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Comparative Analysis of Antimicrobial Activity of the Extracts of *Senna alata* and *Glycyrrhiza glabra* Against Bacterial Pathogens

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ABSTRACT

The misuse of antibiotics has increased the resistance in bacteria against them, which has resulted in the need to develop other sources of antibiotics that should have the ability to kill microbes without giving them the chance to develop resistance against them. **Objective:** To evaluate and compare the antimicrobial activity of methanolic, ethanolic, and chloroform extracts of *Senna alata* and *Glycyrrhiza glabra* against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*, and to assess their potential as alternative agents for the management of bacterial infections. **Methods:** In the current study, antimicrobial activity of methanolic, ethanolic, and chloroform extracts of *Senna alata* and *Glycyrrhiza glabra* was assessed against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. After the collection of plant samples, extracts were prepared by the cold maceration method in laboratory research using different solvents. Further antimicrobial effects of all extracts were determined by using the agar well diffusion assay. **Results:** In contrast, chloroform extracts showed variable activity; *G. glabra* chloroform extract exhibited notable activity against *S. aureus* (18 mm), while *S. alata* chloroform extract showed the lowest overall activity, indicating a species-dependent efficacy of non-polar extracts. **Conclusions:** Chloroform extracts had not shown remarkable activity against any pathogenic strains. According to the above results, it can be deduced that *S. alata* and *G. glabra* can be used to treat various bacterial infections in the future, and they have the potential to serve as complementary or alternative agents that could help reduce the over-reliance on conventional antibiotics.

INTRODUCTION

Antibiotics, drugs, are antimicrobial in nature. These biologically active molecules have been used for decades to combat bacterial infections in humans [1]. But over a period of time, the misuse and excessive use of antibiotics have resulted in the development of antibiotic-resistant bacteria, rendering many of the currently available drugs less effective against bacterial diseases. Antibiotic resistance and the development of multidrug-resistant bacterial strains have become a serious problem nowadays. This has ultimately become really common in hospitals and risks hampering the global control of infectious diseases [2]. In this challenging situation, the focus on the investigation of medicinal plants as alternative sources of antibacterial agents has been accelerated [3]. Traditionally, medicinal plants have been a rich source of various therapeutically active molecules for ages. These molecules are called phytochemicals, produced by plants deliberately as secondary metabolites to survive the harsh environmental conditions such as biotic stress [4]. Plants contain a smaller number of

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Phytochemicals in comparison to carbohydrates, proteins, and fats, and require selective solvents for their extraction [5]. Polar solvents such as methanol, ethanol, ethyl acetate, etc., are used for the extraction of hydrophilic compounds [6]. Different methods are used, such as sonification, heating under reflux, Soxhlet extraction, solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, surfactant-mediated techniques, etc. After these solvents 'extracts of different parts of plants are stored in different manners and then tested for their antimicrobial activity against various bacterial strains. Two medicinal plants, *Senna alata* and *Glycyrrhiza glabra* was used for this study. *Glycyrrhiza glabra*, more commonly known as licorice, is one of the most renowned medicinal plants that has been widely used in various parts of the world and has a history as a traditional medicine dating back over 4,000 years. The roots of this plant are particularly very useful because of their therapeutic effects, such as anti-inflammatory, antiviral, antimicrobial, and anticancerous. The phytochemicals found in the root of Glycyrrhiza are flavonoids, saponins, and phenolic compounds, which main cause behind its medicinal effects. Due to such diverse phytochemical composition, *G. glabra* remains a valuable natural source for the production of plant-based therapeutic products [7]. Whereas, Senna alata, also known as Cassia alata, is of great medicinal potential as its crushed leaves have been a source of several skin diseases like ringworm and eczema treatment. It has been scientifically proven that its extracts have a strong antibacterial and antifungal activity against common pathogens. This therapeutic effect of Senna alata can be credited to its good phytochemical profile, such as phenolics, flavonoids, anthraquinones, and saponins[8].
While the individual antimicrobial properties of *S. alata* and *G. glabra* have been reported, comparative studies evaluating the efficacy of their methanolic, ethanolic, and chloroform extracts under standardized conditions against a common set of bacterial pathogens are scarce. Specifically, a systematic comparison of solvent polarity effects on the extraction of bioactive compounds from these two plants against both Gram-positive and Gram-negative bacteria remains unexplored. This study addressed this gap by providing a direct, comparative analysis under identical experimental conditions. Therefore, the present study aimed to evaluate the antimicrobial activity of methanolic, ethanolic, and chloroform extracts of these plants against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*, and to generate baseline data regarding their effectiveness against these pathogens.

