Phytochemical Profile and Bioactivity of Earleaf acacia (Acacia auriculiformis): Antioxidant, Antibacterial, and Anti-Biofilm Potential

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ABSTRACT

Acacia auriculiformis A. Cunn., commonly known as Earleaf Acacia, is a member of the Fabaceae family and is native to Australia, Papua New Guinea, and parts of Southeast Asia. It is widely cultivated in India, Malaysia, and tropical regions for timber, environmental restoration, and traditional medicine (Sathya & Siddhuraju, 2012). Different parts of the plant like bark, leaves, seeds have been used in folk medicine for the treatment of diarrhoea, infections, inflammation, and skin ailments. Despite its widespread traditional use, the phytochemical and pharmacological profile of A. auriculiformis remains only partially studied, especially in the context of antimicrobial resistance and oxidative stress-related disorders. Earlier studies have indicated the presence of bioactive flavonoids, phenolics, and saponins with promising antioxidant and antimicrobial properties (Singh et al., 2010; Ghosh et al., 1993). However, the bioactivity of extracts may vary based on geographical location, extraction methods, and plant part used, highlighting the need for regional and method-specific evaluations. This study aims to evaluate the phytochemical composition and bioactive properties of ethanol and aqueous extracts of A. auriculiformis leaves collected from Sholinganallur, Chennai. The bioactivities assessed include antioxidant activity using the DPPH assay, antibacterial activity using the agar well diffusion method, minimum inhibitory and bactericidal concentrations (MIC and MBC), biofilm inhibition using crystal violet staining, and compound separation via thin layer chromatography (TLC). The findings

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are discussed in light of prior studies to better understand the plant's therapeutic potential.

INTRODUCTION

Phytochemicals, or plant-derived secondary metabolites, are chemical compounds produced by plants primarily for defence and survival, but many of these compounds have been shown to possess significant pharmacological potential for humans. They include diverse class of chemicals such as alkaloids, flavonoids, tannins, terpenoids, phenolics, saponins, and glycosides, each of which has unique biochemical properties (Crozier et al., 2009). These compounds play vital roles in plant resistance to microbial pathogens, insects, ultraviolet radiation, and oxidative stress. Medicinal plants, particularly those used in traditional systems of medicine such as Ayurveda, Traditional Chinese Medicine, and Siddha, offer a rich reservoir of therapeutic agents that remain underexplored. Systematic phytochemical and pharmacological evaluations of these plants can help validate traditional knowledge and guide the development of novel natural products.

Several studies have focused on the phytochemical and pharmacological properties of Acacia auriculiformis and its relatives in the Fabaceae family. The bark of A. auriculiformis is particularly rich in tannins approximately measuring 12–16% dry weight while the leaves and seeds also contain a variety of phenolic compounds, flavonoids, alkaloids, steroids, glycosides, and saponins (Sathya & Siddhuraju, 2012; Ghosh et al., 1993). Two unique acylated triterpenoid saponins, acaciasides A and B, were isolated from the seed funicles and found to exhibit antimicrobial activity (Ghosh et al., 1993).

In terms of antioxidant potential, Singh et al. (2010) demonstrated that bark extracts of A. auriculiformis possess potent radical scavenging abilities, with DPPH inhibition levels reaching up to 71–72% for the ethyl acetate-water fraction and up to 91.7% inhibition in hydroxyl radical assays for the crude acetone extract. Similarly, Sathya and Siddhuraju (2012) found strong antioxidant activity in both bark and empty pod extracts, reporting IC_{50} values in 10 µg/mL. These findings align with studies on related Acacia species such as A. nilotica, which have also demonstrated high phenolic content and strong antioxidant potential (Baravalia & Chanda, 2011).

There is limited direct research on the anti-biofilm activity of A. auriculiformis. However, related species such as Acacia nilotica and Acacia macrostachya have been shown to inhibit biofilm formation in Staphylococcus aureus, E. coli, and other multidrug-resistant strains (Sasidharan et al., 2012; Salih et al., 2021). This suggests that phytochemicals such as tannins and flavonoids common across Acacia species may also contribute to anti-biofilm effects in A. auriculiformis.

