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Phytochemical Analysis and Antimicrobial Activity of Pakistani Medicinal Plants used as Antibacterial Agents Against Escherichia coli O157:H7

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Medicinal Plants are a rich source of bioactive compounds having anti-microbial properties and effectiveness against infectious diseases, have a greater affinity to mitigate antibiotic resistance, and to produce alternative, economical veterinary therapeutics. This study aimed to determine the phytochemical composition of *Azadirachta Indica, Melia Azedarach, Withania Coagulans,* and *Nigella Sativa,* and their antimicrobial properties against *E. coli O157:H7*. The plants were sequentially extracted using hydro-methanolic solvent. Phenolic acids (Gallic acid, Sinapic acid, Caffeic acid) and Flavonols (Myricetin, Quercetin, Kaempferol) were quantified using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). Antibacterial activity was assessed using the Agar Well diffusion assay at concentrations of 100–400 mg/mL, with ciprofloxacin as the positive control. HPLC revealed gallic acid as the predominant phenolic compound in *A. indica* (83.28%), *M. azedarach* (83.36%), and *W. coagulans* (98.61%), while sinapic acid dominated in *N. sativa* (74.59%). Flavonol composition varied, with kaempferol highest in *A. indica* (80.84%), myricetin in *M. azedarach* (63.08%) and *N. sativa* (100%), and quercetin in *W. coagulans* (65.92%). Antibacterial activity was dose-dependent, with the hydro-methanolic extract showing the highest activity at 400 mg/mL. *M. azedarach* exhibited the largest inhibition zone (12.21 ± 1.16 mm), followed by *N. sativa* (9.03 ± 1.47 mm), *A. indica* (8.98 ± 1.97 mm), and *W. coagulans* (8.81 ± 1.39 mm). The study concluded that the medicinal plants used were rich in phenolic compounds, showing strong antimicrobial properties against *E. coli* O157:H7, and can be used as a promising candidate for an affordable antibiotic alternative.

Introduction

Antimicrobial resistance (AMR) represents one of the most challenging global public health concerns. Indiscriminate use of antibiotics in animals accelerated the emergence of multidrug-resistant pathogens, as reported in a previous study, β-lactam with (86.6%) resistance, followed by Tetracycline (80%), aminoglycosides (66.66%), and Gentamicin (60%) show alarming resistance rates among *E. coli* O157:H7 isolates (Khan et al., 2025; Khan et al., 2026).

AMR has a socio-economic impact on the rural livelihood of Pakistan; the food animals are their only source of income, and dogs are their source of security. During infections, the high cost of antibiotics and emerging issues of multidrug-resistant pathogens greatly ruined their economy, necessitating the production of alternative antibiotic strategies to overcome the burning issues and to relieve their economy (Umair et al., 2024; Muneeb et al., 2026). Pakistan is blessed with a variety of ethnoveterinary medicinal plants and possess significant potential against various infectious agents. Recently, 114 Pakistani medicinal plants have been documented by Muhammad et al. (2025) in Punjab for treating gastrointestinal disorders, Mastitis and wound infections. Conventional antibiotics targeted single bacterial processes, predisposing them to rapid resistance development. Interestingly, plant-derived phytochemicals control through multi-target mechanisms. Phenolic acids and flavonoids simultaneously disrupt bacterial membranes, inhibit efflux pumps, and interfere with quorum sensing (Al-Arnoot et al., 2025). A recent investigation demonstrated that plant-derived phenolic acids can restore tetracycline efficacy against MDR *E. coli* through multi-target disruption of resistance mechanisms, including preferential binding to efflux pump proteins (Peng et al., 2025; Perera et al., 2026; Wiles et al., 2025).

Azadirachta indica (*A. indica*), also called Neem in Pakistan, has been reported to have antimicrobial activity against both Gram-positive and Gram-negative organisms, including *E. coli*, and additionally has activity against drug-resistant infections (Mudenda et al., 2023). *Melia azedarach* is among the plants that have been confirmed to have a greater quantity of Phytochemicals i.e., phenols, flavonoids alkanoids and tannins (Abolarinwa et al., 2024) and is traditionally used for treating various infectious conditions in livestock. *Nigella sativa* yields thymoquinone and other bioactive constituents exhibiting antimicrobial, antioxidant, and immunomodulatory activities. *Withania coagulans* is valued for its therapeutic applications in infectious and inflammatory disorders (Jia et al., 2025; Nand et al., 2025; Khan et al., 2023).