METHODS

An experimental *in vitro* study was conducted from October 2020 to May 2021 at the Research Labs of the Institute of Biochemistry and Biotechnology (IBB), University of the Punjab (PU), Lahore. Pure cultures of *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* were obtained from IBB, PU, Lahore. Senna herb (leaves and stems) and Licorice bark (roots) were procured from a local herbal market in Lahore, Pakistan. The plant specimens were authenticated by a botanist at the Department of Botany, University of the Punjab, where voucher specimens (accession numbers PU-BOT-123 for *S. alata* and PU-BOT-456 for *G. glabra*) were deposited for future reference Maceration is an extractive technique used in this study in which plant material is completely immersed in a solvent in an airtight bottle for a particular time based on plant material and solvent being used. Pure cultures of *B. subtilis* and *S. aureus* were obtained from the Institute of Biochemistry and Biotechnology (IBB), University of the Punjab, Lahore. *E. coli* was procured from the Department of Pathology, General Hospital, Lahore, as it was not available in the IBB collection at the time of the study. All extractions and antimicrobial assays were performed in triplicate (three independent biological replicates) to ensure reproducibility. For the agar well diffusion assay, each extract was tested against each bacterial strain on three separate occasions, with two technical replicates per plate. All extractions and antimicrobial assays were performed in triplicate (three independent biological replicates) to ensure reproducibility. For the agar well diffusion assay, each extract was tested against each bacterial strain on three separate occasions, with two technical replicates per plate. All strains were confirmed for purity and identity using standard microbiological techniques. Senna herb and Licorice bark were ground into powder by pestle and mortar to increase the surface area for extraction. Powdered particles of the samples were sieved, and uniform-sized particles were then stored for the extraction. Chloroform, ethanol, and methanol were used as potential solvents for the extraction of Senna herb and licorice bark. For the present study, 10g of plant samples were taken in a reagent bottle along with 100mL of the solvent. The bottles were kept in the dark by covering them with aluminum foil to avoid degradation of the compounds. The bottles were then placed in a shaking incubator at 37ºC for 5 days. After the extraction process, plant extracts were processed to separate the phytocompounds from the plant wastes and then from the solvent. For the separation of plant waste, the mixtures were passed through muslin cloth. The filtrate was then filtered again through the Whatman filter paper to separate out smaller plant wastes. For the evaporation of the solvents, a water bath was set at the respective

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temperatures of the solvents, and the extract mixtures were placed in the pool. For the evaporation of the methanol, the temperature was set at 67ºC for methanolic extracts. Similarly, for the ethanol and chloroform extracts, the temperature was set at 78ºC and 61ºC, respectively. After the solvent had evaporated, the thick solidified extracts were stored at 4ºC for further analysis. As the extracts are not dried completely, we use liquid nitrogen to freeze them for lyophilization. A negative control consisting of the respective solvent (methanol, ethanol, or chloroform) was included in each assay to account for any solvent-induced inhibition Extracts were lyophilized for 1 day, and thus they are converted to powdered form and stored at -20 ºC. Strains of Gram-positive (*Staphylococcus aureus*), (*Bacillus subtilis*), and Gram-negative (*Escherichia coli*) are used. All extractions and antimicrobial assays were performed in triplicate (three independent biological replicates) to ensure reproducibility. For the agar well diffusion assay, each extract was tested against each bacterial strain on three separate occasions, with two technical replicates per plate. The bacterial strains were provided by the Department of Pathology, General Hospital. The three bacterial strains were sub-cultured overnight at 37 °C on nutrient agar plates, and fresh cultures were used to prepare the inoculum. Antibacterial activity of three different plant extracts (methanolic, ethanolic, and chloroform) was evaluated separately against each bacterial strain using the agar well diffusion method. For each bacterial strain, individual nutrient agar plates were prepared for each extract, so that the effect of all three extracts was tested independently on the same strain. The test organism was uniformly spread on the agar surface using a sterile cotton swab. Three wells of 6 mm diameter were aseptically punched into each plate using a sterile blue tip. Two wells were loaded with 100 µL (1 mg/mL) of the respective plant extract, while the third well contained ampicillin as a positive control. The plates were allowed to stand for 30 minutes to facilitate diffusion of the extracts, followed by aerobic incubation at 37 °C for 24 hours. Antibacterial activity was assessed by measuring the diameter (mm) of the zones of inhibition around the wells.

