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# Exploring the Antimicrobial Potentials of Methanolic Fraction of Ethyl Acetate Extract Derived from Honeycomb Against *Salmonella typhi* and *Paratyphi*

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## Abstract

The study investigates the antimicrobial activity of methanolic fraction $M_E$ of ethyl acetate extract derived from honeycomb against Typhoid and Paratyphoid bacilli, the pathogens responsible for typhoid and paratyphoid fever. Given the rising concern regarding antimicrobial resistance (AMR) among Salmonella strains, this research aims to explore alternative therapeutic approaches utilizing natural products. The methodology encompasses collecting and preparing honeycomb samples, followed by extraction, concentration, and antimicrobial susceptibility testing against standardized bacterial strains. Results indicated that the methanolic fraction of ethyl acetate extract exhibits significant antibacterial properties, potentially inhibiting bacterial growth effectively at $2000\mu g/cm^{3.}$ The Minimum inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of the fractions resistance to *Salmonella typhi* and *paratyphi* was at $2000\mu g/cm^3$. Mechanistic studies utilizing microscopy techniques provide insights into the morphological changes induced by the methanolic fraction of ethyl acetate extract on bacterial cells. Statistical analyses confirm the robustness and significance of the findings. Hence, this research underscores the potential of honeycomb extracts as adjunctive therapies in managing enteric infections, contributing to the search for novel antimicrobial agents in the face of escalating AMR challenges.

**Keywords:** Antimicrobial, Ethyl Acetate Extract, Honeycomb, Methanolic Fraction, *Salmonella typhi, Salmonella paratyphi.*

## 1. Introduction

Typhoid and paratyphoid fever, attributable to *Salmonella typhi* and *Salmonella paratyphi*, respectively, constitute significant global health challenges, especially in areas characterized by inadequate access to clean water and sanitation facilities. These illnesses are principal contributors to both morbidity and mortality rates, particularly within low- and middle-income

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nations (Almasshadany *et al*., 2023; Hooda *et al*., 2023). The increasing prevalence of antimicrobial resistance (AMR) in different *Salmonella* strains heighten the need to create new and effective solution. As efficacy, current therapeutic interventions diminishes due to the increasing resistance, there exists a critical necessity for the formulation of novel and effective strategies to address these enduring public health dilemmas.

Recent studies highlight the increasing prevalence of AMR in *Salmonella* species, underscoring the significant need for alternative treatment strategies (Zhou *et al*., 2023). Natural products are emerging as a valuable candidate for new antimicrobial agents because of their rich chemical compositions and complex biological activities (Gonzalez *et al*., 2023). Among natural products, for century honey has been recognized globally for its healing properties and ability to fight microbes. (Almasaudi *et al.*, 2020). The honeycomb, where honey matures, is a rich reservoir of bioactive compounds derived from beeswax, pollen, propolis, and honey (Guo *et al*., 2022). Recent research has focused on isolating and characterizing bioactive fractions from honeycomb, mainly ethyl acetate extracts. These have demonstrated considerable promise for their antimicrobial properties (Pillai *et al*., 2020).

Specifically, methanolic fractions derived from honeycomb have been shown to exhibit a broader spectrum of antimicrobial activity compared to other fractions. For example, methanolic extracts have demonstrated efficacy against various bacterial pathogens, including those resistant to conventional antibiotics (Hooda *et al*., 2023). This indicates the potential of methanolic extracts from honeycomb as a valuable source of novel antimicrobial agent

Despite the promising antimicrobial potential of ethyl acetate extracts from honeycomb, their effectiveness against *Salmonella typhi* and *Salmonella paratyphi* remains underexplored. The study seek to address this gap by examing the antimicrobial potential of these extracts on the targeted pathogens. The study will elucidate their mechanisms of action and evaluate their potential as adjunctive therapies for managing typhoid and paratyphoid fever. Through the combination of indigenous knowledge with contemporary scientific approaches. This study seeks to uncover novel strategies for tackling the escalating problem pose by antimicrobial resistance.