Thin Layer Chromatography (TLC) has been used in multiple studies as a qualitative method to visualize the complexity of phytochemical mixtures in Acacia species. Chromatograms developed with polar and semi-polar solvents have revealed distinct bands corresponding to flavonoids and phenolics, and DPPH-stained TLC plates have successfully identified antioxidant-active compounds (Sathya & Siddhuraju, 2012; Chew et al., 2011).

Collectively, these studies provide strong support for the pharmacological relevance of A. auriculiformis and justify continued evaluation of its extracts in bioactivity assays relevant to antimicrobial and antioxidant therapy.

METHODOLOGY

Collection and Preparation of Plant Material

Leaves of Acacia auriculiformis were collected from Sholinganallur, Chennai and authenticated from a botanist. The leaves were washed to remove dust and dried in the shade to protect their active compounds. The dried leaves were ground into a fine powder using a mechanical grinder and stored in clean, airtight containers for later use.

Extraction Process

The aqueous and ethanolic extracts were prepared by macerating the powdered leaves with water and ethanol for 72 hours with intermittent stirring. The ethanolic extract was concentrated using a rotary evaporator, and the water extract was dried in an oven. Both extracts were preserved for the analysis in a cool place.

Phytochemical Screening

Preliminary phytochemical analysis was done to check for the presence of alkaloids, flavonoids, phenols, glycosides, saponins, triterpenoids, and phytosterols. The plant extracts were treated with reagents and observed for colour change while interacting with the reagents.

Thin Layer Chromatography (TLC) and Bioautography

TLC was used to separate the different compounds in the extracts. Solvents like chloroform, ethanol, and methanol were used as the mobile phase to help move the compounds on the stationary phase in a TLC plates. The Rf values (movement of spots) were recorded as the ratio of distance travelled by the solute to the distance travelled by the solvent. TLC bioautography was performed to identify the antioxidant compounds by spraying the DPPH solution on the plates. Antioxidant spots appeared as light spots on a purple background.

DPPH Antioxidant Assay
The antioxidant activity of the extracts was measured using the DPPH assay. Different concentrations of the extracts were mixed with DPPH solution, and the absorbance was measured at 517 nm. Ascorbic acid was used as the standard. The percent of free radical inhibition was calculated to show antioxidant potential.

Antibacterial activity by Agar Well Diffusion Method
The antibacterial activity of the extracts was tested against Staphylococcus aureus and Escherichia coli using the agar well diffusion method. Bacteria were spread on agar plates, and wells were filled with different extract concentrations. Plates were incubated at 37°C for 24 hours. The clear zones around the wells were measured. Oxacillin was used as the positive control, and distilled water as the negative control.

Minimum Inhibitory Concentration (MIC)
MIC was found using the broth microdilution method. Different concentrations of the extracts were prepared in nutrient broth and mixed with bacterial cultures in 96-well plates. After 24 hours at 37°C, the lowest concentration with no visible growth (no cloudiness) was recorded as the MIC.

Minimum Bactericidal Concentration (MBC)
After finding the MIC, samples from clear wells were placed on agar plates to check for any remaining bacteria. The lowest concentration that showed no bacterial growth on the agar was recorded as the MBC.

Antifungal Test (Agar Well Diffusion Method)
The antifungal activity was tested against Saccharomyces cerevisiae. Extracts were added to wells in agar plates with the fungus and incubated. The zone of inhibition was measured. Amphotericin B was used as the standard antifungal control.

Biofilm Inhibition Test
The ability of the aqueous extract to stop biofilm formation was tested using crystal violet staining. Bacteria were grown in 96-well plates with different extract concentrations. After incubation, wells were washed, stained with crystal violet, and the absorbance was measured at 570 nm. A lower absorbance meant stronger biofilm inhibition.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical screening for the aqueous and ethanolic extracts of Acacia auriculiformis leaves were qualitatively assessed. Both extracts tested negative for alkaloids, saponins, tannins, and carbohydrates. Phytosterols are detected in both aqueous and ethanolic extracts. They are the plant-based sterols structurally similar to cholesterol. They are known for their cholesterol-lowering effects and also possess anti-inflammatory and anticancer properties. Phenolic compounds are strong antioxidants. Their presence in the aqueous extract suggests a capacity to neutralize free radicals and reduce oxidative stress. This contributes to the therapeutic potential of the plant in managing degenerative diseases and inflammation. Flavonoids are well-established antioxidants with additional antimicrobial, antiviral, and anti-inflammatory properties. Their presence supports the use of A. auriculiformis in traditional medicine for treating infections and inflammation. Glycosides often act as precursors to active aglycones with notable pharmacological actions. They may contribute to antimicrobial, cardioprotective, or anti-inflammatory effects depending on their structural class. Triterpenoids are bioactive compounds with a wide range of activities including anti-inflammatory, antiviral, hepatoprotective, and anticancer effects. Their dual presence reinforces the broad therapeutic potential of both extract types.