All data are presented as mean ± standard deviation (SD). Statistical significance was determined using a two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Differences were considered statistically significant at $p<0.05$. All statistical analyses were performed using GraphPad Prism version 8.0.

RESULTS

The antimicrobial activity of plant extracts was assayed in vitro by agar well diffusion method against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. The antimicrobial activity of plant extracts was compared with standard antibiotic ampicillin (1mg/1ml). All the plant extracts, methanolic, ethanolic, and chloroform, have exhibited antimicrobial activity towards each bacterium. The effect of methanolic extracts of both plants and ampicillin can be observed clearly for all the bacterial strains. Susceptibility was determined by measuring the diameters of the zone of inhibition. In the same way, the antibacterial properties of ethanolic and chloroform extracts of both plants were also determined by the same procedure, and a comparative evaluation of their inhibitory capacity against the chosen microorganisms was made (Figure 1).

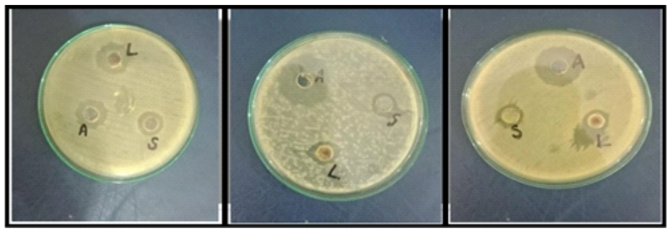

Figure 1: Comparative Antibacterial Effect of Methanolic Extracts of Both Plants and Ampicillin Against Selected Bacterial Strains, Measured as Zones of Inhibition (mm)

The methanolic extracts of the *Cassia alata* (Senna herb) and *Glycyrrhiza glabra* showed a significant antibacterial effect on all the bacterial strains tested. Readings were taken for 3 days. The greatest susceptibility was seen with *Staphylococcus aureus*, where the average zone of inhibition was 18 mm with both plant extracts, which shows a great potential for antibacterial activity. It was moderate towards *Escherichia coli* with the average inhibition areas of 14 mm of *Cassia alata* and 16 mm of *Glycyrrhiza glabra*. Both extracts gave the least inhibition with *Bacillus subtilis* (14 mm). All in all, both Gram-positive and Gram-negative bacteria were susceptible to the methanolic extracts, which proved their wide-spectrum antibacterial properties as previously established (Table 1).

Table 1: Antibacterial Activity of Methanolic Extracts of *Cassia alata* (Senna Herb) and *Glycyrrhiza glabra* Against Selected Bacterial Strains, Expressed as Zones of Inhibition (mm)

Bacterial Strains	*Cassia alata*				*Glycyrrhiza glabra*			
	Day 1	Day 2	Day 3	Average	Day 1	Day 2	Day 3	Average
Staphylococcus aureus	15mm	23mm	16mm	18mm	19mm	19mm	17mm	18mm
Escherichia coli	12mm	15mm	14mm	14mm	17mm	16mm	14mm	16mm
Bacillus subtilis	14mm	15mm	13mm	14mm	13mm	15mm	13mm	14mm

The antibacterial properties of the ethanolic extract of *Cassia alata* and *Glycyrrhiza glabra*. The *Staphylococcus*

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aureus was once again proved to be the most susceptible microorganism with an average zone of inhibition of 17 mm and 18 mm against *Cassia alata* and *Glycyrrhiza glabra*, respectively. The ethanolic extracts exhibited moderate inhibitory activities against *Escherichia coli*, where the average zones of the two plants were 14 mm. Conversely, *Bacillus subtilis* exhibited relatively low sensitivity, especially to *Glycyrrhiza glabra* (12 mm). These results indicate that ethanolic extracts have strong antibacterial properties, but marginally less than methanolic extracts against a few bacterial strains (Table 2).

