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IN-VITRO ANTIBACTERIAL ACTIVITY OF ARQE AJEEB ON *ENTEROCOCCUS HIRAE, ESCHERICHIA COLI, PSEUDOMONAS AERUGINOSA*, AND *STREPTOCOCCUS MUTANS*

S. Nagaraj [*1], S. N. Basar [2], L. Banu [1] and N. Sultana [3]

Department of Mahiyatul Amraz (Pathology) [1], Department of Ilmul Saidala (Pharmacy) [2], Department of Kulliyat (Principles of Unani Medicine) [3], Government Unani Medical College Bangalore - 560079, Karnataka, India.

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Antimicrobial, Arq e ajeeb, *Enterococcus hirae, Escherichia coli, Pseudomonas aeruginosa, Streptococcus mutans*

Correspondence to Author:
Dr. Suma Nagaraj

Professor,
Department of Mahiyatul Amraz (Pathology), Government Unani Medical College Bangalore - 560079, Karnataka, India.

E-mail: drsumagumc@gmail.com

ABSTRACT: The increased use of antibiotics and the rise of antibiotic resistance among microorganisms pose significant public health concerns. While several herbal drugs have been validated for their antimicrobial activity, identification of additional antimicrobial agents is necessary. The Unani system of medicine comprises numerous herbs and formulations used for treating various infections. Arq e Ajeeb (AA) is one such formulation, historically utilized during epidemics to address conditions such as cholera (Haiza), tuberculosis (Sil), plague(Taoon), and seasonal fevers. Additionally, AA has been used as an anti-inflammatory and analgesic. This study aimed to evaluate the *in-vitro* antimicrobial activity of AA on four different bacteria, considering antibacterial properties demonstrated by its ingredients. The antibacterial activity of the test sample was evaluated using the minimum inhibitory concentration (MIC) method. Bacterial strains (*P. aeruginosa, E. hirae, E. coli, S. mutans)* were inoculated in 100µl sterile MHB broth. A test sample underwent double dilutions ranging from 100 to 0.048828mg. Ciprofloxacin (0.1%) served as the standard antibiotic. The plates were incubated at 32.5 ± 2.5°C for 24 to 48 h. Resazurin dye was added, and a color change was observed. MIC value, representing the lowest concentration without color change, was determined. AA showed antibacterial activity at concentration 100 to 0.048828 mg. MIC for *E. hirae, E. coli, S. mutans* was 0.048828mg, while that for *P. aeruginosa* was 1.562 mg. AA demonstrated significant antibacterial activity and may serve as a better alternative to conventional drugs. Furthermore, its antifungal and antiviral properties anticipate further investigation into *in-vitro* antimicrobial activity against other microorganisms.

INTRODUCTION: The use of antibiotics and drug-resistance among microorganisms has been increasing, thereby posing a major threat to public health. Herbal formulations may be potential treatment alternatives to conventional antibiotics for various ailments.

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The Unani system of medicine encompasses numerous herbs and formulations used for the treatment of various infections. Although several herbal drugs have been validated for their antimicrobial effects, there is a need to identify additional antimicrobial agents. One such formulation is Arq e Ajeeb (AA).

AA is a liquid preparation obtained by mixing camphor (Kafoor), menthol (Jauhar-e-Pudina), thymol (Jauhar-e-Ajwain) in the ratio 2:2:1 [1, 2, 3, 4]. AA is a traditional medicinal drug that has been used for centuries to treat various ailments such as Nafakh-e-Shikam (flatulence of the stomach),

Ghisyan (nausea), Qai (vomiting), Sue hazm (indigestion), Waja-ul-Meda (stomach ache), Ishal (diarrhea), Waja ul Fawad (cardialgia), Haiza (cholera), Qaulanj (colic), Nazla (catarrha), Zukham (coryza), Lazae Hashrat (irritation by insect bite), Shaqiqa (migraine), and Suda (headache) [2]. AA has been administered orally in a dose of 2–5 drops with warm water and also used locally through application or inhalation.