## 2. Methodology

This methodology outlines the step-by-step process for exploring the antimicrobial potentials of $M_E$ of ethyl acetate extract from honeycomb against *Salmonella typhi* and *Paratyphi* following standard international acceptable procedure from sample collection to data analysis and interpretation based on the obtained results.

### 2.1. Collection, Preparation and extraction of Honeycomb Samples

Honeycomb samples were meticulously collected from local beekeepers (apiaries), ensuring the harvesting process minimizes contamination risks. These samples were stored in clean, airtight containers to preserve their integrity. Subsequently, the honeycomb samples were conveyed to the laboratory under regulated conditions to preserve the integrity transported to the laboratory under controlled conditions to maintain their quality and prevent any external contamination (Svečnjak *et al*., 2023). Honeycomb samples were grounded and crushed to increase surface area, facilitating more efficient extraction of bioactive compounds. The $M_E$ of Ethyl acetate extract was performed using a solvent extraction method and the process involved multiple rounds of solvent extraction and filtration to maximize the yield and purity of the bioactive extracts (Qahtani *et al*., 2022; Guo *et al*., 2022). The ethyl acetate extract was condensed using low pressure to maintain the integrity of the bioactive molecules in the honeycomb, thus preparing the extract for later antimicrobial analysis (Arif *et al*., 2022)

## 2.2. Antimicrobial Susceptibility Testing

Standardized *Salmonella typhi* and *Paratyphi* bacterial strains were obtained from reputable clinical isolates. The antimicrobial susceptibility of the $M_E$ of ethyl acetate extract was assessed using standard microbiological methods, including agar diffusion and broth microdilution assays (Jabeen *et al*., 2023). Test strains were inoculated onto agar plates and broth media containing varying concentrations $M_E$ of ethyl acetate extract, followed by incubation at optimal temperature and humidity conditions to facilitate bacterial growth and accurately measure the antimicrobial effects of the extracts and fractions (Juninda *et al*., 2023).

The diameter of inhibition zones on agar plates, minimum inhibitory concentration (MIC), and minimum bacteriocidal concentration (MBC) in broth microdilution and Agar were assayed and measured to evaluate the antimicrobial potency of the ethyl acetate fractions. The results were then compared with those of standard antimicrobial agents as control groups to assess the relative efficacy of the fractions. MIC values were determined as the lowest concentration of Methanol fraction ethyl acetate extract, which visibly inhibits bacterial growth, quantitatively measuring their antimicrobial activity. MBC values were determined as the lowest concentration of Methanolic fraction $M_E$ of ethyl acetate extract, which visibly kills bacterial growth (Nisa, 2024).

## 2.3. Mechanistic Studies

Vacuum Liquid Chromatography (VLC) and GC-MS were carried out to determine the fraction's bioactive and non-bioactive components. The duo offers valuable insights by enabling the antimicrobial potentials induced by the fractions. Toxicological studies were carried out to determine the fraction's acute and sub-chronic toxicity. Biochemical assays were used to investigate their impact on cellular processes like membrane integrity and enzyme activity, providing a deeper understanding of their antimicrobial effects and potential therapeutic potential against *Salmonella typhi* and *Paratyphi.*

Data obtained from antimicrobial susceptibility testing and mechanistic studies were subjected to rigorous statistical analysis using appropriate methods to clarify the robustness and reliability of the findings. Statistical significance is assessed through analysis of variance (ANOVA). These statistical analyses help identify any significant differences between experimental groups and control conditions, providing insights into the effectiveness of the Methanolic fraction of ethyl acetate extract against *Salmonella typhi* and *Paratyphi* and elucidating their potential mechanisms of action.

# 3. Results

## 3.1.1. Antibacterial activity of fractions obtained from crude ethyl acetate extract of honeycomb

The Microbial activity of methanolic fractions obtained from crude ethyl acetate extract of honeycomb at 2000 µg/cm$^3$ shows that $H_E$ and $C_E$ fractions of ethyl acetate extract demonstrated no inhibitory effect against *S. typhi* and *S. paratyphi* A, B, and C at 2000 µg/cm$^3$. Thus, $EA_E$ inhibited *S. paratyphi* A and C at 2000 µg/cm$^3$ concentration. $M_E$ fraction exhibited a broader spectrum of activity at 2000 µg/cm$^3$ concentration against *S. typhi* and *S. paratyphi* A, B, and C, as shown in Table 1.