Due to the evidence-based medicinal effects of *Azadirachta indica, Melia azedarach, Withania coagulans,* and *Nigella sativa,* The present study was designed to comprehensively investigate the phytochemical analysis and antibacterial activity of four ethnomedicinal plants with specific objectives: (1) to quantify phenolic acid (gallic, Sinapic, caffeic) and flavonol (myricetin, quercetin, kaempferol) contents using RP-HPLC. (2) to evaluate in vitro antibacterial activity of extracts prepared with hydro-methanolic solvent against *E. coli O157:H7*. (3) to establish correlations between phytochemical profiles and antibacterial efficacy.

Material and Methods

Samples Collection and Identification

Fresh leaves of *Azadirachta indica* (AI) and *Melia azedarach* (MA) were collected from the Pattoki campus, a sub-campus of UVAS, Lahore, Pakistan. Dust-free seeds of *Nigella sativa* (NS) and *Withania coagulans* (WC) were purchased from a local market in Lahore, Pakistan. The plants were identified by Dr. Zaheer-ud-din Khan at the Department of Botany, University of Lahore, Pakistan. Voucher specimens were deposited in the herbarium with the following numbers: GC. Herb. Bot. 3668 (AI), GC. Herb. Bot. 3669 (MA), GC. Herb. Bot. 3670 (NS), and GC. Herb. Bot. 3671 (WC).

Chemicals

Standards of phenolic acids (sinapic acid, caffeic acid, gallic acid) and flavonoids (myricetin, quercetin, kaempferol) were provided by Sigma Chemicals Co. (St. Louis, MO, USA). Hydrochloric acid, ethanol, acetonitrile, and methanol were supplied by Merck (Darmstadt, Germany). Stock solutions of flavonoids and phenolic acids were prepared in methanol at a concentration of 10 µg/ml and stored in the dark at 4 °C. Calibration curves were constructed by comparing peak area to concentration.

Test Microorganism

The bacterial strain used in this study, *Escherichia coli O157:H7*, previously isolated (Khan et al., 2022), was obtained from the Animal Research and Health Laboratory in the Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan.

Medicinal Plant Extraction

Leaves were washed with distilled water, and seeds were sieved to remove debris. Both were shade-dried at room temperature for at least 15 days. The dried materials were weighed and ground separately into a coarse powder using an electric blender and stored in air-tight containers. The hydro-methanolic extracts of AI, MA, NS, and WC were extracted as per the technique of (Daba et al. 2026)

HPLC Analysis

High-performance liquid chromatography (LC-110A) coupled with a CTO-10A column oven, SPD-10A UV-vis detector, and data acquisition class LC-10A software was employed to quantify phenolic acids and flavonols in the hydro-methanolic extracts of AI, MA, NS, and WC, as detailed by Ullah et al. (2022). Column chromatography using silica gel was used to separate the extracts by increasing solvent polarity.

Antimicrobial Activity

The microbial susceptibility assay was carried out using the agar well diffusion method on nutrient agar against *E. coli O157:H7*. Nutrient agar (14 g in 500 mL distilled water) was prepared, and 15-20 mL was poured per Petri dish. The culture density for the antimicrobial assay was adjusted to 0.5 McFarland standard, and 0.5 mL of pure culture was swabbed onto sterile nutrient agar plates using a sterile cotton swab. Four wells of 5 mm diameter were made in each plate using a sterile borer. The wells were filled with herbal extracts at different concentrations (100, 200, 300, and 400 mg/mL). A ciprofloxacin (5 µg) disk was used as a positive control. The plates were incubated for 24 hours at 37 °C. The zones of inhibition were measured using a Vernier caliper.

Statistical Analysis

The results from each sample were analyzed in triplicate, and the average (n=3 ± SD) is presented. Analysis of variance (ANOVA) was conducted using Minitab 2000 Version 13.2 (Minitab Inc., PA, USA). The threshold for a statistically significant difference was set at $P < 0.05$.

Results

Antibacterial Activity

The results of antimicrobial effectiveness (zones of inhibition) of *Azadirachta indica*, *Melia azedarach*, *Withania coagulans*, and *Nigella sativa* were found to be promising against *E. coli* O157:H7, as shown in Figure 1. The result showed that the zone of inhibition was significantly higher in the control group (18.0±1.30 mm) compared to the treatment group. At a concentration of 100 mg/ml, the extract exhibited very little antibacterial activity against *E. coli* O157:H7, while activity was enhanced by increasing the concentration to 400 mg/ml. However *Melia azadarach* showed higher efficacy against *E. coli* O157:H7, while the antimicrobial effect of it was concentration-dependent.

