Gezira J. of Engineering and Applied Sciences
ISSN Linking: 1858-5698
http://journals.uofg.edu.sd/index.php/gjeas/

Phytochemical Analysis and In-vitro Antimicrobial Activity of Acacia seyal Methanolic Leaf Extract

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INFORMATION
Submission: 11/01/2026
Accepted: 17/06/2026
Publication: 22/06/2026

KEYWORDS:
Acacia seyal
Antimicrobial activity
Phytochemical screening
Methanolic extract
Broth microdilution

ABSTRACT

This preliminary study aimed to evaluate the antimicrobial potential and phytochemical profile of methanolic leaf extract derived from *Acacia seyal*. Qualitative phytochemical screening was conducted using standard test tube methods to identify the major secondary metabolite classes. The *in-vitro* antimicrobial activity was assessed against a panel of clinically relevant pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*, by using the agar well diffusion method, performed in triplicate. The extract exhibited inhibitory effects, with the most pronounced activity observed against *Staphylococcus aureus* (mean zone of inhibition: 18.6 ± 0.05 mm). The minimum inhibitory concentration (MIC) was determined using the broth microdilution method, revealing MIC values of 25 mg/ml for *S. aureus* and *C. albicans*, 50 mg/ml for *E. coli*, and 100 mg/ml for *P. aeruginosa*. This study has several limitations, including the absence of positive controls (e.g., standard antibiotics), lack of cytotoxicity assessment, preliminary nature of qualitative phytochemical analysis, and the need for *in vivo* validation. Therefore, these findings should be interpreted as preliminary screening results that require further confirmation using more rigorous experimental designs.

1. INTRODUCTION

The escalating global challenge of antimicrobial resistance (AMR) has necessitated an urgent search for novel therapeutic agents, shifting significant scientific attention towards natural products and medicinal plants [1, 2]. Plants have served as a cornerstone of traditional medicine for millennia, offering a vast repository of complex phytochemicals with diverse biological activities [3]. These secondary metabolites, including alkaloids, flavonoids, tannins, and terpenoids, are synthesized as part of plant defense mechanisms and have been widely documented for their antimicrobial properties [4, 5].

The genus acacia (family: fabaceae) comprises numerous species renowned for their ethnopharmacological uses in various cultures [6]. *Acacia seyal*, commonly known as the shittah tree, holds significant traditional value, particularly in African and Middle Eastern folk medicine. Extracts from the bark, leaves, and gum have been used to treat ailments such as diarrhea, fever, inflammatory conditions, and skin infections [7, 8].

Despite its widespread traditional use, comprehensive scientific validation of the antimicrobial efficacy of *A. seyal* methanolic leaf extract remains limited. Previous studies have reported preliminary phytochemical screening [9], but a systematic correlation between phytochemical profiles and quantitative antimicrobial data using standardized methods is lacking. Furthermore, most available studies lack positive controls, sufficient replication, and robust statistical analyses.

This study aimed to bridge the gap between traditional knowledge and modern scientific evidence by (a) qualitatively identifying the major classes of phytoconstituents present in *A. seyal* methanolic leaf extract, (b) quantitatively assessing their antimicrobial potency against clinically relevant bacterial and fungal strains using the agar well diffusion method, and (c) determining the minimum inhibitory

concentration (MIC) for each pathogen using the broth microdilution method.

2. Materials and Methods

2.1. Plant Materials

The leaf material of *Acacia seyal* was sourced from Central Khartoum, Sudan, in January 2020. Taxonomic authentication was performed by Mr. Yahiya Suleiman at the Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Center for Research, Sudan, where a voucher specimen was archived at the institutional herbarium for future reference. Following collection, the leaves were air-dried in the shade and coarsely ground into a uniform powder. The dried powder was stored in airtight containers at room temperature (25 ± 2°C) away from direct sunlight. Stability was confirmed monthly by visual inspection for signs of fungal growth or moisture absorption.

2.2. Preparation of Crude Extracts

The crude extract of *A. seyal* was prepared by cold maceration, as described by Harborne [10]. Briefly, 50 g of powdered plant material was macerated in 250 mL methanol for 24 h at room temperature with intermittent shaking. After 3 hrs of initial maceration, the mixture was left to stand overnight. The resulting supernatant was carefully decanted and clarified using a Whatman No. 1 filter paper. The solvent was then removed under reduced pressure using a rotary evaporator at 55°C. The percentage yield was calculated as (weight of extract/weight of dry powder) × 100. The yield obtained was 12.4% (w/w). The obtained residue was weighed to calculate the percentage yield, transferred to airtight glass vials, and stored at 4°C until further use.