AA Contains Three Key Ingredients: Camphor (*Cinnamomum camphora*), Menthol (*Mentha arvensis*), and Thymol (*Trachyspermum ammi*). Camphor, obtained from the distillation of camphor tree wood, has diverse biological properties, including insecticidal, antimicrobial, antiviral, anticoccidial, anti-nociceptive, anticancer, and antitussive effects [5] menthol is extracted from partially dried leaves of *Mentha arvensis* and has moderate antibacterial activity against both Gram-positive and Gram-negative bacteria [6, 7]. Thymol (*Trachyspermum ammi*), found in the oil of thyme and extracted from Ajwain, has strong antiseptic properties, and inhibits the growth of Gram-positive and Gram-negative bacteria. These compounds also possess antifungal and antibiofilm effects [8].

Although AA-oil has been utilized since ancient times, data on its efficacy against different bacterial infections is lacking. The present study aimed to assess the antibacterial efficacy of AA using the minimum inhibitory concentration (MIC) test. MIC refers to the lowest concentration of an antimicrobial agent that effectively inhibits the growth of a bacterial culture.

In this study, we evaluated AA-oil for its antibacterial activity against *Enterococcus hirae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus mutans*. These organisms were selected because they are known to be associated with various infections and diseases.

E. hirae is a Gram-positive coccus, considered as a crucial model for evaluating the efficacy of various compounds due to its susceptibility to antimicrobial agents. *E. hirae* was selected in our experiment due to its intriguing characteristics and potential significance in clinical scenarios. While *E. hirae* is relatively less common among human clinical samples, it has been recognized as a notable cause of infections, particularly in individuals with certain risk factors such as diabetes, liver cirrhosis, and chronic kidney disease. Notably, its association with urinary tract infections, biliary tract infections, and infective endocarditis highlights its clinical relevance. The rise of advanced diagnostic techniques like MALDI-TOF MS has led to increased reports of E. hirae infections, urging clinicians to consider it as a potential pathogen in urinary tract infections among patients with predisposing conditions [9].

Escherichia coli, a Gram-negative bacterium, is a common cause of urinary tract infections (UTIs) and enterocolitis. It is a multidrug-resistant bacteria that may cause various human disorders inside and outside the gastrointestinal system. These infections include gastrointestinal infections such as diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [10]. Extraintestinal complications caused by E.coli include UTIs and newborn meningitis [11]. Furthermore, some *E. coli* strains can cause pneumonia, peritonitis, mastitis, and septicemia [12]. *E. coli accounts* for 17.3% of clinical infections requiring hospitalization [13].

Pseudomonas aeruginosa are Gram-negative bacteria that can cause several infections in both community and clinical settings, particularly among critically ill patients. These infections include infections of the respiratory tract, bloodstream, wounds, and burns, as well as those of the urinary tract. Additionally, *P. aeruginosa* can form biofilms to protect itself from antibiotics and the host immune system. Antibiotic resistance is its prominent characteristic, and it can exhibit multidrug, extensive drug, and pan drug resistance [14, 15].

Streptococcus mutans is a facultatively anaerobic, Gram-positive, opportunistic pathogen. It coexists with humans in a carriage state; however, it causes disease under appropriate conditions. *S. mutans* was specifically targeted in our study due to its major role in initiating dental caries [16]. Additionally, *S. mutans* in the oral cavity has been linked to systemic diseases such as cardiovascular disorders, stroke, preterm birth, diabetes, and pneumonia [17].

Overall, these organisms were selected based on their relevance to different medical and dental conditions, and the potential of AA-oil to inhibit their growth and activity was investigated. We used Ciprofloxacin, the conventional antimicrobial, as a standard for comparing the MIC values. This study evaluated the antibacterial activity of AA against selected microorganisms using MIC testing, thus contributing to the discovery of novel alternative therapies in the face of rising antibiotic resistance.