**Table 1: Antibacterial activity of fractions obtained from crude ethyl acetate extract of honeycomb**

| Fraction concentration (2000µg/cm$^3$) | | | | |
|---|---|---|---|---|
| Test organism | Hexane fraction of ethyl acetate extract ($H_E$) | Chloroform fraction of ethyl acetate extract ($C_E$) | Ethyl acetate fraction of ethyl acetate extract ($EA_E$) | Methanolic fraction of ethyl acetate extract ($M_E$) |
| *Salmonella typhi* | - | - | - | + |
| *Salmonella paratyphi A* | - | - | + | + |
| *Salmonella paratyphi B* | - | - | - | + |
| *Salmonella paratyphi C* | - | - | + | + |

.+: Activity, -: No Activity

Figure 1 shows the antibacterial activity distribution among the various solvent fractions (Hexane – HE, Chloroform – CE, Ethyl Acetate – EAE, and Methanolic – ME) of honeycomb extract tested at a concentration of 2000 µg/cm³. The figure illustrates a clear dominance of the methanolic fraction (ME), which contributed 50% of the total observed antibacterial activity, exhibiting inhibition against all four tested *Salmonella* species — *S. typhi*, *S. paratyphi A*, *B*, and *C*. The ethyl acetate fraction (EAE) accounted for 25% activity, showing inhibitory action against *S. paratyphi A* and *C*. In contrast, both the hexane (HE) and chloroform (CE) fractions demonstrated no observable antibacterial activity (0%), confirming their inability to extract or retain active antibacterial compounds at the tested concentration.

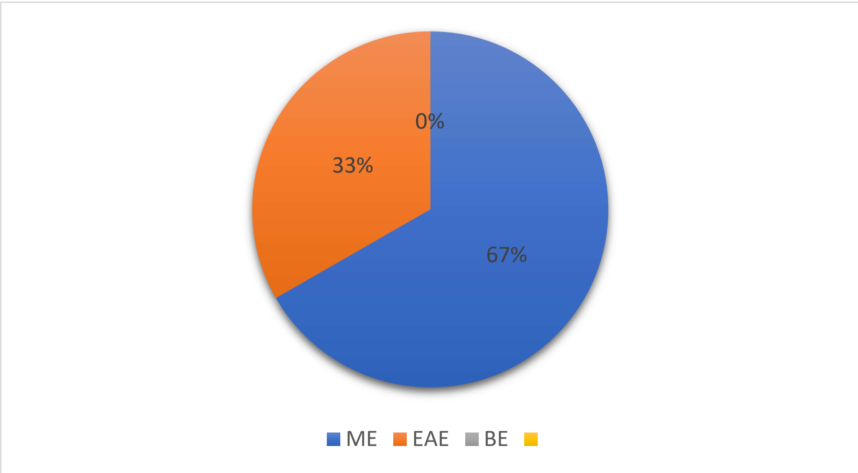

**Figure 1**: Antibacterial activity distribution among fractions of honeycomb extract (2000 µg/cm³)

### 3.1.2. Minimum inhibitory and minimum bactericidal concentration of methanolic fraction of ethyl acetate extract derived from honeycomb

The antibiogram in Table 2 presented MIC and MBC of the $M_E$ of ethyl acetate extract derived from honeycomb against *S.typhi* and *S. paratyphi* C which was at 2000 µg/cm$^3$. The MIC and MBC of the fraction against *S. paratyphi* B was at 4000 µg/cm$^3$ while MIC of fraction against *S. paratyphi* A was at 4000 µg/cm$^3$. However, $M_E$ fraction was not bactericidal against *S. paratyphi* A, it was only effective in inhibiting the growth.

