±0.006	14.50±0.542	8.05±0.150
	Methanol	415±0.093	16.90±0.250	12.50±0.521
	Aqueous	31±0.007	8.25±0.251	10.00±0.251
C. gigantean	Acetone	215±0.004	12.90±0.100	10.25±0.230
	Chloroform	357±0.006	11.00±0.461	15.50±0.503
	Methanol	725±0.002	17.50±0.560	20.00±0.081
	Aqueous	54±0.004	12.25±0.250	7.00±0.100

Note: Data given are mean of three replicates ±SE, P< 0.005

4. DISCUSSION

The use of medicinal flora to treat various diseases is well documented in the history of all civilizations. They have been serving as the major sources of therapeutic agents for the maintenance of human health. Apart from small molecules of medicinal chemistry, natural products are still important sources of simple therapeutic agents against various infectious diseases [21].

Antibiotic resistance is a serious problem worldwide. The emergence and spread of multidrug resistant microbes is a challenge for current antimicrobial therapy. Therefore, medicinal plants are an important source for the discovery of new antimicrobial substances [22, 23]. In this direction, this study has been conducted to evaluate the antimicrobial activity of various solvent extracts of two medicinal plants *A. aspera* and *C. gigantean.* Both plants are reported to have many medicinal properties and have been used in traditional medicine [6, 7, 9, 24, 25]. The present study was primarily designed to explore the antibacterial potential of the phytochemicals present in the leaves of both these plants. Thus, four different solvents (chloroform, acetone, methanol, and water) were used to prepare plant extract.

Polarity of organic solvents play an important role in extraction of various group of phytochemicals, which exhibit different bioactivity due to their ability to dissolve or diffuse in the media used in evaluating antibacterial activity. In the present investigation, the disk diffusion method was used to evaluate the antibacterial activity of *A. aspera* and *C. gigantean* extracts. The results of this study showed that the methanol extracts of leaves of both plants exhibited the highest antimicrobial potential among all solvent extracts against both bacterial strains. The aqueous extract at the same time showed the least antimicrobial activity in our study. Reports in the literature also suggest that methanol is a better solvent for the extraction of antimicrobial compounds from medicinal plants [26, 27]. All extracts exhibited concentration dependent antibacterial activity.

The study was further extended to evaluate the MIC, MBC concentrations of all extracts. Based on individual extract and tested bacterial strain, MIC values for *A. aspera* ranged from

0.75 mg/ ml to 5.00 mg/ml and for *C. gigantean* this value ranged between 0.50mg/ml to 10.00 mg/ml. The MBC values were obtained in the range of 1.00 mg/ml to 14.00 mg/ml. At the same time, the MBC/MIC ratio demonstrated that both plant extracts are bactericidal in nature. This study exhibited that all extracts of both plants have the ability to kill both gram-negative (*E. coli*) and gram-positive (*B. subtilis*) bacteria.

It is well documented in literature that polyphenols possess antibacterial property [28]. Thus, total phenolic content was also measured in all extracts of both plants. Total phenolic content ranged from 38.00 to 725 mgGAE/gm PM. Highest phenolic content was observed in methanol extracts of both plants. We also observed a direct correlation between polyphenol concentration and antibacterial activity with methanolic extracts of both plants. Whereas, acetone, chloroform and aqueous extracts did not show such correlation. Thus, it was confirmed that antimicrobial activity does not necessary be credited only to total phenolic concentrations [29]. Other bioactive secondary metabolites are also known for their antimicrobial activity [3, 30, 31].

The results of this study revealed antimicrobial potential of both plants against gram-negative *E. coli* and gram-positive *B. subtilis* and indicated the presence of broad spectrum of antibiotic phytoconstituents.

5. CONCLUSION

In present study we have evaluated the antimicrobial of the leaf extracts of both selected medicinal plants namely *A. aspera* and *C. gigantean*. Results indicate that all solvent extracts of both plants possess significant antimicrobial activity at tested concentrations. The data obtained in this study provides a scientific validation for the traditional uses of these plants in cold, cough, wound healing and various other ailments. Thus, leaves of *A. aspera* and C. gigantean can be a promising source for new antimicrobial agents. Further studies on active principals may also increase the efficacy of these findings.

6. ACKNOWLEDGEMENTS

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7. CONFLICTS OF INTEREST

The authors declare no conflicts of interest relevant to this article.

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