MATERIAL AND METHODS:

***Preparation of Arq e Ajeeb*:** The ingredients were purchased from Sana herbals, Kottigepalya, Bangalore, and were authenticated from FRLHT, Bangalore (voucher specimen no.: 5563-Kafoor (*Cinnamomum camphora*), 5564-Satte Ajwain (*Trachyspermum ammi*), 5565-Satte pudina (*Mentha piperita*). Furthermore, AA was prepared by crushing crystals of each ingredient separately, mixing in an airtight container and allowed to liquefy. Later, it was filtered, and homogenous transparent liquid was obtained, which was stored in moisture-free bottles [2].

Preparation and Standardization of Microbial Stock Cultures and Reagents:

Preparation and Standardization of Stock Cultures: A loopful of *P. aeruginosa, E. hirae, E. coli, S. mutans* cultures were added to 10mL of sterile saline each, and the total number of cells was adjusted to 10^6 CFU/mL at 620nm using a digital colorimeter.

Preparation of Resazurin and Standard Antibiotic Solution: The stock resazurin solution was prepared by dissolving 2.7 mg in 4mL of sterile saline. Further, a working solution was prepared by dissolving 1ml of stock solution in 5ml of sterile saline. The standard antibiotic, Ciprofloxacin, was prepared in sterile distilled water at 0.1% concentration.

Preparation of Test Sample: AA-oil was diluted to prepare different concentrations ranging from 100 to 0.048828 mg/ml.

Methodology: The research was conducted at Dept. of Ilmul Amraz (Pathology), Government Unani Medical College, Bangalore, Karnataka, India. Arq e Ajeeb (AA) was prepared by combining camphor, menthol, and thymol in a ratio of 2:2:1. These ingredients were procured from Sana Herbals, Kottigepalya, Bangalore, and authenticated by the Foundation for Revitalization of Local Health Traditions (FRLHT), Bangalore. The microbial strains (*Enterococcus hirae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus mutans*) were obtained as stock cultures and standardized to a concentration of 10^6 colony-forming units per milliliter (CFU/mL) using a digital colorimeter at 620nm.

The antibacterial activity of AA was assessed using the minimum inhibitory concentration (MIC) method. Double dilutions of AA were prepared, ranging from 100 to 0.048828 mg/ml.

The MIC method is a widely recognized and standardized approach for evaluating antimicrobial efficacy. The selected bacterial strains (*E. hirae, E. coli, P. aeruginosa, S. mutans*) were selected due to their clinical relevance and association with various infections. Ciprofloxacin, a conventional antibiotic, served as a standard for assessing the antibacterial activity of AA. The use of resazurin dye provided a reliable indicator of microbial growth inhibition.

Determination of Antimicrobial Efficacy:

Minimum Inhibitory Concentration (MIC) by Microculture Method: The minimum inhibitory concentration (MIC) of Arqe Ajeeb was determined using the broth microdilution method as described by Balouiri M *et al.* Bacteria were cultured in Mueller Hinton Broth (MHB) while fungi were cultured in RPMI-1640 medium.

The experiment was repeated for positive control comprising 0.1% Ciprofloxacin (Standard antibiotic). The growth control included bacterial suspension and 100µl broth medium, while broth control had only the broth medium. The plates were incubated at 32.5 ± 2.5°C for 24 to 48 h. Further, resazurin dye was added, and incubated again for 1 h. The color change was noted. Any color change from purple to pink or colorless was recorded as positive (growth). The MIC value was determined as the lowest concentration, which showed no color change

RESULTS AND DISCUSSION: AA showed antibacterial activity at all concentrations from 100 to 0.048828mg against *S. mutans, E. coli, and E. hirae*, whereas *P. aeruginosa* showed antibacterial

activity from 100 to 1.562mg **Table 1**. These findings demonstrate that AA has antibacterial activity against *S. mutans, E. coli and E. hirae and P. aeruginosa*.