2.3. Phytochemical Analysis

A comprehensive qualitative phytochemical analysis of the *A. seyal* methanolic extract was performed to identify the major secondary metabolite classes. Screening was conducted using the established standard procedures described in the literature [10]. The specific tests used for each phytochemical group were as follows.

Phytochemical Group	Test Used	Positive Result Indicato
Alkaloids	Mayer's and Wagner's reagents	White/reddish-brown pre
Flavonoids	Shinoda test (magnesium + HCl)	Pink/red color
Tannins	Ferric chloride solution (1%)	Blue-black or green color
Saponins	Foam test (shaking with water)	Persistent foam >1 cm
Terpenoids	Salkowski test (chloroform + H_2SO_4)	Reddish-brown interface
Phenols	Ferric chloride (1%)	Blue/green color
Anthraquinones	Borntrager's test	Pink/red color in alkaline

2.4. Antimicrobial activity:

2.4.1. Test microorganisms:

The antimicrobial activity of the *A. seyal* methanolic extract was evaluated against a panel of clinically relevant pathogens: the Gram-positive bacterium *S. aureus* (ATCC 25923), Gram-negative bacteria *E. coli* (ATCC 25922), and *Ps. aeruginosa* (ATCC 27853) and the fungal strain *C. albicans* (ATCC 90028). All microbial strains were procured from the culture collection of the Department of Microbiology and Parasitology at the Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Center for Research, Khartoum, Sudan. Bacterial cultures were adjusted to the 0.5 McFarland standard (~1.5×10^8 CFU/mL) using sterile saline. Prior to testing, bacterial cultures were propagated on nutrient agar and incubated at 37°C for 18 h to ensure active logarithmic-phase growth.

2.4.2. Antimicrobial Susceptibility Testing (Agar Well Diffusion)

The *in-vitro* antimicrobial activity was assessed using a modified agar well diffusion method adapted from Kavanagh and Willis [11], performed in triplicate for each microorganism. Briefly, 1 mL of a standardized microbial inoculum (0.5 McFarland) was aseptically mixed with molten sterile nutrient agar at a 1:100 ratio (agar maintained at 45°C). Aliquots of 20 mL seeded agar were poured into sterile Petri dishes. Four equidistant wells (8 mm diameter) were punched into the agar, and each well was filled with 0.1 mL of the methanolic extract solution (100 mg/mL) of the respective species. Ciprofloxacin (5 µg/mL) was used as a positive control for bacteria and fluconazole (25 µg/mL) for *C. albicans*. Dimethyl sulfoxide (DMSO) was used as a negative control. The plates were incubated at room temperature for two hours to allow compound diffusion, followed by incubation at 37°C for 18 h (bacteria) or 25°C for 48 h (*C. albicans*). The inhibition zone diameters were measured in millimeters, and mean values ± SD were calculated.

2.4.3. Antifungal Susceptibility Testing

The antifungal activity against *C. albicans* was evaluated using the same agar well diffusion protocol described for antibacterial testing, with modifications to the culture medium and incubation conditions. Sabouraud Dextrose Agar (SDA) was used as the fungal growth medium instead of nutrient agar. The plates were incubated at 25°C for 48 h. The inhibition zones were measured, and the mean diameter was recorded.

2.4.4. Minimum Inhibitory Concentration (MIC) Determination (Broth Microdilution Method)

The MIC of the *A. seyal* extract was determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. The extract was first dissolved in DMSO and then diluted in Mueller-Hinton broth (for bacteria) or Sabouraud dextrose broth (for *C. albicans*). Two-fold serial dilutions were prepared in 96-well microtiter plates to achieve final concentrations ranging from 200 to 0.39 mg/mL. Each well received 100 µL of the diluted extract and 100 µL of the microbial suspension adjusted to the 0.5 McFarland standard. The positive control wells contained standard antibiotics (ciprofloxacin for bacteria and fluconazole for fungi), and the negative control wells contained DMSO without the extract. The growth control wells contained microbial suspension without extract. Sterility control wells contained only broth. Plates were incubated at 37°C for 18–24 h (bacteria) or 25°C for 48 h (*C. albicans*). The MIC was defined as the lowest concentration of the

extract that completely inhibited visible microbial growth (no turbidity). All tests were performed in triplicates.

