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IN VITRO ANTIMICROBIAL ACTIVITY SCREENING OF *HELIOTROPIUM EUROPAEUM* AGAINST WIDE RANGE OF MICROORGANISMS AND MULTI DRUG RESISTANT (MDR) BACTERIA

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ABSTRACT
Since ancient times several diseases have been treated with natural products, such as plants. *Heliotropium europaeum* L., commonly known as Akrepotu, is an important taxa of the Boraginaceae family. There are antimicrobial researches about *H. europaeum* in the literature and results are promising. Therefore this medical plant investigated against 41 microorganisms by using disk diffusion method according to show its antimicrobial potential more clearly. A wide range of Gram positive and Gram negative bacteria and yeast were selected to test the antimicrobial effect of *H. europaeum*. Most of these strains are standard and MDR, some of the strains are clinic isolated and the rest are food isolated. These microbial species are *Bacillus subtilis* DSMZ 1971, *Candida albicans* DSMZ 1386, *Enterobacter aerogenes* ATCC 13048, *Enterococcus faecalis* ATCC 29212, *Esherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSM 5071, *Pseudomonas fluorescens* P1, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* DSMZ, *E. coli* (MDR), *K. pneumonia* (MDR), *A.baumannii* (MDR), *E. aerogenes* (MDR), *S. odorifera* (MDR), *P. vulgaris* (MDR), *S. pneumonia* (MDR), *S. aureus* MRSA, *S. aureus* MRSA+MDR, *P. rustigianii* (MDR), *A. species* (MDR), *Staphylococcus hominis* (CI), *Staphylococcus haemolyticus* (CI), *Staphylococcus lugdunensis* (CI), *Shigella boydi* (CI), *Acinetobacter boumanii* (CI), *Shigella flexneri* (CI), *Staphylococcus aureus* (CI), *Enterococcus faecalis* (CI), *Klebsiella pneumoniae* (CI), *Candida tropicalis* (CI), *Enterococcus durans* (FI), *Enterococcus faecium* (FI), *Klebsiella pneumoniae* (FI), *Listeria innocua* (FI), *Salmonella infantis* (FI), *Salmonella kentucky* (FI), *Esherichia coli* (FI), *Staphylococcus aureus* (FI). The results were presented that *H. europaeum* ethanol extract has antimicrobial activity against some tested microbial strains.

KEYWORDS: *Heliotropium europaeum, antimicrobial activity, MDR, disc diffusion method, ethanol extract.*

I. INTRODUCTION
It is known that many plants in the world have been used for medicinal purposes since antique ages. The first information on medicinal plants and their uses come from the history of China, Egypt and Greece. It is known that some drugs were produced and exported in Anatolia during the Hittite period.[1]

The plants that have been used primarily for therapeutic purposes together with the existence of humanity have been the foundation of many of today's synthetic drugs. However, in the last 25 years of the 20th century there was a return to herbal remedies due to the considerable side effects of synthetic drugs.[2]

Heliotropium is one of the important genera of the Boraginaceae family. Boraginaceae has hundred genera and eighteen hundreds species which are distributed through temperate regions but more abundantly in the Mediterranean region.[3]

The genus *Heliotropium* is represented by 18 taxa in the Turkish flora and four of which are endemic. *H. europaeum* L. is an annual herb distributed in European Turkey and North, South, East and Inner Anatolia.[4]

World Health Organization (WHO) has predicted increasing antimicrobial resistance as a major threat for the public health for the 21st century. In order to prevent spreading of antibiotic resistant infections, scientists have been conducting intensive researches to determine new antimicrobial agents. One way to prevent antibiotic resistance of microorganisms is by using new compounds that are not based on existing antimicrobial agents.[5-6]

In recent years, antimicrobial activity related experiment have been applied by using plant extracts.[7-13] Although the antimicrobial activity of many natural plant species were determined until today, the broad range antimicrobial activity of *H. europaeum* ethanol extract haven't been analysed by disk diffusion method yet.