SN	PHYTOCHEMICAL	OCCURANCE	
		AQUEOUS EXTRACT	ETHANOLIC EXTRACT
1	Phytosterol	Present	Present
2	Phenols	Present	Absent
3	Flavonoids	Present	Present
4	Glycosides	Present	Present
5	Triterpenoids	Present	Present
6	Alkaloids	Absent	Absent
7	Saponins	Absent	Absent
8	Tannins	Absent	Absent
9	Carbohydrates	Absent	Absent

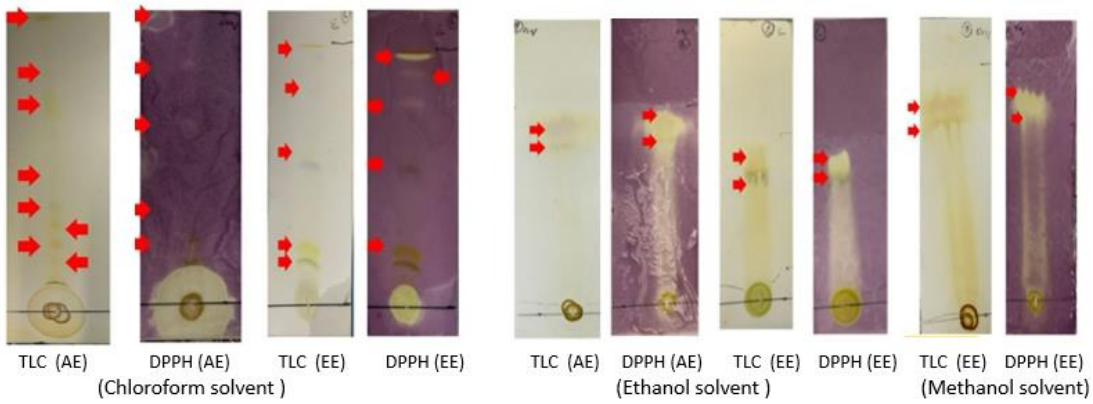
Table 1: Detection of phytochemicals

Bioautography Assay using TLC

The Thin Layer Chromatography (TLC) was performed to analyze the phytochemical constituents in the aqueous (AE) and ethanol (EE) extracts of Acacia auriculiformis using chloroform, ethanol, and methanol as mobile phases. The number of separated bands and their Rf values are presented in table 2 and visualized in Figure 1. Among all tested solvent systems, the aqueous extract developed in chloroform showed the highest number of spots with 8 distinct bands having Rf values ranging from 0.09 to 1.00. This indicates the presence of a wide variety of phytochemicals with differing polarities. The ethanol extract in chloroform revealed 5 spots, suggesting it was also able to solubilize a narrow range of compounds. Both extracts developed fewer bands in ethanol and methanol solvents, highlighting that chloroform was the most effective mobile phase for compound separation in this study.

To assess antioxidant activity, in-vitro DPPH bioautography was performed on the TLC plates (Figure 5). The aqueous extract in chloroform revealed 5 DPPH-active spots, while the ethanol extract in the same solvent showed 4 active antioxidant bands. Ethanol and methanol extracts from both AE and EE yielded only 2 active antioxidant spots, and the ethanolic extract (EE-Methanol) in methanol solvent did not show any compound, may be because of the highly polar solvent systems. Chloroform as a mobile phase resolved more compounds which highlighted for antioxidant activity. Aqueous extracts yielded more diverse antioxidant-active spots in chloroform, indicating that certain water-soluble phytochemicals may possess strong radical scavenging potential. The antioxidant bands revealed by DPPH staining validate the presence of phenolics and flavonoids, which are known to donate electrons and neutralize free radicals. The TLC and bioautography data complement the DPPH assay findings and confirm that both aqueous and ethanol extracts of A. auriculiformis contain multiple antioxidant-active phytochemicals. The variety of compounds detected supports the plant's pharmacological relevance and underscores the value of using solvent systems with a wide polarity range for phytochemical.

