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Investigation of the *in vitro* antibacterial potential of a polyherbal extract against *Escherichia coli*

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Abstract
Antimicrobial resistance is a cosmopolitan issue for poultry industry. Avian pathogenic *E. coli* (APEC) is causative agent for the avian collibacillosis which is resistant to many antibiotics (exa. β-lactams and aminoglycosides). Alternative to antimicrobial drugs such as polyherbal can act as natural antimicrobial source to lessen the antimicrobial resistant. Under this aim present study was conducted to evaluate *in vitro* antimicrobial activity of polyherbal extract against *E. coli* O78. Minimum Inhibitory Concentration (MIC) of polyherbal hydromethanolic extract (50:50) was determined with using standard protocol of micro broth dilution technique with some modification. The Polyherbal formulation included Clove (*Syzygium aromaticum*), Amla (*Emblica officinalis*), Saunf (*Foeniculum vulgare*), Sunth (*Zingiber officinale*), Ajwain (*Trachyspermum ammi*), Garlic (*Allium sativum*), Tulsi (*Ocimum sanctum*), Ashwagandha (*Withania somnifera*) and Galo (*Tinospora cordifolia*). The Minimum inhibitory concentration of polyherbal hydromethanolic extract (50:50) against *E. coli* was found at 6.25 mg/mL, which is indicative of *in vitro* effect of current polyherbal formulation. The presence of different phytochemicals from different plants are responsible for the antimicrobial activity of the polyherbal formulation.

Keywords: Polyherbal extract, *in vitro*, antimicrobial activity, *E. coli* O78

1. Introduction
The poultry industry faces different challenges, ranging from environmental strain to pathological disorders. Among all pathological conditions, broiler production in the India faces higher losses every year because of colibacillosis (Lutful and Kabir, 2010) [1]. *Escherichia coli* (*E. coli*) is a group of bacteria which are a normal part of poultry microflora. *Escherichia coli* is a member of the genus Escherichia and is a gram-negative, facultative anaerobe, rod-shaped, motile, catalase-positive, oxidase negative, coliform bacteria. *E. coli* is mostly found on the skin, feathers, upper respiratory tract, lower intestine (Tenaillon *et al*., 2010; Panth *et al*., 2019) [2, 3]. Avian pathogenic *Escherichia coli* (APEC) infection causes significant financial losses (Christensen *et al*., 2021) [4]. Colibacillosis defined as a localized or systemic infection caused by an APEC which is major cause of morbidity and mortality in the global poultry farming (Guabiraba and Schouler, 2015) [5].
Antibiotics such as tetracyclines, sulfonamides and aminoglycosides are commonly used to treat colibacillosis in chickens. Increasing resistance to APEC for various classes of antibiotics which includes some important antibiotics like betalactams, colistin, and carbapenems indicates that controlling APEC is challenging in future (Kathayat *et al*., 2021) [6].
For centuries, a significant proportion of India's population has utilised traditional medicines (Pandey and Singh, 2011) [7]. To combat a variety of Gram-positive and Gram-negative bacteria, thousands of plant species have also been employed *in vitro* as novel therapeutic medications (Elmowalid *et al*., 2019) [8].
Herbal plants which possess antimicrobial activity reported by various researchers *viz*. *Syzygium aromaticum* (Han and Parker, 2017) [9], *Emblica officinalis* (Hasan *et al*., 2016) [10], *Foeniculum vulgare* (Rather *et al*., 2016) [11], *Zingiber officinal* (Ashraf *et al*., 2017) [12], *Trachyspermum ammi* (Hyder *et al*., 2022) [13], *Allium sativum* (El-Saber Batiha *et al*., 2020) [14], *Ocimum sanctum* (Kumar *et al*., 2022) [15], W*ithania somnifera*

(Paul *et al*., 2021) [16] and *Tinospora cordifolia* (Sharma *et al*., 2019) [17].

2 Materials and Methods

2.1 Herbs utilized to prepare polyherbal formulation

Ingredients used to prepare polyherbal formulation were purchased from open market, identified and validated by botanist at Department of Botany, College of Forestry, Navsari Agricultural University, Navsari, Gujarat-396450. Ingredients include Clove (*Syzygium aromaticum*), Amla (*Emblica officinalis*), Saunf (*Foeniculum vulgare*), Sunth (*Zingiber officinale*), Ajwain (*Trachyspermum ammi*), Garlic (*Allium sativum*), Tulsi (*Ocimum sanctum*), Ashwagandha (*Withania somnifera*) and Galo (*Tinospora cordifolia*) (Figure 1).