The purpose of this research was to detect the antimicrobial activity of *H. europaeum* ethanol extract against 41 microorganisms by disk diffusion method.

II. MATERIALS AND METHODS

Plant sample

H. europaeum L., commonly known as Akrepotu, is an important taxa of the Boraginaceae family.[4] Flowers were used to give relief from constipation and piles. Powder of leaves was used to treat skin problems.[14] The juice of the crushed *H. europaeum* was used topically to treat dermatophytosis of hair, nails and skin in domestic animals, while boiled leaves were applied on skin to treat pimples and eruption.[15-17] *H. europaeum* were collected from Canakkale/Turkey, and identified by Dr. Mustafa Eray Bozyel.

Extraction procedure

All *H. europaeum* samples were dried after collection and the samples were ground by a mortar and a pestle. In order to extract active substances, ethanol (Sigma-Aldrich) was chosen as an extraction solvent. Ground samples were shaken in ethanol at 140 rpm for 2 days at room temperature.[11, 18, 19] All the extracts were filtered through Whatman No. 1 filter paper into evaporation flasks. The filtrate was evaporated by a rotary evaporator (Heidolph Hei-Vap Value HL/HB-G1) at 45°C.[10] After evaporation the residues were collected and used to prepare 6, 12 and 24 mg extracts.

Microorganisms

A wide range of Gram positive and Gram negative bacteria and yeast were selected to test the antimicrobial effect of *H. europaeum*. The pathogenic microorganisms were chosen for the analyses on the basis of their significance because of potential for contamination of food and human infection. Most of these strains are standard and MDR, some of the strains are clinic isolated and the rest are food isolated. *Bacillus subtilis* DSMZ 1971, *Candida albicans* DSMZ 1386, *Enterobacter aerogenes* ATCC 13048, *Enterococcus faecalis* ATCC 29212, *Esherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSM 5071, *Pseudomonas fluorescens* P1, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* DSMZ, *E. coli* (MDR), *K. pneumonia* (MDR), *A.baumannii* (MDR), *E. aerogenes* (MDR), *S. odorifera* (MDR), *P. vulgaris* (MDR), *S. pneumonia* (MDR), *S. aureus* MRSA, *S. aureus* MRSA+MDR, *P. rustigianii* (MDR), *A. species* (MDR), *Staphylococcus hominis* (CI), *Staphylococcus haemolyticus* (CI), *Staphylococcus lugdunensis* (CI), *Shigella boydi* (CI), *Acinetobacter boumanii* (CI), *Shigella flexneri* (CI), *Staphylococcus aureus* (CI), *Enterococcus faecalis* (CI), *Klebsiella pneumoniae* (CI), *Candida tropicalis* (CI), *Enterococcus durans* (FI), *Enterococcus faecium* (FI), *Klebsiella pneumoniae* (FI), *Listeria innocua* (FI), *Salmonella infantis* (FI), *Salmonella kentucky* (FI), *Esherichia coli* (FI), *Staphylococcus aureus* (FI) were used in the study.

Preparation of inoculum

All bacterial strains were incubated at 37°C for 24 hours. But since the requirements for *C. albicans* and *C. tropicalis* are different, *C. albicans* and *C. tropicalis* were inoculated at 27°C for 48 hours. Inoculum were prepared by transferring morphologically similar colonies of each organism into 0.9% sterile saline solution until the visible turbidity was equal to 0.5 McFarland, thus standard inoculum is adjusted to contain approximately 10^8 cfu/mL for bacteria and 10^7 cfu/mL for *C. albicans* and *C. tropicalis*.[12, 20]

Disk diffusion method

Disk diffusion test was performed as described previously by Andrews.[21] The culture medium was poured into 120 mm sterile petri dish to give a mean depth of 4.0 mm ± 0.5 mm.[22] 30 µL, 60 µL and 120 µL aliquots of each extract was applied on sterile paper disks of 6 mm diameter end up with sample on each disk. To get rid of any residual solvent which might interfere with the results, disks were left to dry overnight at 30°C in sterile conditions. The surface of the plates was inoculated using previously prepared inoculum containing saline suspension of microorganisms. Inoculated plates were then left to dry for 5 min at room temperature before applying the disks.[23,24] Disks were firmly applied to the surface of the plate which had an even contact with the agar. Plates were incubated and inhibition zone diameters were expressed in millimetres.