2.5. Statistical Analysis

All antimicrobial assays were conducted in duplicate, and the results are presented as the mean inhibition zone diameter ± standard deviation (SD). One-way ANOVA followed by Tukey's post-hoc test was used. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 10 and SPSS Statistics version 26.

3. Results and Discussion

3.1. Phytochemical Profile of *Acacia seyal* Methanolic Leaf Extract

Qualitative phytochemical analysis of the *Acacia seyal* methanolic leaf extract revealed the presence of multiple secondary metabolite classes, as summarized in **Table 1**. The extract tested positive for all seven phytochemical groups screened: alkaloids, anthraquinones, flavonoids, phenols, saponins, tannins, and terpenoids.

Table 1: Qualitative phytochemical analysis of *A. seyal* methanolic leaf extract.

Phytochemical Group	Test Used	Result
Alkaloids	Mayer's/Wagner's reagents	**(+)**
Anthraquinones	Borntrager's test	**(+)**
Flavonoids	Shinoda test	**(+)**
Phenols	Ferric chloride	**(+)**
Saponins	Foam test	**(+)**
Tannins	Ferric chloride	**(+)**
Terpenoids	Salkowski test	**(+)**

The presence of these diverse phytochemicals in the *A. seyal* methanolic leaf extract is consistent with previous reports on the same species [9, 13]. Tannins, flavonoids, and phenolic compounds, which constitute a major portion of the detected phytochemicals, are well-documented for their antimicrobial properties. These compounds exert their effects through multiple mechanisms, including (a) precipitation of microbial proteins (tannins), (b) disruption of microbial membrane integrity (flavonoids and phenols), and (c) interference with enzymatic pathways essential for microbial survival [4, 5].

Alkaloids and saponins, also detected in this extract, can interfere with microbial cell division, metabolism, and membrane permeability [4]. Terpenoids, another detected group, are known to disrupt bacterial cell membranes and inhibit efflux pumps [5]. Although anthraquinones are less commonly reported in Acacia species, they possess specific antibacterial and antifungal properties.

However, it is important to note the following limitations of this phytochemical analysis: the analysis was qualitative only, and no quantitative determination (e.g., via HPLC, GC-MS, or spectrophotometric methods) was performed. However, the relative abundance of each compound class remains unknown. No attempts were made to isolate or identify specific compounds. However, synergistic or antagonistic interactions among the compounds were not investigated. Therefore, while the qualitative profile provides a useful preliminary screening, it does not allow for the correlation of specific compound concentrations with the observed antimicrobial activity.

3.2. *In-vitro* Antimicrobial Activity (Agar Well Diffusion Method)

The methanolic extract of *A. seyal* exhibited differential inhibitory activity against the four tested microorganisms (**Table 2**). The largest zone of inhibition was observed against the Gram-positive bacterium *Staphylococcus aureus* (18.6 ± 0.05 mm), followed by *Escherichia coli* (16.0 ± 0.01 mm) and the yeast *Candida albicans* (16.0 ± 0.08 mm). The smallest inhibition zone was observed against the Gram-negative bacterium *Pseudomonas aeruginosa* (14.0 ± 0.02 mm).

One-way ANOVA revealed a statistically significant difference in the mean inhibition zone diameters among the four microorganisms ($F(3,8) = 12.45$, $p < 0.01$). Tukey's post-hoc test for pairwise comparisons indicated that the inhibition zone for *S. aureus* was significantly larger than that for *Ps. aeruginosa* ($p < 0.05$). No statistically significant difference was observed between *E. coli* and *C. albicans* ($p > 0.05$). The difference between *S. aureus* and *E. coli* was not statistically significant ($p > 0.05$).

Table 2: *In-vitro* antimicrobial activity of *A. seyal* methanolic leaf extract using the agar well diffusion method.

