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The antibacterial effect of *Anethum graveolens* (dill) on *Bacteroides fragilis* isolated from clinical samples in Mosul city, Iraq

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Abstract

Bacteroides fragilis is a Gram-negative, non-motile, non-spore-forming, and obligate anaerobic bacterium commonly found in the human colon and other body sites. the *leuB* specific gene, known as β-isopropyl malate dehydrogenase, was utilized as a target for PCR amplification to detect *Bacteroides fragilis* spp. The *leuB* gene plays a crucial role involved in the leucine biosynthesis pathway and catalyzed the conversion of β-isopropyl malate to α-Ketoiocaproate. Sialidase, also known as neuraminidase, is a group of enzymes that cleave sialic acid, a carbohydrate found on the surface of mammalian and microorganism cells. The nanH gene encodes this enzyme. β-isopropyl malate dehydrogenase found with 100% sequence identity but lacked the sialidase enzyme gene (nanH). we investigated the antibacterial activity of *Anethum graveolens* on *B. fragilis* growth. The bacterium displayed high sensitivity to concentrations of 100 µg/ml, resulting in inhibition zones of 25mm and 24mm for the two isolates, respectively. Decreasing concentrations of 50 µg/ml (15mm, 19mm), 25 µg/ml (14mm, 14mm), and 12 µg/ml (4mm, 9mm) also exhibited sensitivity.

Keywords: *Bacteroides fragilis*, LeuB gene, nanH gene, *Anethum graveolens*

Introduction

First identified *Bacteroides fragilis* in the late 1800s, it is a type of gram-negative, obligate anaerobic bacteria that appears as coccobacilli in different sizes ranging from 1.5 to 6 in shape. It does not produce spores or move and thrives in anaerobic conditions. Although recent studies indicate that some strains can grow in environments with very low oxygen concentrations, as well as avoid exposure to UV radiation. (Kowal, 2017) [1].

B. fragilis is a relatively uncommon species, comprising just 4-13% of all Bacteroidetes species and less than 1% of all microorganisms. It typically resides within the mucus layer of the lower gastrointestinal tract of a wide range of mammalian species (Troy and Kasper, 2010; Kowal, 2017 [1]) [2]. It's believed that humans acquire *B. fragilis* during birth. During the first few months of life, the bacterial flora in the intestine undergoes significant changes, and by the age of one year, the microbiota transitions toward an adult microbiota profile (Ballini, 2023). [3]

Compared to other Bacteroides species, *B. fragilis* displays distinct characteristics. It's indole-negative, and catalase-positive, and does not ferment the sugars trehalose and arabinose. Furthermore, it exhibits unique traits such as 20% bile resistance, Esculin hydrolysis, and resistance to Vancomycin, Gentamicin, and Colistin, making it distinguishable from other anaerobic bacteria (Wexler, 2007 [4]; Mohan and Lehman, 2022)

Phenotypically, members of the *B. fragilis* group are very similar. However, *B. fragilis* is the most clinically relevant species, and its isolates often exhibit antibiotic resistance. Selective media, such as Bacteroides Bile Esculin agar (BBE), are commonly used when isolating individuals from the *B. fragilis* group (Narimani, 2016 [6] and Amiri, 2022) [7].

Bacteroides fragilis is the most common anaerobic bacterium found in endogenous infections, and multi-microbial infections, especially in the bowel cavity. It has been associated with a kill rate exceeding 19% (Yekani *et al.*, 2020) [8].

In spite of an opportunistic pathogen but can cause several infections. Typically, contamination occurs after perforation of the colon, which is the natural habitat of gut bacteria. This allows bacteria to access both the clean peritoneal cavity and the gastrointestinal tract. Facultative anaerobes like *Escherichia coli* colonize early and destroy vital tissue, lowering oxygen pressure, which then creates a suitable environment for anaerobic bacteria like *B. fragilis* to colonize (Wexler, 2007 [4] and Dhyiaa, 2020). The presence of *B. fragilis* in the peritoneal cavity and its attachment to the peritoneal wall can lead to the formation of an abscess. If left untreated, the abscess can cause blockage and destruction, leading to an increase in the prevalence of *B. fragilis* in the bloodstream. (Wexler, 2007 [4]; Kowal, 2017).

B. fragilis is present in human feces and comprises 1-2% of the resident gut bacteria. Although it is normally harmless in the gut, *B. fragilis* can cause infections such as peritonitis, vaginal infection, lung, soft tissue infections, brain abscesses, and pelvic. In addition, infection with *B. fragilis* can result in diarrhea due to the release of toxins when it infects different anatomical sites (Salipante *et al.*, 2015 [10]; Yekani *et al.*,2020 and Amiri, 2022) [7].