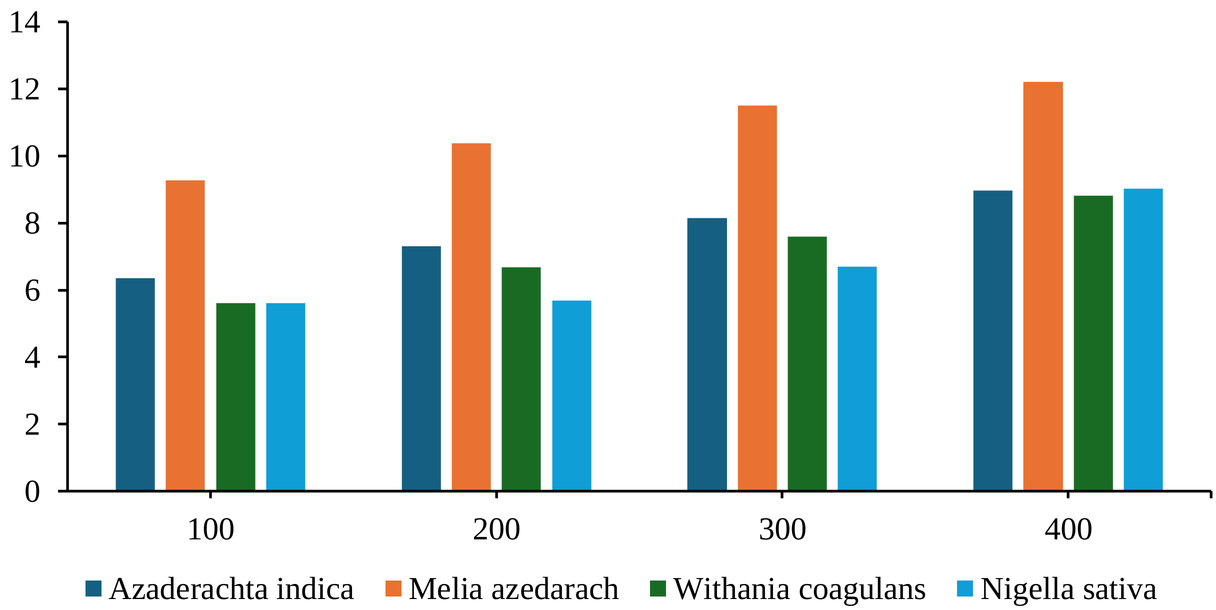

Fig. 1. In Vitro Antibacterial Sensitivity Analysis of Plant Extracts Against *E. coli O157:H7*

Phytochemical Analysis of Hydro-Methanolic Plant Extracts by HPLC

The hydro-methanolic extract obtained from the leaves of *Azadirachta indica*, *Melia azedarach*, *Withania coagulans*, and seeds of *Nigella sativa* were screened for various phytochemicals using reverse-phase high-performance liquid chromatography (RP-HPLC). The analysis targeted three phenolic acids (gallic acid, sinapic acid, and caffeic acid) and three flavonols (kaempferol, myricetin, and quercetin). The retention times (RT) and percent peak areas for each compound were recorded to determine their relative abundance in the plant samples.

Phenolic Acids

The HPLC analysis revealed the presence of all three phenolic acids in most of the plant extracts, with considerable variation in their concentrations.

For *Azadirachta indica*, caffeic acid exhibited the highest retention time (8.277 min), followed by sinapic acid (6.573 min) and gallic acid (3.644 min). The percent peak area was highest for gallic acid (83.280%), followed by caffeic acid (12.498%) and sinapic acid (4.222%).

In *Melia azedarach*, a similar elution order was observed, with caffeic acid showing the highest retention time (8.266 min), followed by sinapic acid (6.536 min) and gallic acid (3.639 min). Gallic acid again constituted the major phenolic compound (83.358%), with caffeic acid (11.762%) and sinapic acid (4.880%) present in lower proportions.

The chromatogram of *Withania coagulans* showed sinapic acid with the highest retention time (7.843 min), while gallic acid eluted earlier (3.643 min). Gallic acid was overwhelmingly dominant, comprising 98.605% of the detected phenolic acids, with sinapic acid accounting for only 1.395%.