Fig 1: TLC chromatogram of aqueous extract (AE) and ethanol extract (EE) in solvent system



SN	SOLVENT SYSTEM	EXTRACTS	Rf -VALUE	Rf -VALUE FOR TLC BIOAUTOGRAM
1	Chloroform	Aqueous	1.0, 0.67, 0.34, 0.31, 0.22, 0.17 ,0.11, 0.09	1.0, 0.67, 0.31, 0.22, 0.17
		Ethanol	0.97, 0.91, 0.60, 0.43, 0.16	0.97, 0.91, 0.60, 0.61
2	Ethanol	Aqueous	0.85, 0.80	1.0, 0.80
		Ethanol	0.87, 0.74	0.87, 0.74
4	Methanol	Aqueous	0.82, 0.74	0.82, 0.78
		Ethanol	-	-

Table 2: Rf values of all the spots observed on chromatogram

Antibacterial Activity

The antibacterial activity of aqueous and ethanol extracts of Acacia auriculiformis was evaluated using the agar well diffusion method against two bacterial strains: Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative). However, no zone of inhibition was observed for the tested concentration of 10 µg/µL – 40 µg/µL for either extract against both organisms. This indicates that the crude extracts were ineffective under the tested conditions in inhibiting the growth of the selected bacterial strains.

No antibacterial activity was observed against Escherichia coli for either extract at any of the tested concentrations of 10 µg/µL – 40 µg/µL. This is consistent with existing literature, which often reports reduced efficacy of plant extracts against Gram-negative organisms due to the presence of an outer membrane that limits the penetration of bioactive compounds (Chew et al., 2011; Singh et al., 2010).

Extract	Zone of Inhibition in mm									
	Controls		*Staphylococcus aureus*				*E.coli*			
	Negative	Oxacillin	10 µg	20 µg	30 µg	40 µg	10 µg	20 µg	30 µg	40 µg
Aqueous	-	31	-	-	-	-	-	-	-	-
Ethanol	-	35	-	-	-	-	-	-	-	-

Table 3 Zones of inhibition observed against Staphylococcus aureus and Escherichia coli in mm

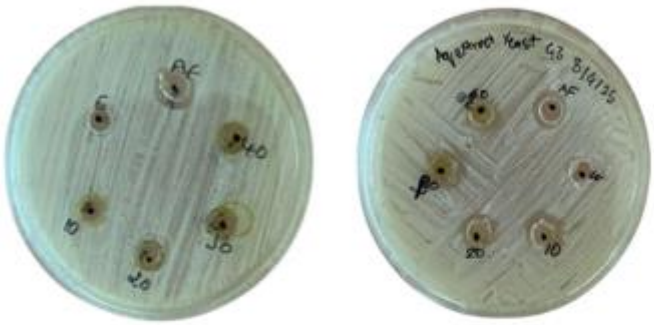
Antifungal Activity

The antifungal activity of Acacia auriculiformis extracts was evaluated against Saccharomyces cerevisiae using the agar well diffusion method. The ethanol extract produced inhibition zones ranging from 8 mm to 12 mm, while the aqueous extract consistently showed 10 mm zones at higher concentrations of 30 µg/µL and 40 µg/µL. These results suggest that A. auriculiformis possesses moderate antifungal potential, with the ethanol extract exhibiting slightly greater efficacy than the aqueous counterpart.

Extract	Zone of inhibition in mm					
	Saccharomyces cerevisiae					
	Negative	Amphotericin B	10 µg	20 µg	30 µg	40 µg
Aqueous	-	12	-	-	10	10
Ethanol	-	11	8	10	10	12

Table 4: Zones of inhibition observed against Saccharomyces cerevisiae

Fig 2: Antifungal activity by agar well diffusion method against Saccharomyces cerevisiae