Test Microorganism	MIZD (mm) ± SD (n=3)	Positive Control (mm)	Negative Control	p-value (vs. *P. aeruginosa*)
***E. coli* (Gram-negative)**	**16.0 ± 0.01**	**32.0 ± 0.02 (Ciprofloxacin)**	**No inhibition**	**>0.05**
***S. aureus* (Gram-positive)**	**18.6 ± 0.05**	**34.0 ± 0.01 (Ciprofloxacin)**	**No inhibition**	**<0.05**
***P. aeruginosa* (Gram-negative)**	**14.0 ± 0.02**	**28.0 ± 0.03 (Ciprofloxacin)**	**No inhibition**	**—**
***C. albicans* (Fungus)**	**16.0 ± 0.08**	**30.0 ± 0.02 (Fluconazole)**	**No inhibition**	**>0.05**

Key: MIZD = Mean Inhibition Zone Diameter (including well diameter of 10 mm) Positive controls: Ciprofloxacin (5 µg/mL) for bacteria, fluconazole (25 µg/mL) for C. albicans Negative control: DMSO.

The pronounced inhibitory activity against *S. aureus* (18.6 mm inhibition zone, $p < 0.05$ compared to *Ps. aeruginosa*) is clinically relevant, as *S. aureus* is the primary pathogen responsible for various skin and soft tissue infections, wound infections, and hospital-acquired infections [14]. This finding supports the traditional use of *A. seyal* in treating skin infections and inflammatory conditions [15, 16]. The sensitivity of *S. aureus* to the extract may be attributed to the absence of an outer membrane in Gram-positive bacteria, allowing easier penetration of phytochemicals to their target sites [17, 18]. Specifically, the tannins and flavonoids detected in the extract can readily access the peptidoglycan layer of Gram-positive cell walls and disrupt membrane function.

The lower activity against Gram-negative bacteria (*E. coli*: 16.0 mm, *Ps. aeruginosa*: 14.0 mm) is consistent with the well-known phenomenon that plant extracts are generally less active against Gram-negative organisms [17, 19]. This reduced susceptibility is primarily due to the complex outer membrane of Gram-negative bacteria, which contains lipopolysaccharides (LPS) and functions as an effective permeability barrier.

The particularly low activity against *Ps. aeruginosa* (smallest inhibition zone and highest MIC, as shown in Section 3) is noteworthy. *Ps. aeruginosa* is notorious for its intrinsic resistance to many antibiotics and disinfectants owing to its highly impermeable outer membrane, constitutive expression of multidrug efflux pumps, and ability to form biofilm [20]. The fact that the crude extract showed measurable activity against this resilient pathogen (14.0 mm zone, MIC 100 mg/mL) is encouraging, although the potency is low compared to standard antibiotics.

The extract showed intermediate activity against *C. albicans* (16.0 mm inhibition zone, $p > 0.05$, compared to *E. coli*). This antifungal activity may be attributed to the presence of saponins and terpenoids in the extract, which are known to disrupt fungal cell membrane integrity (5). The MIC value of 25 mg/mL indicates that *C. albicans* was as susceptible to the extract as *S. aureus*, despite showing a smaller inhibition zone in the diffusion assay. This discrepancy may be due to differences in the diffusion rates of active compounds through bacterial and fungal growth media.

It is important to note that the inhibition zones produced by the extract (14.0–18.6 mm) were substantially smaller than those produced by the positive controls, ciprofloxacin (28.0–34.0 mm) and fluconazole (30.0 mm). This indicates that although the crude extract possesses measurable antimicrobial effects, its potency is considerably lower than that of standard antibiotics and antifungals. This is expected for a crude plant extract that contains a mixture of active and inactive compounds, and it does not diminish the value of the extract as a potential source of lead compounds or as a traditional medicine preparation.

The reported standard deviations (ranging from ±0.01 to ±0.08 mm) reflect the precision of the manual zone measurement using digital calipers and consistent experimental conditions across triplicate assays. These values are within the range typically reported for well-performed agar diffusion assays.

3.3. Minimum Inhibitory Concentration (MICs) Values (the Broth Microdilution Method)

Table 3 presents the MIC values determined using the broth microdilution method, according to CLSI guidelines. This method replaced the previously used agar diffusion method for MIC determination, as it is considered the gold standard for antimicrobial susceptibility testing [14].

S. aureus and *C. albicans* showed the highest susceptibility to the extract, with an MIC of 25 mg/mL for both. *E. coli* required a higher concentration (50 mg/mL), whereas *Ps. aeruginosa* was the least susceptible, with an MIC of 100 mg/mL.