2.2 Preparation of Polyherbal Extract

Herbal ingredients were subjected to air shade drying, pulverization and used to prepare polyherbal formulation. The polyherbal mixture was subjected to extraction with hydromethanol (50:50). The extract was concentrated with using rotary vacuum evaporator thereafter preserved in refrigerator at -20°C and used for evaluation of *in vitro* antibacterial activity by standard assay.

2.3 Minimum Inhibitory Concentration (MICs) by Microbroth Dilution Technique

2.3.1 Preparation of Drug Stock Solution and Serial Dilutions

The polyherbal formulation was prepared at a stock concentration of 400 mg/mL using sterile distilled water as the solvent. Serial two-fold dilutions were made to obtain the following concentrations: 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.12 mg/mL, and 1.56 mg/mL. These dilutions were used for MIC testing.

2.3.2 Bacterial strain

The *Escherichia coli* O78 strain was procured from MTCC-CSIR, Chandigarh, and used as the test organism for evaluating antibacterial activity.

2.3.3 Preparation of bacterial suspension

A loopful of *E. coli* O78 culture was inoculated into sterile nutrient broth and incubated overnight at 37°C. The culture turbidity was adjusted to match 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) by measuring optical density (OD) at 625 nm and correcting with either additional culture or sterile broth. To prepare the final inoculum, 100 µL of the adjusted culture was added to 9.9 mL of sterile nutrient broth, yielding a final bacterial concentration of approximately 9×10^6 CFU/mL.

2.3.4 Procedure for broth dilution technique

A sterile 96-well microtiter plate was labeled and arranged appropriately. 100 µL of each polyherbal dilution was added to designated test wells (A1-A8). 100 µL of sterile distilled water (vehicle control) was added to well A10. 100 µL of enrofloxacin (250 µg/mL) was added to well A9 (positive control). 100 µL of the bacterial inoculum (9×10^6 CFU/mL) was dispensed into all wells from A1 to A11. All tests were performed in triplicate to ensure reproducibility. Well A12 served as the sterility control, containing 200 µL of sterile nutrient broth only (no inoculum or drug). All tests were performed in triplicate to ensure reproducibility.

2.3.5 Quality control and colony enumeration

To verify the initial bacterial concentration, 10 µL was withdrawn from the growth control well (A11) immediately after inoculation. This was added to 990 µL of sterile nutrient broth, followed by serial 1:10 dilutions. From the final dilution, 100 µL was spread on nutrient agar plates and incubated at 37 °C for 18-20 hours. Colony-forming units (CFU) were enumerated to confirm the viable bacterial count and match with the McFarland 0.5 standard.

2.3.6 Resazurin-Based Viability Detection and MIC Determination

After 18-20 hours of incubation, 30 µL of resazurin solution (2.5 mg/mL in distilled water) was added to all wells. Plates were then re-incubated for 30 minutes at 37°C. A colour change from blue (resazurin) to pink (resorufin) indicated bacterial metabolic activity and hence growth. The lowest concentration of the polyherbal extract that showed no color change (blue color retained) was recorded as the MIC value. All results were confirmed by repeating the assay three times.