For *Nigella sativa*, caffeic acid demonstrated the highest retention time (8.280 min), followed by sinapic acid (6.373 min) and gallic acid (3.760 min). In contrast to the other plants, sinapic acid was the predominant phenolic compound in *N. sativa* (74.592%), followed by caffeic acid (18.568%) and gallic acid (6.840%). A detailed summary of the phenolic acid profiles is presented in Figure 2 and 3.

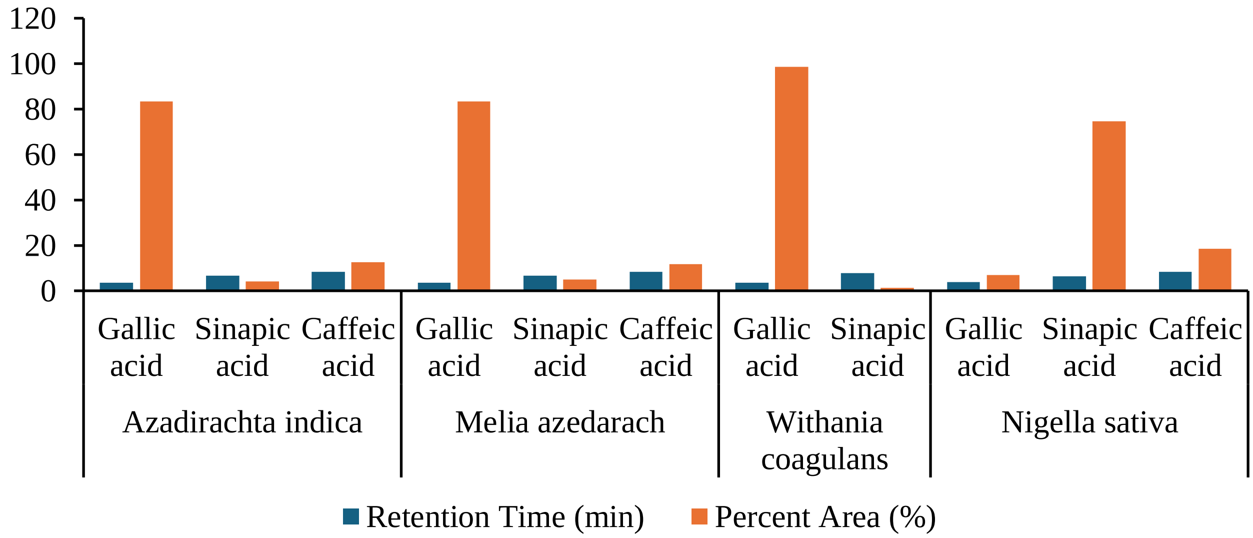

Fig. 2. Phenolic Acid Composition of Hydro-Methanolic Plant Extract by HPLC

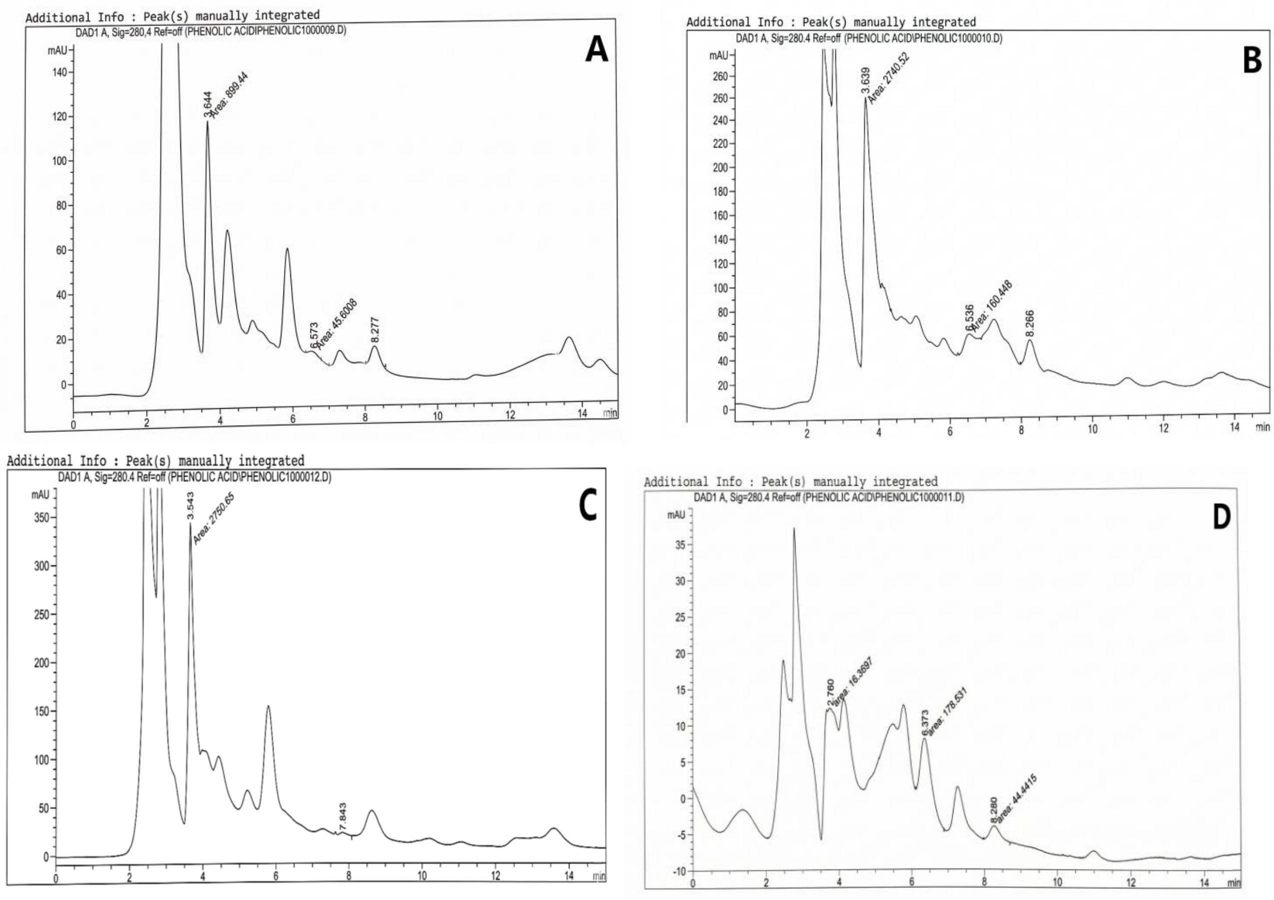

Fig. 3. Graph shows phenolic contents in herbs through HPLC

Flavonols

The hydro-methanolic extract was analyzed for the presence of three flavonols: myricetin, quercetin, and kaempferol. The flavonol composition varied considerably among the four plant species (Figure 4 and 5).

In *Azadirachta indica*, kaempferol exhibited the highest retention time (15.907 min) and was the dominant flavonol, comprising 80.841% of the total detected flavonols. Quercetin was also present, eluting at 9.860 min and accounting for the remaining 19.159%. Myricetin was not detected in this sample.

The chromatogram of *Melia azedarach* revealed the presence of myricetin (RT: 5.927 min) and quercetin (RT: 9.870 min). Myricetin was the predominant flavonol (63.079%), followed by quercetin (36.921%). Kaempferol was not detected.

For *Withania coagulans*, both myricetin (RT: 6.447 min) and quercetin (RT: 9.627 min) were detected. Quercetin was the major component (65.918%), while myricetin constituted 34.082% of the flavonol content. Kaempferol was absent.

Nigella sativa showed the presence of only one flavonol, myricetin, which eluted at 6.407 min and represented 100% of the detected flavonols.