**Table 2**: **Minimum inhibitory and minimum bactericidal concentration of methanolic fraction of ethyl acetate extract derived from honeycomb**

| **Fraction concentration (2000µg/cm$^3$)** | | | | | |
|---|---|---|---|---|---|
| Test organism | 4000 | 3000 | 2000 | 1000 | 500 |
| *Salmonella typhi* | + | + | +*# | - | - |
| *Salmonella paratyphi A* | +* | + | + | - | - |
| *Salmonella paratyphi B* | +*# | + | + | - | - |
| *Salmonella paratyphi C* | + | + | +*# | - | - |

.+: Activity, *: MIC

Figure 2 infers the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the methanolic fraction of the honeycomb extract resist the tested *Salmonella* strains from the distribution. It is evident that 50% of the total antibacterial response (comprising *S. typhi* and *S. paratyphi C*) recorded a concentration of 2000 µg/cm³, indicating strong inhibitory and bactericidal effects at relatively low doses. The remaining 50%, represented by *S. paratyphi A* and *B*, required a higher concentration of 4000 µg/cm³ to achieve inhibition or bactericidal action. This gradient demonstrates the varying sensitivity of different *Salmonella* strains to the methanolic fraction.

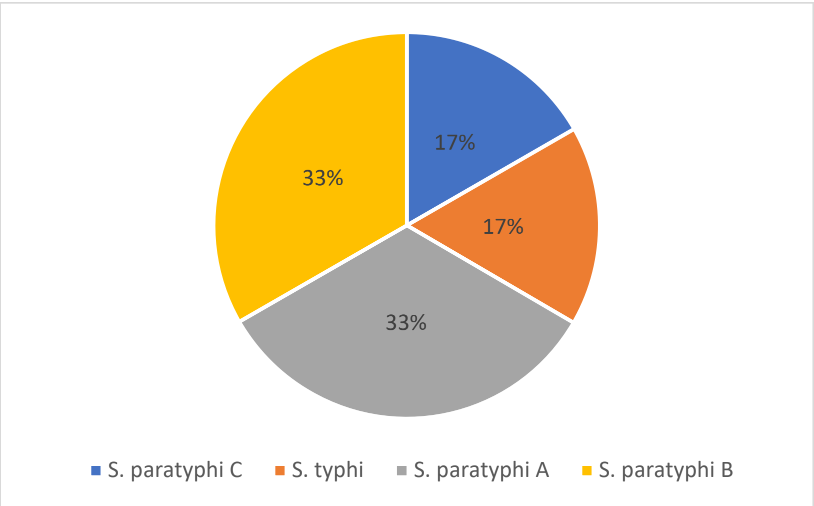


**Figure 2**: MIC and MBC levels of Methanolic fractions against Salmonella Strains (µg/cm³)

## 4. Discussion and Limitations

The observed efficacy of the $M_E$ fraction aligns with existing literature that reports methanolic extracts from various natural sources as effective against a range of bacterial pathogens. For instance, methanolic extracts from different plant species have been documented to exhibit significant antibacterial activity against *Salmonella* species (Arage *et al.*, 2022 : Ngemenya *et a*l., 2022). The broad-spectrum activity of the $M_E$ fraction suggests that it contains bioactive compounds capable of targeting multiple bacterial strains, consistent with studies highlighting methanol as a superior solvent for extracting antimicrobial agents.

The absence of antibacterial activity in the $H_E$ and $C_E$ fractions may be attributed to antimicrobial compounds' solubility properties and extraction efficiencies. Previous research has indicated that hexane and chloroform may not effectively extract certain antimicrobial agents such as flavonoids compared to methanol (Sani, 2022). This selective efficacy observed with the $EA_E$ fraction further supports the notion that different solvents can yield distinct antimicrobial profiles, emphasizing the importance of solvent choice in the extraction process. The results revealed that the methanolic fraction holds promise as a potential source of antimicrobial agents against *Salmonella* infections.

*S. typhi*, both the MIC and MBC were determined to be 2000 µg/cm³, indicating that this concentration is effective for both inhibiting and killing the bacteria. This is consistent with previous studies demonstrating that methanolic extracts from various natural sources can exhibit strong antibacterial activity at comparable concentrations (Duyen *et al.,* 2024 : El- Sawi *et a*l., 2024 : Ngemenya *et al.,* 2022). The findings revealed that the methanolic fraction contains potent antimicrobial compounds capable of targeting *S. typhi* effectively.