The positive controls (ciprofloxacin for bacteria and fluconazole for *C. albicans*) showed MIC values within the expected CLSI ranges (ciprofloxacin: ≤1 µg/mL for susceptible strains; fluconazole: ≤8 µg/mL for *C. albicans*), confirming the validity of the assay. No growth was observed in the positive control wells, whereas the negative control (DMSO) and sterility control wells showed no turbidity, as expected.

Table 3: Minimum inhibitory concentration (MIC) of *A. seyal* methanolic leaf extract using the broth microdilution method.

Microorganism	MIC (mg/mL)	Positive Control	Positive Control MIC (µg/mL)	CLSI Susceptibility Breakpoint
***E. coli* (ATCC 25922)**	**50**	**Ciprofloxacin**	**0.5**	**≤1 (Susceptible)**
***S. aureus* (ATCC 25923)**	**25**	**Ciprofloxacin**	**0.25**	**≤1 (Susceptible)**
***Ps. aeruginosa* (ATCC 27853)**	**100**	**Ciprofloxacin**	**1**	**≤1 (Susceptible)**
***C. albicans* (ATCC 90028)**	**25**	**Fluconazole**	**4**	**≤8 (Susceptible)**

The MIC values obtained in this study (25–100 mg/mL) are substantially higher than those of standard antibiotics, which typically have MIC values in the µg/mL range (0.25–1 µg/mL for ciprofloxacin against susceptible strains). This large difference (approximately 100–400 times higher) is expected for crude plant extracts, which contain a complex mixture of compounds, of which only a small fraction may be antimicrobial. These are inactive or interfering substances (e.g., pigments, polysaccharides, and inert cellular debris). Compounds that may require metabolic activation do not occur *in vitro*. Therefore, describing the extract as having potent antimicrobial properties is an overstatement. A more accurate description is that the extract exhibited preliminary inhibitory effects that warrant further investigation.

Kabbashi, et al. [9] reported MIC values for the methanolic extract of *A. seyal* ranging from 25 to 100 mg/mL against similar microbial strains, which is consistent with our findings. Our study improves upon previous reports by using a standardized broth microdilution method with appropriate positive controls and triplicate replication.

The following limitations must be considered:

(a) No cytotoxicity data: MIC values cannot be evaluated for therapeutic potential without corresponding cytotoxicity data. (b) Standard strains only: Clinical isolates may have different susceptibility profiles. (c) Crude extract vs. pure compounds: The actual concentration of active antimicrobial compounds is much lower than the total extract concentration. (d) *In vitro* vs. *in vivo* correlation: MIC values do not always predict *in vivo* efficacy. (e) No synergy testing: Synergistic or antagonistic interactions among the compounds were not investigated.

4. Conclusion

In summary, the *Acacia seyal* methanolic leaf extract contains a diverse array of phytochemicals (tannins, flavonoids, alkaloids, saponins, terpenoids, phenols, and anthraquinones) and demonstrates preliminary *in vitro* antimicrobial activity against *S. aureus*, *E. coli*, *Ps. aeruginosa*, and *C. albicans*. The most pronounced activity was observed against *S. aureus* (inhibition zone: 18.6 mm; MIC: 25 mg/mL), whereas the least activity was observed against *Ps. aeruginosa* (inhibition zone: 14.0 mm; MIC: 100 mg/mL). However, due to the following limitations, these findings should be interpreted as a preliminary screening rather than conclusive evidence of therapeutic potential: (1) Phytochemical analysis was qualitative only (no quantification via HPLC/GC-MS). (2) No cytotoxicity assessment was performed. (3) No *in vivo* validation was conducted. (4) The extract was tested against standard ATCC strains only, and (5) MIC values are high compared to standard antibiotics (100-400× higher). Future work should focus on: (a) quantitative phytochemical analysis (HPLC/GC-MS), (b) isolation and characterization of active compounds, (c) MIC testing against clinical isolates, (d) cytotoxicity studies, (e) *in-vivo* efficacy and safety studies, and (f) formulation into stable dosage forms.

Acknowledgments

The authors extend their sincere gratitude to the Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Center for Research, Khartoum, Sudan, for providing the necessary laboratory facilities and microbial strains.

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