3. Results & Discussion

In the present study, the Minimum Inhibitory Concentration (MIC) of the polyherbal hydromethanolic extract (50:50) was evaluated using a standardized microbroth dilution method, with modifications as described by Wiegand *et al*. (2008) and Sanhueza *et al.* (2017) against *Escherichia coli* O78 (MTCC strain). The MIC was defined as the lowest concentration of extract that inhibited visible bacterial growth, as indicated by the resazurin color change assay. As shown in Figure 2**,** the color transition from blue to pink represents metabolic activity of *E. coli*. In all three replicates (Rows A, B, and C), wells corresponding to concentrations ≥ 6.25 mg/mL retained the blue color, indicating no bacterial growth**,** whereas lower concentrations (3.12 mg/mL and 1.56 mg/mL) turned pink, showing active bacterial metabolism**.** The MIC of the polyherbal extract was consistently observed at 6.25 mg/mL across three independent replicates. The positive control (Enrofloxacin, 250 µg/mL) inhibited bacterial growth completely, whereas the vehicle control showed no inhibitory effect. The observed MIC value of 6.25 mg/mL demonstrates substantial *in vitro* antibacterial activity of the polyherbal extract. The findings of this study align with prior research that highlights the antimicrobial potential of individual and combined plant extracts. Mussarat *et al.* (2021) [28], who reported MIC of 6.25 mg/mL using a methanolic extract of the *Podeena qehwa* formulation, closely matching the present study's MIC. Conversely, Yavanarani and Selvakumar (2024) [30] found a significantly higher MIC (150 mg/mL) for a siddha polyherbal formulation, indicating variability depending on formulation and extraction. Comparatively, Kuncha *et al*. (2019) [27] reported a MIC of 15.62 µg/mL for a different polyherbal extract, and Pandey & Singh (2011) [25] noted a 2.31 mg/mL MIC for methanolic clove extract alone. These discrepancies may arise from variations in plant combination, extraction solvents, bacterial strains, or assay techniques. Ramamoorthy *et al.* (2019) [29] reported a MIC of 50 µg/mL using an MTT assay for a polyherbal extract containing *Areca catechu*, *Azadirachta indica*, and *Syzygium cumini*. A methanolic polyherbal extract containing *Acacia ferruginea*, *Chloroxylon swietenia*, and others showed a 2.92 cm zone of inhibition at 50 mg/mL against *E. coli* MTCC 41 (Kota *et al*., 2017) [26].

The MIC of the polyherbal extract indicates the effective *in vitro* antibacterial activity of the formulation against *E. coli* O78. The effectiveness of this polyherbal extract is likely due to the synergistic effect of multiple bioactive phytoconstituents such as eugenol (Clove), gallic acid (Amla), allicin (Garlic), and withanolides (Ashwagandha). These compounds may exert antimicrobial effects through various mechanisms such as disruption of bacterial cell membranes, inhibition of DNA replication, protein synthesis, and interference with microbial metabolic pathways. The multitarget mode of action of such a complex formulation is particularly advantageous, as it may reduce the probability of resistance development in pathogens like *E. coli*. The choice of hydromethanol (50:50) as the solvent likely enhanced the extraction of both polar and semi-polar compounds, contributing to the antimicrobial activity. The consistency of MIC across replicates supports the reliability of the formulation and the method.