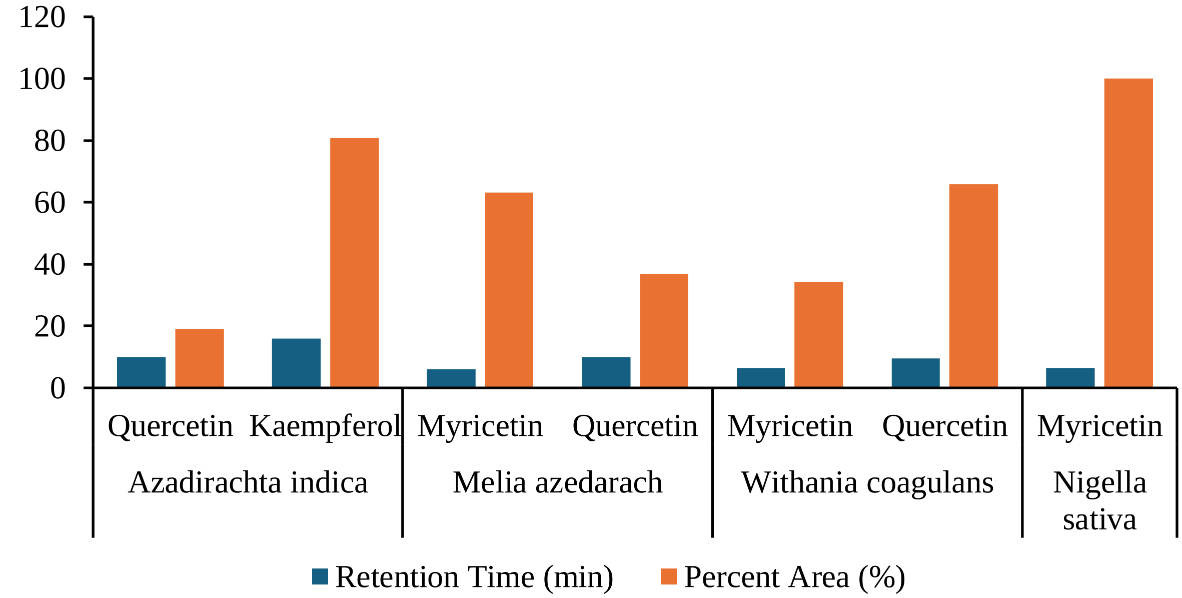

Fig. 4. Flavonol Composition of Hydro-Methanolic Plant Extracts by HPLC

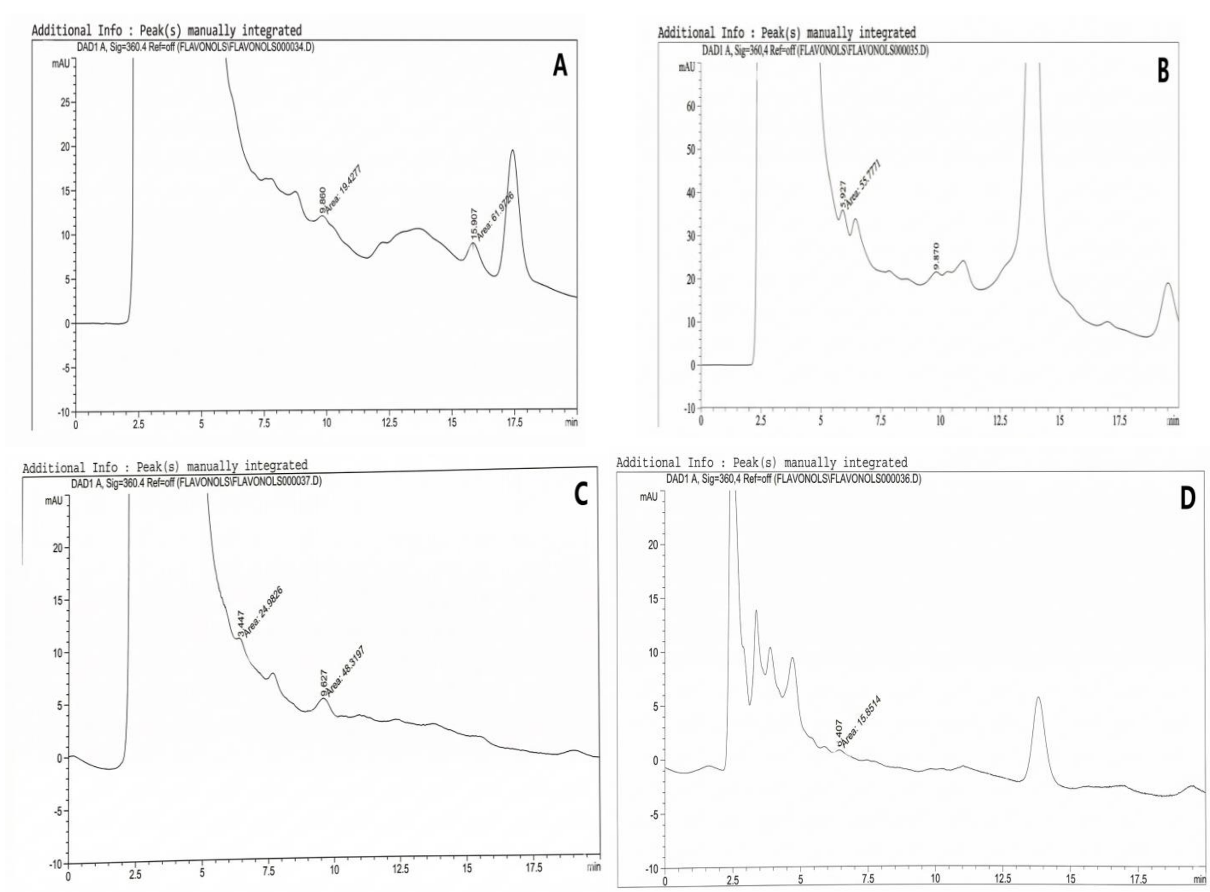

Fig. 5. Graph represents Flavonols contents in different herbs through HPLC

Discussion

This study investigated the total phenolics and flavonols, including Sinapic acid, Caffeic acid, Gallic acid, kaempferol, Myricetin, and Quercetin in the *Melia azedarach*, *Azadirachta indica*, *Withania coagulans*, and *Nigella sativa* samples (Tables 2 & 3). The presence of these bioactive compounds provides a scientific basis for the traditional use of these plants in managing infectious diseases. The variation in the quantity and composition of total phenolics and flavonols observed in this study is consistent with findings reported for medicinal plants from other regions, including China, Brazil, and Taiwan, highlighting the influence of genetic and environmental factors on secondary metabolite profile. Such chemotypic variations are expected and underscore the importance of standardizing plant materials for medicinal use.

Melia azedarach leaves revealed richness in phenolic acids (gallic acid, sinapic acid, caffeic acid) and flavonols (myricetin, quercetin). Javid et al. (2025) similarly stated that phytochemical compounds, including phenols, flavonoids, alkaloids, tannins, glycosides, steroids, saponins, and terpenoids in *M. azedarach* are responsible for its antimicrobial properties. The high concentration of these phytochemicals in our samples explains the pronounced antibacterial activity observed against *E. coli O157:H7*. The antibacterial activity observed in this study can be directly correlated with the phytochemical constituents identified through HPLC analysis. This correlation between chemical composition and biological activity strengthens the validity of our findings.

In *Azadirachta indica*, the presence of gallic acid, sinapic acid, and caffeic acid as phenolic compounds, along with quercetin and kaempferol as flavonols, supports the findings of Dash et al. (2017), who reported that leaves of *A. indica* contain high levels of flavonoids, alkaloids, and glycosides contributing to their