In this study, the *leuB*-specific gene, known as β-isopropyl malate dehydrogenase, was utilized as a target for PCR amplification to detect Bacteroides fragilis spp. The *leuB* gene plays a crucial role involved in the leucine biosynthesis pathway and catalyzed the conversion of β-isopropyl malate to α-Ketoiocaproate. The complete nucleotide sequence of the gene *LeuB* coding for β-isopropyl malate dehydrogenase of Bacteroides fragilis determined, an open reading frame of 1,061 nucleotides was detected that could encode a polypeptide of 353 amino acid residues with a calculated molecular mass of 39,179 Da.

Anethum graveolens (dill)

A. graveolens is an annual aromatic herb commonly known as dill. The plant comes from the family Umbelliferae the origin in West Asia and Mediterranean countries. The name of the medical plant is derived from the Greek word another, but the common name comes from the Norse word dilla or Dylla it means soothe (Chahal *et al*., 2016) [11].

Dill (Shapt) or (dillweed), this plant has a long history of cultivation because used as a medicinal herb, and contain essential oil possesses several active components useful against antispasmodic, hypolipidemic activities anti-inflammatory, antifungal, antibacterial, antioxidant, insecticidal, antidiabetic, etc. taxonomic of *Anethum graveolens* below (Warrier *et al*.,1994) [12]:

Kingdom: Plantae, Division: Magnoliophyta,

Class: Magnoliopsida, Order: Apiales, Family: Apiaceae, Genus: Anethum, Species: Graveolens.

Material and methods

Isolation of *Bacteroides fragilis*

We were collected 150 clinical bacterial samples from females (Urine, high vaginal and colon biopsy) and males (urine, and biopsy) samples all of them previously diagnosed by a physician, the age of females between (18-50 years), and others (20-70years) all outpatients came to AL-salam General Teaching Hospital, Al-Khansaa Hospital, Ibn Sena General Hospital and Albatool teaching hospital in Mosul city -IRAQ, from 1/October /2022 to 1/ March/2023.

Bacterial culture and initial diagnosis

Two swabs and biopsies are getting of each sample, incubation samples on anaerobic media, (BHIB), tryptose soy broth in anaerobic condition by using an anaerobic generator (gas bag) and use *Pseudomonas* spp. as indicators for anaerobic and incubation in anaerobic jar for 2 days 37ºC. so the suspected colonies identified morphologically and biochemically and then are cultured on bile esculin agar, and MacConkey agar for detection of the association between *B. fragilis* and facultative anaerobic bacteria.

Molecular detection of *Bacteroides fragilis* by using LeuB gene

Whole genome DNA extracted from suspected isolates of *B. fragilis* under study using the DNA extraction kit supplied by (Geneaid, USA). DNA was extracted according to the steps recommended by the company (8). The PCR assay was used to amplify the 440 bp fragment of the *LeuB* gene utilizing the primers F and R and the condition were: initial denaturation at 94 C for 5 minutes followed by 40 amplification cycles that included a denaturation step at 94 C for 45 second and annealing at 58 C for 45 second and extension at 72C for 45 second. Final extension step was set at 72 C for 10 minute. PCR products were analyzed on 1% agarose gel, visualized using a transilluminator then the bands were purified by GFX PCR DNA and Gel band Purification Kit and sent for sequencing at the Psomagene sequencing company (USA).

Molecular detection of sialidase enzyme by using nan H gene

PCR assay was used to amplify the 528 bp fragment of the *LeuB* gene utilizing the primers F and R and the condition were: initial denaturation at 95 C for 5 minutes followed by 40 amplification cycles that included a denaturation step at 95 C for 30 second and annealing at 60 C for 30 second and extension at 72C for 30 seconds. final extension step was set at 72 C for 1 minute. PCR products were analyzed on 1% agarose gel, visualized using a transilluminator then the bands were purified by GFX PCR DNA.

Plant material collection and extract preparation of *Anethum graveolens* (AGME):

A. graveolens plants were obtained from the local market (Erbil, Iraq) and washed in running tap water, and shade-dried. 10 g of *A. graveolens* dried and put in one hundred ml of ethanol for 24 hr., then filtered by using the Whatman filter paper (No.1), after that, centrifuged at 3000 rpm /15 min to get filtrated extracts, then were stored at −20◦C for further experiments (Salini and Pandian, 2015) [13].

Antibacterial activity of Anethum graveolens (AGME) extracts.

A 24-hr old culture of bacterial isolates was transferred into 2 ml of normal saline to make a suspension for each bacterial isolate (1.5×10^8 CFU/ml), then bile esculin agar plates were cultured by the bacterial isolate suspension, left at room temperature for 15 minutes until dry.