TABLE 1: MINIMUM INHIBITORY CONCENTRATION OF ARQE AJEEB

Sample name	Sample code	Concentration tested	MIC (mg/ml)			
			S. mutans	*P. aeruginosa*	*E. coli*	*E. hirae*
AA oil	RR200319	100 to 0.048828 mg	0.0488	1.562	0.0488	0.0488
Ciprofloxacin	Standard	0.1 0.000000012mg		0.000048mg		

AA, Arq e Ajeeb; MIC, minimum inhibitory control; mg, milligrams; mg/ml, milligrams per milliliter

Owing to an increasing risk of multi-drug resistance to pathogenic microbes, there is a need for the identification of novel antimicrobials that are organic and herbal in nature. Therefore, a popular Unani formulation AA, was initially obtained for antibacterial studies on two Gram-positive (*E. hirae, S. mutans*) and two Gram-negative (*E. coli, P. aeruginosa*) organisms.

In the present study, the formulation of AA has shown significant inhibitory activity against four pathogenic organisms that cause various diseases. Microorganisms like *S. mutans, E. coli, E. hirae, and P. aeruginosa* are known to cause different ailments in the human body. *E. coli* causes pneumonia, urinary tract infection, diarrhea, *E. hirae*-related pyelonephritis and cholangitis[18].*P. aeruginosa* mounts infection in weak host defense mechanisms. It is an opportunistic pathogen of humans. *P. aeruginosa* infects the dermis, soft tissue, bacteremia, bone and joint, gastrointestinal and blood, urinary tract, respiratory system, particularly in patients with severe burns, tuberculosis, cancer and AIDS. Moreover, it causes a significant problem in patients hospitalized with cancer, cystic fibrosis and burns, with 50% fatality rate [19] S. *mutans* are associated with pyogenic and other infections in various sites, including mouth, heart, joints, skin, muscle, and central nervous system. It can also cause dental plaque [20].

Our findings showed that *S. mutans, E. coli, E. hirae* were susceptible to AA similar to that for Ciprofloxacin, the standard antibiotic. However, *P. aeruginosa* was comparatively more susceptible, based on the MIC test.

MIC is an effective antimicrobial susceptibility test that is performed as per the most recognized standards provided by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Moreover, broth micro-or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. Interpretation of the microdilution method was performed by adding the resazurin dye and observing the color change. The active bacterial cells reduce the blue colored resazurin to pink-colored resorufin. The lowest concentration with no color change was noted as the MIC value.

AA is the combination of three essential oils: camphor, menthol, and thymol. Several studies have reported the properties of these ingredients. Wang et al. evaluated the prominent antibacterial activity and anti-QS activities of *C. camphora* essential oil and confirmed it as a potential antibacterial agent and Quorum sensing inhibitor. In another study, Taylor et al. has reported the antiviral effects of Menthol on Coxsackievirus B, a common human enterovirus. In another study, Ardestani *et al.* reported that the essential oil of *Trachyspermum ammi* fruit may inhibit vaginal pathogens growth.

The synergistic action of three essential oils may have been the reason for the potential antibacterial effects of AA, which validates it as a better alternative to conventional antibiotic drugs.

This study was limited to the evaluation of the in vitro antibacterial activity of AA against only four species, namely *Enterococcus hirae, Escherichia coli, Pseudomonas aeruginosa*, and *Streptococcus mutans*. The results of *in-vitro* investigations may not always predict the outcomes of in vivo experiments. Another limitation is that the study did not investigate the mechanism of action of AA against the four microorganisms. Moreover, the safety and toxicity of AA were not investigated in this study.

Regardless of these limitations, this research shows that AA has strong antibacterial action against a variety of microorganisms.

CONCLUSION: The new approach of using AA as an antibacterial agent may be considered safe for internal use with good antibacterial activity and minimal side effects and may be used for the benefit of mankind. However, to check whether the formulation AA has a broad spectrum antibacterial activity, further studies are necessary.

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