significant antibacterial activity. The dominance of gallic acid (83.28%) in *A. indica* is particularly noteworthy, as gallic acid is known for its strong antimicrobial properties through membrane disruption and enzyme inhibition. The present study revealed the presence of myricetin, quercetin, gallic acid, and sinapic acid in *Withania coagulans* through HPLC analysis. Previous studies by Qureshi et al. (2019) and Arif et al. (2025) also reported the presence of gallic acid and quercetin in *W. coagulans*, supporting our findings. The overwhelming predominance of gallic acid (98.61%) in *W. coagulans* suggests that this compound may be the primary contributor to its antibacterial activity. Seeds of *Nigella sativa* were found to contain gallic acid, sinapic acid, caffeic acid, and myricetin. Interestingly, sinapic acid (74.59%) was the dominant phenolic in *N. sativa*, in contrast to the other plants where gallic acid predominated, indicating species-specific phytochemical profiles. These findings are consistent with Akhtar et al. (2021), who identified gallic acid as a phenolic acid in methanolic extracts of *N. sativa*, and with Kumar et al. (2019), who stated that flavonoids and phenolic compounds in *N. sativa* are responsible for its antibacterial activity.

A key observation of this study was that the antibacterial efficacy varied considerably depending on the solvent used for extraction. Although we ultimately focused on hydro-methanolic extracts for detailed analysis, preliminary comparisons confirmed that Organic extracts, particularly methanolic ones, demonstrated significantly more potent antibacterial activity compared to aqueous extracts. This phenomenon can be attributed to the polarity of secondary metabolites such as phenolic acids and flavonols, which are more soluble in organic solvents than in water. Organic solvents not only extract these bioactive compounds more efficiently but also preserve their bioactivity against bacterial species. This finding has practical implications for the preparation of plant-based therapeutics, suggesting that hydro-methanolic extraction is optimal for harnessing the full antimicrobial potential of these plants.