Table 2: Antibacterial Activity of Ethanolic Extracts of *Cassia alata* (Senna herb) and *Glycyrrhiza glabra* Against Selected Bacterial Strains, Expressed as Zones of Inhibition (mm)

Bacterial Strains	*Cassia alata*				*Glycyrrhiza glabra*			
	Day 1	Day 2	Day 3	Average	Day 1	Day 2	Day 3	Average
Staphylococcus aureus	15mm	20mm	18mm	17mm	19mm	19mm	17mm	18mm
Escherichia coli	15mm	15mm	14mm	14mm	15mm	13mm	14mm	16mm
Bacillus subtilis	14mm	15mm	13mm	14mm	13mm	11mm	12mm	12mm

The antibacterial effect of chloroform extracts of *Cassia alata* and *Glycyrrhiza glabra*. In general, the lowest antibacterial activity of the solvent used was with chloroform extracts of *Cassia alata*, with small areas of inhibition against the *Staphylococcus aureus* (11 mm), *Escherichia coli* (10 mm), and *Bacillus subtilis* (9 mm). Conversely, the chloroform extract of the *Glycyrrhiza glabra* was relatively more active, especially against *Staphylococcus aureus* (18 mm) and *Bacillus subtilis* (16 mm). Nevertheless, chloroform extracts were not as effective as methanolic and ethanolic extracts, which means that a smaller number of bioactive antimicrobial compounds were extracted in the chloroform solvent (Table 3).

Table 3: Antibacterial Activity of Chloroform Extracts of *Cassia alata* (Senna herb) and *Glycyrrhiza glabra* Against Selected Bacterial Strains, Expressed as Zones of Inhibition (mm)

Bacterial Strains	*Cassia alata*				*Glycyrrhiza glabra*			
	Day 1	Day 2	Day 3	Average	Day 1	Day 2	Day 3	Average
Staphylococcus aureus	13mm	12mm	9mm	11mm	19mm	19mm	17mm	18mm
Escherichia coli	10mm	13mm	8mm	10mm	10mm	13mm	14mm	12mm
Bacillus subtilis	8mm	10mm	9mm	9mm	15mm	17mm	16mm	16mm

DISCUSSION

Antibiotics were once thought to be the most effective chemotherapeutic agents against bacterial infections, but overuse and widespread use have resulted in the rapid development of antimicrobial resistance, which presents a significant global public health issue. This increased resistance has aroused the quest to develop alternative antimicrobial drugs, and in this regard, the application of medicinal plant extracts is of interest. Various phytochemicals, such as phenolics, flavonoids, glycosides, and tannins, are present in medicinal plants and have an antibacterial effect that works by disrupting bacterial cell walls, modulating membrane permeability, and interrupting vital metabolic pathways of bacteria, which they need to survive and replicate [9]. The objective of the present study was the assessment of the antimicrobial activity of two traditional medicinal plants, *Cassia alata* and *Glycyrrhiza glabra*, against Gram-positive *Bacillus subtilis*, *Staphylococcus aureus*, and Gram-negative *Escherichia coli* bacteria of clinical interest [10]. Maceration technique, a proven procedure, was used for the extraction of bioactive compounds from both plants. It enables the extraction of bioactive compounds efficiently and avoids the degradation of heat-sensitive phytochemicals [11]. The extraction was compared using solvents with different polarities: methanol, ethanol, and chloroform, and thus, allowed an insight into the effect of solvents on antimicrobial activity. Moreover, the agar well diffusion test was chosen because it is reliable, simple, and commonly used in antimicrobial screening research, which enables easy visualization and quantification of inhibition zones [12]. In the present study, methanolic extracts were observed to be the most effective on all bacterial strains. The highest inhibition zone was measured against Staphylococcus aureus (18 mm), and therefore, this bacterium was more susceptible to it, probably because of its simpler and easier cell wall structure, enabling entry of polar bioactive compounds. This is due to the better performance of methanol, which is more polar and can extract phenolics and flavonoids, which have antimicrobial potential, and this is a trend that has been observed in other studies, too, such as the study done by [13]. Ethanolic extracts exhibited an intermediate level of antibacterial activity, and with lower inhibition zones specifically targeting *Bacillus subtilis*, which is viewed as resistant, indicating that the solvent polarity is modulating the extract activity; comparable solvent-specific trends have been observed in Senna species, where methanolic extracts invariably exceed the efficacy of ethanolic extracts [14]. Conversely, the lowest antibacterial activity was observed in chloroform extracts, particularly against *B. subtilis*, and it may be explained by the low solubility of polar antimicrobial compounds in non-polar solvents, which Mushtaq *et al.* also reported [15]. Nevertheless, the comparatively increased potency of *G. glabra* chloroform extracts against *S. aureus* indicates that some non-polar antibacterial components in the licorice exist, and they are species-specific, with phytochemical diversity. In the case of *E. coli*, even though it is a Gram-negative bacterium, it exhibited relatively lower susceptibilities, presumably because of the presence of an outer lipopolysaccharide membrane; measurable zones of inhibition could be observed, which indicated that some phytochemicals in the extracts of both plants could penetrate or disrupt the