Wells were made into bile esculin agar plate, then extracts were diluted to four concentrations (100,50,25,12.5 µg/ml), with 50 µL of each concentration added to wells. The negative control was sterilized distilled water. Plates incubated at 37 for 24 hr.

Result and discussion

Sample collection and identification

Among 150 samples from high vaginal swab, urine, biopsy from colonoscopy infection. Upon analysis, indicated that 74 (49.3%) out of the 150 samples exhibited positive growth of anaerobic bacteria under anaerobic conditions. and the result of used specific *LeuB* gene identification of *B. fragilis* result of two isolates (2.7%) as shown in fig1-1.

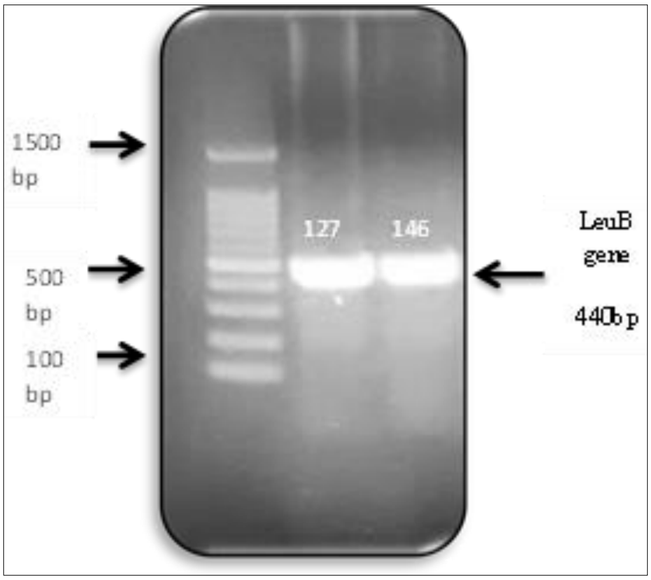

Fig 1-1: amplicon of *LeuB* gene at 440bp

The identification of *B. fragilis* using the *LeuB* gene yielded two isolates with a fragment size of 440bp. This finding is consistent with a previous study conducted in Lebanon, where the prevalence rate was reported to be only one isolate (1.96%) (Yehya *et al*., 2013) [14]. Other studies have reported varying rates of isolation, with some obtaining 10% (Al-gbory, 2018) [15] and others obtaining 19% (Dhyiaa, 2020) [9]. However, it is important to note that the results obtained by Ignacio *et al*. (2015) [6] differed, as they identified 9.5% of isolates containing this gene using the 16S-23S rRNA region for bacterial identification.

Detection of Sialidase enzyme (*nanH* gene)

Sialidase, also known as neuraminidase, is a group of enzymes that cleave sialic acid, a carbohydrate found on the surface of mammalian and microorganism cells. The nanH gene encodes this enzyme. The PCR amplification of the Sialidase enzyme, resulting in a 528bp fragment, is depicted in Figure 1-2

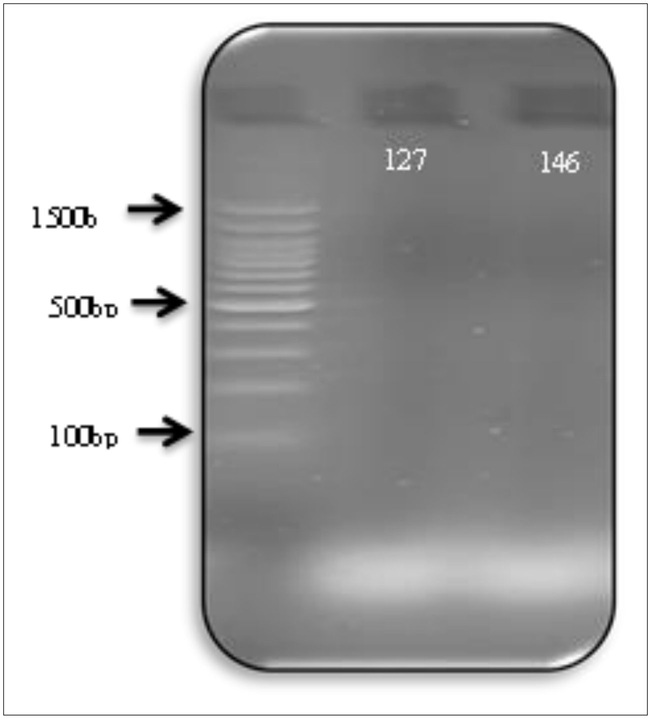

Fig 1-2: Amplicon of *nanH* gene at 528bp.