The present study confirmed the inhibitory effect of hydro-methanolic extract of leaf of *Melia azadarach* through the agar well diffusion method against *E. coli O157:H7*. *Melia azedarach* exhibited maximum antibacterial activity at a 400 mg/mL concentration. The largest inhibitory activity of *Melia azadarach* through hydro-methanolic extract was 12.212 ± 1.159 mm. This dose-dependent response confirms that the antibacterial effect is concentration-related, a characteristic feature of plant-derived antimicrobials. These findings are consistent with Sen and Batra (2012), who reported a larger inhibition zone for methanolic extract of *M. azedarach* against *E. coli* (21.5 ± 0.86 mm) compared to aqueous extract (8.9 ± 0.41 mm). Munir et al. (2017) reported an even higher inhibition zone of 39 mm for methanolic extract of *M. azedarach*, which exceeds our observations, while Touzout et al. (2023) reported a slightly lower value of 14.5 mm. These quantitative variations among studies may be attributed to differences in extraction methods, plant chemotypes, geographical origin, or test conditions. Despite these quantitative variations, our results corroborate the findings of Touzout et al. (2023) and Hemdan et al. (2023), who stated that leaves of *M. azedarach* are effective against Gram-negative bacterial strains. This consistency across multiple studies reinforces the reliability of M. azedarach as a potential antibacterial agent.

Our results are in agreement with Paray et al. (2018), who reported that neem leaf water extract exhibited strong antibacterial activity against *E. coli* with inhibition zones ranging from 10.0 ± 0.0 mm to 15.5 ± 0.71 mm. The zone of inhibition observed for our *A. indica* extract (8.98 ± 1.97 mm at 400 mg/mL) falls within the lower range of these values, which may be due to differences in extraction solvents or plant material potency. The pronounced antibacterial activity of *A. indica* can be attributed to its rich content of phenolic acids and flavonoids (Hemdan et al., 2023), which are known to exert antibacterial effects through their ability to form complexes with bacterial cell wall. This mechanism of action is particularly advantageous as it may slow the development of bacterial resistance compared to single-target synthetic antibiotics.

Earlier studies by Khan et al. (2023) suggested that *W. coagulans* exhibits moderate activity against *E. coli*, which is in confirmation with our findings. Our observed inhibition zone of 8.81 ± 1.39 mm at 400 mg/mL confirms this moderate activity level. Additionally, our results align with previous studies reporting antibacterial activity of *Withania coagulans* against *Pseudomonas aeruginosa* (Gupta et al., 2022), *Staphylococcus aureus* (Asghar et al., 2021), and *Vibrio cholerae* (Khan et al., 1993). This broad-spectrum activity suggests that W. coagulans contains bioactive compounds with activity against diverse bacterial genera. Liaqat et al. (2018) reported that methanolic extracts of *Nigella sativa* possess superior antimicrobial activity compared to aqueous extracts, a finding consistent with our observations. However, our findings diverge from those of Derbal and Niar (2019), who concluded that methanolic extracts of *N. sativa* were ineffective against *E. coli*. This discrepancy may be explained by differences in bacterial strains, extract preparation methods, or the geographical origin of plant materials. In the present study, the highest activity (9.025 ± 1.471 mm) was observed for *N. sativa*. Khan et al. (2013) reported a higher inhibition zone of 23 mm for *N. sativa* against *E. coli*. Such variations highlight the need for standardized protocols in evaluating plant-based antimicrobials. Our findings correlate well with Shafodino et al. (2022), who demonstrated that methanolic extracts of *N. sativa* effectively inhibit *E. coli*. Sulaiman and Muhammad (2023) also reported that both methanolic and aqueous extracts of *N. sativa* are effective against Gram-positive and Gram-negative bacteria, though lower concentrations may be ineffective against Gram-negative strains. This concentration-dependent efficacy against Gram-negative bacteria, which typically exhibit higher intrinsic resistance due to their outer membrane, underscores the potential of *N. sativa* as a source of effective antimicrobial compounds.

Overall, the consistent correlation between phytochemical richness and antibacterial activity across all four plant species validates their traditional use and supports their potential as affordable, plant-based alternatives for managing E. coli infections in veterinary medicine. The dose-dependent activity observed for all extracts suggests that therapeutic efficacy can be optimized through appropriate dosing, while the species-specific phytochemical profiles indicate that each plant may offer unique advantages for particular applications.

Conclusion

In conclusion, the research has established that *Melia azedarach*, *Azadirachta indica, Withania coagulans*, and *Nigella sativa* procured from district Lahore Pakistan, comprise phenolics, which consist of flavonoids and phenolic acids. The anti-microbial activity of these plants were concentration-dependent. *Melia azedarach* exhibits the highest antimicrobial activity against *E. coli* O157:H7. The possibility exhist due to their antimicrobial activity it could be inspire the development of novel pharmaceutical drugs intended to combat *E. coli* Infections.

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