Ethanol extract Aqueous extract

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) assay was conducted to determine the lowest concentration of Acacia auriculiformis extract required to inhibit visible growth of E. coli and Staphylococcus aureus. However, the results showed no inhibitory activity at any tested concentration from 25 µg/µL up to 100 µg/µL for both ethanol and aqueous extracts. This finding contrasts with several published reports that observed moderate MIC values for A. auriculiformis extracts. For example, Singh et al. (2010) reported MIC values ranging from 2–6 mg/mL against S. aureus and E. coli, and Pennacchio et al. (2006) found antimicrobial activity in methanolic leaf extracts. The absence of activity in the current study could be due to several factors: differences in extract concentration, extraction efficiency, solvent polarity, plant chemotype (regional variation), or strain-specific bacterial resistance. Additionally, the lack of MIC effect may suggest that the bioactive compounds present in the crude extracts are either not sufficiently concentrated, or require synergistic mechanisms that are not strong enough in diluted conditions. These findings emphasize the importance of compound isolation and extract standardization, as crude extracts often show variable activity depending on their chemical complexity and preparation methods.

	E.coli				*S.aureus*			
Extract(µg/µL)	25	50	75	100	25	50	75	100
Aqueous	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-

Table 5: MIC of AE and EE against E. coli and S. aureus

Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) assay was conducted to evaluate the bactericidal potential of the extracts. Both ethanol and aqueous extracts showed bacterial growth at all tested concentrations 25 µg/µL –100 µg/µL against Staphylococcus aureus and Escherichia coli. This indicates a lack of bactericidal activity under the tested conditions, suggesting the need for extract optimization or the use of alternative extraction methods to enhance efficacy.

Extract	Concentration(µg/µL)	*S. aureus*	*E.coli*
Aqueous	25	+++	+++
	50	++	++
	75	+	+
	100	+	+
Ethanol	25	+++	+++
	50	++	+++
	75	+	++
	100	+	++

Table 6: MBC of AE and EE against E. coli and S. aureus

Biofilm Inhibition Assay

The anti-biofilm activity of the aqueous extract of Acacia auriculiformis was assessed using the crystal violet staining method against two bacterial strains: Staphylococcus aureus and Escherichia coli. The results, shown in Figure 3, revealed a differential and concentration-dependent inhibition pattern for each organism. For S. aureus, the aqueous extract exhibited steadily increasing inhibition with rising concentration. At 25 µg/µL, the inhibition was approximately 9–10%, increasing to about 20% at 50 µg/µL, ~28% at 75 µg/µL, and reaching a maximum of ~34–35% at 100 µg/µL. This clear dose-dependent response indicates that the aqueous extract contains bioactive compounds capable of interfering with biofilm formation, likely through disruption of early

adhesion or extracellular matrix production. In contrast, for E. coli, the inhibition response was less consistent. The extract showed a moderate inhibition of ~6% at 25 µg/µL, which increased to ~15% at 50 µg/µL. However, the inhibition declined at higher concentrations, falling to ~10% at 75 µg/µL and ~9% at 100 µg/µL. This suggests a weaker or possibly non-specific anti-biofilm effect on Gram-negative E. coli, potentially due to the protective nature of its outer membrane or reduced extract penetration. These results highlight the extract's greater efficacy against Gram-positive biofilms, particularly S. aureus, which is consistent with trends seen in antibacterial activity. Similar anti-biofilm effects have been observed in related Acacia species, such as A. nilotica and A. macrostachya, where phenolic compounds and tannins disrupt microbial communication and biofilm integrity (Sasidharan et al., 2012; Salih et al., 2021). Overall, the aqueous extract of Acacia auriculiformis demonstrated moderate but meaningful biofilm inhibition, especially against S. aureus, supporting its potential in the development of anti-biofilm therapies.