The PCR amplification results indicate the absence of the sialidase enzyme in the two isolate samples obtained from the colon. In the colon, there exists a thick mucus bilayer that acts as a barrier, separating the epithelial surface from the diverse content of commensal and potentially pathogenic bacteria. The inner layer of the mucus is composed of a combination of at least seven different epithelial cell surface mucins, along with antimicrobial proteins and other secreted substances. This inner layer forms a tightly connected structure, effectively preventing the entry of the majority of microorganisms into the underlying epithelial cells. (Lewis and Lewis, 2012) [17]. Bacteria that possess the sialidase enzyme have the ability to hydrolyze sialic acid when interacting with the gastrointestinal, respiratory, oral, and reproductive tracts. The presence of sialidase allows bacteria to control their survival in the mucosal environment through various mechanisms, as outlined by Lewis in 2012 [17].

The results obtained are in disagreement with the findings reported by Salman *et al*, (2017) [18] and Liu (2011) [19]. These studies suggested that the presence of the sialidase enzyme could be used to identify and detect the presence of *B. fragilis*. However, in our study, the gene encoding the sialidase enzyme was not detected. Instead, we used other genes to detect the presence of *B. fragilis*.

The absence of the sialidase enzyme indicates that *B. fragilis* present in the patient sample does not play a pathogenic role. This finding aligns with the study conducted by Lewis in 2012 [17], which investigated the presence of sialidase and its role in biofilm formation. In our research on biofilm formation, we observed that the two isolates did not form biofilms, further supporting the absence of pathogenicity.

Antibacterial activity of *Anethum graveolens*. Dill (AGME) extracts.

The findings indicated a noticeable inhibitory impact on the growth of *Bacteroides fragilis*, a bacterial genus when exposed to various concentrations of the ethanol extract derived from *Anethum graveolens*. Among the concentrations tested, the strongest inhibitory effect was observed at a concentration of 100 µg/ml (25mm, 24mm), followed by 50 µg/ml (15mm, 19mm), and then 25 µg/ml (14mm, 14mm). However, the concentration of 12.5 µg/ml (4mm, 9mm) consecutively exhibited minimal significant effects. The results are summarized in Fig 1-3 below.

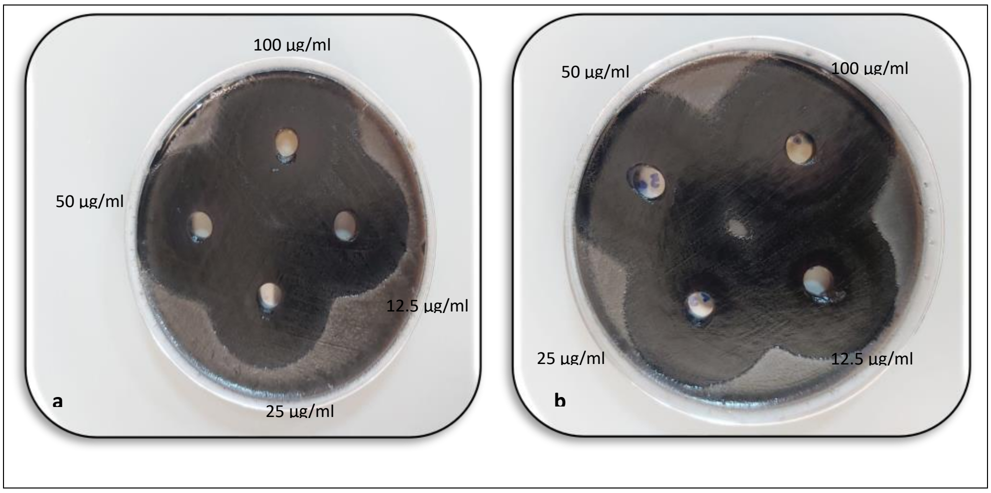

Fig 1-3: showed inhibition growth by Anethum graveolens in a-isolate no.127 and b- isolate no.146 on bile Esculin agar.

This result is due to the properties of medical active compound plants which effect on influence biofilm formation by inhibiting peptidoglycan synthesis, damaging microbial membrane structures, and modulating quorum sensing.

Our findings align with the study conducted by Kaur and Arora (2009) [20], which also demonstrated significant antibacterial activity. The essential oil and extracts derived from the dill plant exhibit promising properties, including antibacterial, antifungal, antioxidant, insecticidal, anti-inflammatory, antidiabetic, antispasmodic, and hypolipidemic activities because have the activities compound carvone, limonene, dill apiol and α-phellandrene (Shahein *et al.*, 2015) [21]. GC-MS analysis of the dill extract in the other study revealed that Eugenol is a major active compound in dill extract (49.62 %) which affect quorum sensing inhibition furthermore the presence another effective component (Makhfian *et al*.,2015) [22].

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