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protective barrier [16]. So, studies show that methanolic extracts are more valuable as antimicrobial substances. Moreover, it was also found that *Glycyrrhiza glabra* had greater antibacterial activity compared to *Cassia alata* in most extracts. This improved effectiveness can be explained by the presence of bioactive phenolic acids like chlorogenic acid and p-coumaric acid, which were noted to have a strong antibacterial effect [17]. Wahab *et al.* also reported the antimicrobial potential of *Glycyrrhiza glabra* against Gram-positive and Gram-negative bacteria, which is in agreement with the findings of the present study [18]. Furthermore, antibacterial activity may be attributed to the synergistic interactions among multiple phytoconstituents present in the plant extracts. Compared to traditional antibiotics, which in many cases focus on one molecular target, plant-based compounds may have a multi-target effect and, therefore, will be less likely to induce resistance. Moreover, the differences in susceptibility between bacterial strains can also be related to the differences in the cell wall structure, the activity of efflux pumps, and the mechanisms of enzymatic degradation. Such results demonstrate the urgency of the research on plant-based antimicrobials as alternative therapeutic agents, as well as supplements to the current antibiotics. Nevertheless, more research, including purification of active compounds, calculation of minimum inhibitory concentrations (MIC), and in vivo-confirmation are required to ascertain their relevance in clinical practice [19, 20].

The limitations of this study include *in vitro* design, which does not account for pharmacokinetic and pharmacodynamic factors in a living system. The use of crude extracts prevents the identification of the specific bioactive compounds responsible for the observed activity. Furthermore, only three bacterial strains were tested, and the absence of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) data limits the quantitative comparison of potency. Finally, the study did not investigate the underlying mechanisms of action or the potential cytotoxic effects of the extracts on human cells, which are critical for any therapeutic application. Further investigations are required in the form of in vivo experiments, an extended number of pathogenic bacteria, and purification of active compounds to ascertain their therapeutic nature and to maximize procedures of extraction in clinical practice. While this study demonstrates antimicrobial activity, the underlying mechanisms were not experimentally investigated. Future work should focus on elucidating the mode of action, such as assessing cell membrane permeability, inhibition of efflux pumps, or disruption of biofilm formation, using techniques like flow cytometry or electron microscopy.

CONCLUSION

In conclusion, *Cassia alata* and *Glycyrrhiza glabra* were found to be significantly lethal against both Gram-positive and Gram-negative bacteria. Among all three tested solvents, methanolic extracts were observed to exhibit the highest antibacterial activity, followed by ethanolic extracts, whereas chloroform extracts showed comparatively lower efficiency against all the bacterial strains. *Staphylococcus aureus* was the most susceptible bacterial strain, while *Bacillus subtilis* showed comparatively lower sensitivity to all three solvents. This observed variation is attributed to the polarity of each solvent. Overall, the results of the study support the potential use of these two medicinal plants as natural sources of antibacterial agents.

Authors' Contribution

Conceptualization: MR
Methodology: MR
Formal analysis: MR, AA, AT
Writing and Drafting: AA, AT
Review and Editing: MR, AA, AT, MA

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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