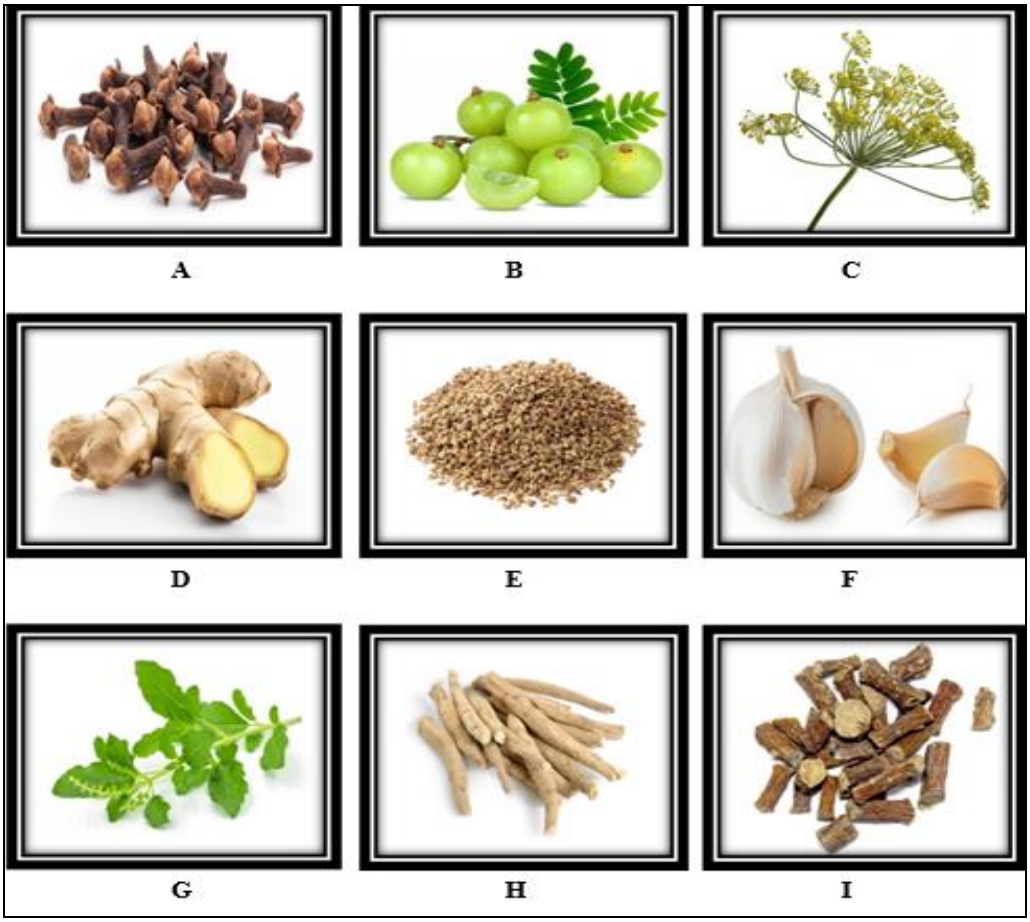

Fig 1: Herbs utilized in the creation of Polyherbal formulation A: Clove (*Syzygium aromaticum*), B: Amla (*Emblica officinalis*), C: Saunf (*Foeniculum vulgare*), D: Sunth (*Zingiber officinale*), E: Ajwain (*Trachyspermum ammi*), F: Garlic (*Allium sativum*), G: Tulsi (*Ocimum sanctum*), H: Ashwagandha (*Withania somnifera*), I: Galo (*Tinospora cordifolia*).

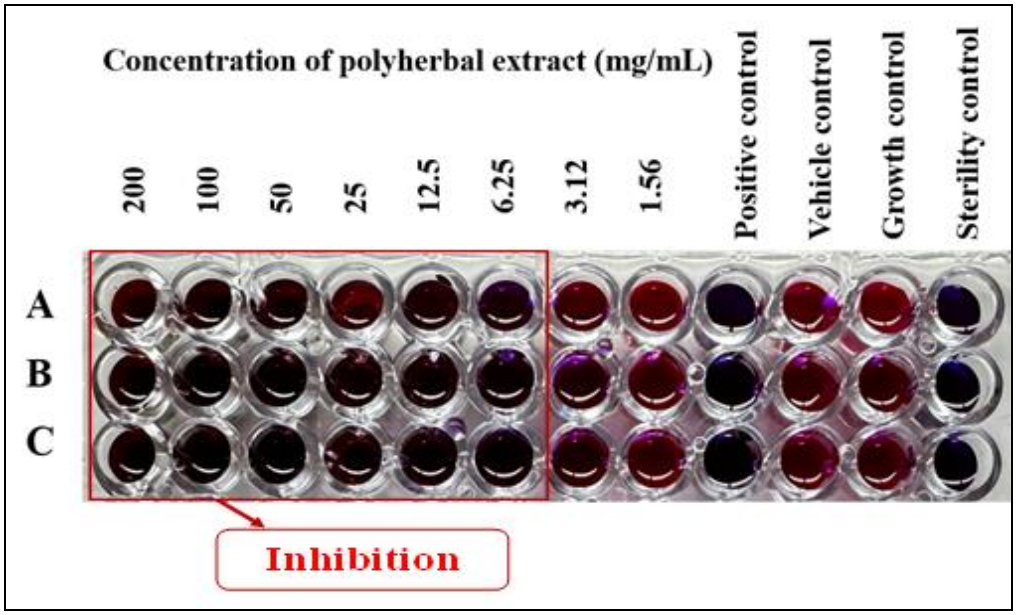

Fig 2: Minimum inhibitory concentration estimation of polyherbal hydromethanolic extract with microbroth dilution technique

5. Conclusion

The polyherbal hydromethanolic extract demonstrated effective *in vitro* inhibitory activity against *Escherichia coli* O78, with a MIC of 6.25 mg/mL. This formulation could serve as a potential phytotherapeutic agent to combat AMR in poultry. Further *in vivo* validation, toxicity profiling, and mechanistic studies are recommended before clinical application.

Conflict of Interest
Not available

Financial Support
Not available

6. Reference

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