Similarly, against *S. paratyphi* C, the MIC and MBC values were also at 2000 µg/cm³, showing effective inhibition and bactericidal activity. This result aligns with the observed broad-spectrum activity of methanolic extracts in other research, where such extracts have been reported to exhibit significant antimicrobial properties against various bacterial strains (Arage, *et al.*, 2022).

In contrast, the MIC and MBC values for *S. paratyphi* B were higher, at 4000 µg/cm³. This suggests reduced potency of the $M_E$ fraction against this strain compared to *S. typhi* and *S. paratyphi* C. Similar variability in antimicrobial efficacy among different bacterial strains has been documented, indicating that the effectiveness of antimicrobial agents can vary widely depending on the target organism (Dahiru, *et al.,* 2023; Sheikh, 2023).

The $M_E$ fraction effectively inhibited the growth of *S. paratyphi* A at 4000 µg/cm³ but did not exhibit bactericidal activity. This finding indicates that while the $M_E$ fraction can suppress the growth of *S. paratyphi* A, it does not kill the bacteria at the tested concentration. Such results highlight the importance of distinguishing between bacteriostatic and bactericidal effects, as noted in previous studies (Nisa, *et al.*, 2024; Phrompanya, *et al.*, 2024). This limitation may affect the therapeutic application of the $M_E$ fraction for *S. paratyphi* A infections.

Specifically, *S. typhi* and *S. paratyphi C* exhibited both MIC and MBC at 2000 µg/cm³, this suggest high susceptibility to the extract. These results align with previous findings by Duyen *et al.* (2024) and El-Sawi *et al*. (2024), who reported potent bactericidal action of methanolic extracts at similar concentrations. In contrast, *S. paratyphi B* showed reduced susceptibility, with MIC and MBC observed at 4000 µg/cm³, while *S. paratyphi A* was inhibited (MIC = 4000 µg/cm³) but not killed, indicating a bacteriostatic rather than bactericidal effect.

Therefore, these results underscore the variable effectiveness of the methanolic fraction against different *Salmonella* strains. The differences in MIC and MBC values suggest strain-specific responses, consistent with findings from similar research where the efficacy of antimicrobial agents varied significantly among different bacterial species (El - Sawi, *et al*., 2024 : Duyen, *et al.*, 2024).

## 5. Conclusions and Future Work

Based on the findings of this study, it was revealed that methanolic fraction $M_E$ of ethyl acetate extract derived from honeycomb shows the most remarkable Antibacterial activity on *Salmonella typhi* and *Paratyphi* A, B, and C, respectively with Significant Correlation to its bioactive Constituents. The phytochemical constituents in the fractions may be responsible for the antibacterial activity. In the present study, the percentage (%) yield of extract decreases as the polarity of solvents increases. Antimicrobial activity was high at 2000 µg/cm$^3$ concentration of extract and fractions. The research revealed that the honeycomb extract and fractions is a promising antibacterial agents. Based on these, the following recommendations were proferred:

1. Refine extraction methods and perform detailed analyses to identify and enhance the bioactive compounds responsible for antimicrobial activity.
2. Study the specific ways in which the methanolic fraction interacts with bacterial cells to improve understanding and effectiveness.
3. Conduct experimental trials to evaluate the tolerability and efficiency of the methanolic fraction as an adjunctive therapy for typhoid and paratyphoid fever.

## Conflict of Interest

The authors have no conflict of interest.

## Author Contributions

**Mustapha Aishatu:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Hafsat Babayi:** Methodology, Validation, Supervision, Resources, Writing – review & editing. **Olusayo Oyeronke Kolo:** Statistical analysis, Interpretation of data, Writing – review & editing, Resources.

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## Ethical Statements

Animal experiments were approved by the Department of Microbiology, Federal University of Technology, and all procedures, including honeycomb sample collection, were conducted according to institutional guidelines and standard biosafety practices.

## Data and Code Availability

The authors do not have the permission to share the data.

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