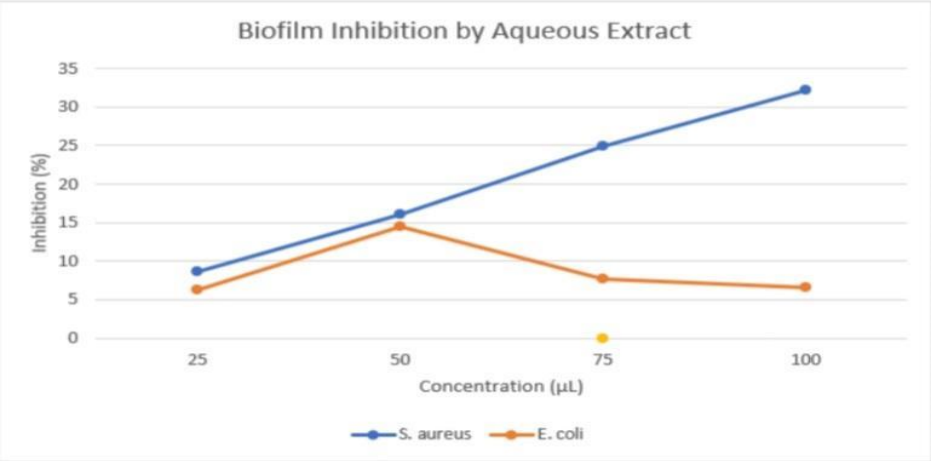


Fig 3: Biofilm inhibition by aqueous extract against S. aureus and E. coli

Antioxidant Activity (DPPH Assay)

The antioxidant potential of the aqueous and ethanol extracts of Acacia auriculiformis was assessed using the DPPH radical scavenging assay, and results are presented in Figure 4. Ascorbic acid was used as the standard reference. The ethanol extract (EE) demonstrated a concentration-dependent increase in DPPH radical inhibition. At the lowest concentration 0.5 µg/µL, it showed 11.05% inhibition, which increased to 15.08%, 26.34%, and peaked at 42.46% inhibition at 2.0 µg/µL. In contrast, the aqueous extract (AE) showed much lower activity, beginning with just 0.19% inhibition at 0.5 mL and reaching only 15.21% at the highest concentration tested 2.0 µg/µL. The ascorbic acid standard, as expected, exhibited strong antioxidant activity, showing 31.53% inhibition at 0.5 µg/µL and increasing to 89.52% at 2.0 µg/µL. While both plant extracts fell short of the standard, the ethanol extract demonstrated meaningful antioxidant

capacity compared to the aqueous extract. These results confirm that the ethanol extract is more effective in extracting antioxidant phytochemicals such as flavonoids and phenolic compounds, which are known to neutralize free radicals. The relatively low activity in the aqueous extract may be due to limited solubility of certain bioactive compounds in water. When compared to existing literature, Singh et al. (2010) and Sathya & Siddhuraju (2012) also reported that ethanol or acetone extracts of A. auriculiformis bark exhibited higher antioxidant activity, with IC_{50} values typically in the tens of µg/mL. The trend observed in the present study aligns with these findings and supports the use of ethanol as a preferred solvent for isolating antioxidant compounds from this species. Overall, the antioxidant assay results underscore the potential of A. auriculiformis ethanol extract as a moderate natural antioxidant source, validating its ethnomedicinal relevance and suggesting further investigation into purified fractions or isolated compounds.

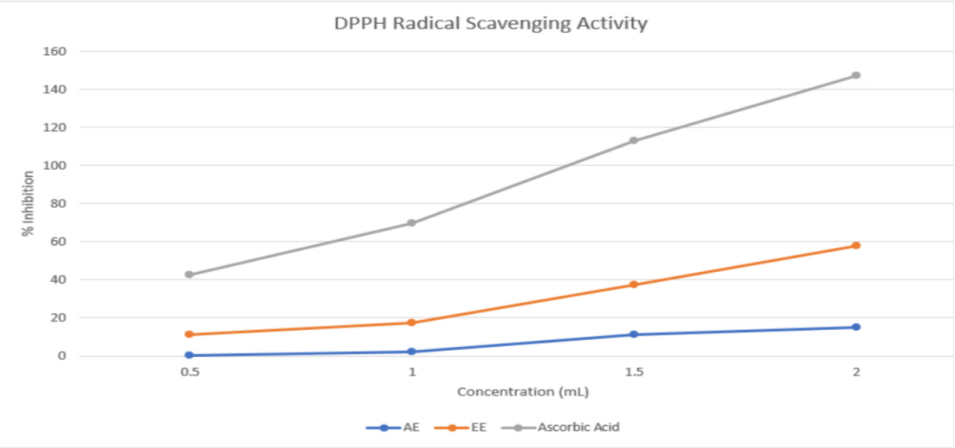


Fig 4: DPPH radical scavenging activity by aqueous and ethanol extracts

DISCUSSIONS

This study aimed to investigate the phytochemical composition and evaluate the biological activities of aqueous and ethanol leaf extracts of Acacia auriculiformis collected from Sholinganallur, Chennai. A range of in vitro assays were employed, including thin-layer chromatography (TLC), TLC-DPPH bioautography, antioxidant activity via DPPH assay, antibacterial activity (agar well diffusion), minimum inhibitory and bactericidal concentration (MIC and MBC), and biofilm inhibition testing. These methods collectively assessed the plant's potential for antimicrobial and antioxidant applications. The results revealed that both extracts contain multiple phytochemical constituents, as evidenced by TLC separation and bioautography assays. The chloroform system yielded the highest number of separated compounds and antioxidant-active spots, particularly for the aqueous extract. Ethanol extracts consistently showed superior performance in antibacterial and antioxidant assays. The DPPH scavenging assay confirmed moderate antioxidant activity, especially for the ethanol extract, which reached 42.46% inhibition at the highest concentration. Meanwhile, the aqueous extract showed lower but measurable antioxidant activity.

The biofilm inhibition assay showed a gradual, concentration-dependent inhibition against S. aureus by the aqueous extract (up to ~35% at 100 µL), while inhibition of E. coli biofilms was weak and non-dose-dependent. This again highlights the extracts' greater efficacy toward Gram-positive bacteria. Overall, the results align with and expand upon previous literature on A. auriculiformis, confirming that its leaf extracts contain phenolic and flavonoid compounds responsible for moderate antioxidant. These findings support its traditional use in treating infections and inflammatory conditions and offer insight into its potential application as a source of plant-based antioxidant agents. A more detailed analysis and interpretation of the individual assays is provided in the subsequent subsections that follow.

The findings of this study lend scientific support to the traditional medicinal use of Acacia auriculiformis in treating microbial infections and inflammatory conditions. The presence of flavonoids, tannins, and saponins — all known for their antimicrobial and antioxidant properties — provides a biochemical rationale for its ethnopharmacological applications in wound healing, gastrointestinal disturbances, and skin infections (Sathya & Siddhuraju, 2012; Singh et al., 2010). The demonstrated antibacterial and antioxidant activities, particularly from ethanol extracts, highlight the potential of A. auriculiformis as a source of herbal-based antimicrobial agents and natural antioxidant supplements. In an era of increasing antibiotic resistance and demand for safer alternatives, phytochemically rich plant extracts offer a promising route to develop polyherbal formulations or as lead candidates in pharmaceutical development (Newman & Cragg, 2020). Importantly, phytochemicals often exert multi-targeted mechanisms of action. For example, flavonoids not only disrupt microbial membranes but also chelate metal ions and inhibit nucleic acid synthesis. Tannins can inactivate microbial enzymes, disrupt membrane integrity, and prevent biofilm formation (Crozier et al., 2009). Such multi-functional behaviour contrasts with many synthetic antibiotics that act on a single

target, making phytochemicals valuable in both mono- and combinatorial therapies. Additionally, antioxidant activity associated with these phytochemicals can reduce oxidative stress, a major factor in the pathogenesis of chronic diseases such as cancer, neurodegeneration, and cardiovascular disorders (Kris-Etherton et al., 2002). Thus, the results not only support the therapeutic claims of traditional medicine but also open pathways for future clinical applications. While the findings are promising, several limitations must be acknowledged. Firstly, the study was conducted using in vitro assays only. Although these provide critical initial insights, they do not account for the complex interactions that occur in living organisms. Therefore, in vivo studies in animal models or clinical settings are necessary to validate the pharmacological relevance of the observed effects. Secondly, the study did not include quantitative phytochemical analysis (e.g., total phenolic content, flavonoid concentration, or high-performance liquid chromatography profiling). Such data could have allowed more precise correlation between bioactivity and phytochemical content. Thirdly, the biofilm inhibition assay was conducted only for the aqueous extract, and only one microbial strain (S. aureus) was tested. Testing across multiple clinically relevant, biofilm-forming strains — including Gram-negative species — would provide a broader understanding of the plant's anti-biofilm spectrum. Finally, isolation and structural characterization of the specific active compounds were not performed. While TLC and bioautography suggest the presence of active constituents, confirming their identity through techniques such as GC-MS, LC-MS, or NMR spectroscopy is crucial for drug development. These limitations underscore the need for comprehensive follow-up studies, including phytochemical standardization, mechanism-of-action studies, and toxicity profiling, to fully unlock the therapeutic potential of Acacia auriculiformis.

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