Controls

Empty sterile disks and extraction solvent (ethanol) loaded on sterile disks which were dried at sterile conditions to remove solvent as done in the study were used as negative controls.

Statistics

The statistical analysis was executed using a non-parametric method Kruskal-Wallis which is one-way analysis of variance with $p < 0.05$.

III. RESULTS AND DISCUSSION

Antimicrobial activity of *H. europaeum* ethanol extract was analysed. In order to load extracts, empty sterile disks were used. These disks were applied on a Mueller Hinton Agar, after they were inoculated with microorganism. Inhibition zone was observed, when the extracts had activity against these microorganisms. The diameter of these zones were measured in millimetres as Table 1.

Table 1: Disk diffusion test results for *H. europaeum* (Inhibition zones in mm).

	30 µL	60 µL	120 µL
B. subtilis DSMZ 1971	7	7	8
C. albicans DSMZ 1386	-	-	-
E. aerogenes ATCC 13048	-	-	-
E. faecalis ATCC 29212	-	-	-
E. coli ATCC 25922	7	8	8
L. monocytogenes ATCC 7644	-	-	-
P. aeruginosa DSM 5071	7	7	8
P. fluorescens P1	-	8	8
S. enteritidis ATCC 13076	-	-	7
S. typhimurium SL 1344	-	-	-
S. aureus ATCC 25923	7	8	9
S. epidermidis DSMZ	7	8	8
E. coli (MDR)	-	-	-
K. pneumonia (MDR)	-	-	-
A.baumannii (MDR)	7	7	7
E. aerogenes (MDR)	7	7	7
S. odorifera (MDR)	7	7	8
P. vulgaris (MDR)	7	7	8
S. pneumonia (MDR)	7	7	7
S. aureus MRSA	8	8	9
S. aureus MRSA+MDR	7	8	8
P. rustigianii (MDR)	7	7	7
A. species (MDR)	7	7	7
S. hominis (CI)	-	-	7
S. haemolyticus (CI)	-	-	-
S. lugdunensis (CI)	-	-	-
S. boydi (CI)	-	-	-
A. boumanii (CI)	-	-	-
S. flexneri (CI)	-	-	-
S. aureus (CI)	7	7	7
E. faecalis (CI)	7	7	7
K. pneumoniae (CI)	-	-	8
C. tropicalis (CI)	-	-	-
E. durans (FI)	-	7	7
E. faecium (FI)	7	8	8
K. pneumoniae (FI)	7	7	7
L. innocua (FI)	-	7	7
S. infantis (FI)	7	7	7
S. kentucky (FI)	7	7	7
E. coli (FI)	7	7	7
S. aureus (FI)	7	8	8

"-": No inhibition, MDR: Multiple drug resistant, CI: Clinic isolated, FI: Food isolated

In our study, *H. europaeum* ethanol extract antimicrobial activity was determined against 41 microorganisms with disc diffusion method at 6 mg 12 mg and 24 mg. According to our result, *H. europaeum* has antimicrobial activity against *B. subtilis* DSMZ 1971 (8 mm), *E. coli* ATCC 25922 (8 mm), *P. aeruginosa* DSM 5071 (8 mm), *P. fluorescens* P1 (8 mm), *S. aureus* ATCC 25923 (9 mm), *S. epidermidis* DSMZ (8 mm), *S. odorifera* (MDR) (8 mm), *P. vulgaris* (MDR) (8 mm), *S. aureus* MRSA (9 mm), *S. aureus* MRSA+MDR (8 mm), *K. pneumoniae* (CI) (8 mm), *E. faecium* (FI) (8 mm) and *S. aureus* (FI) (8 mm) at 24 mg. However there is no activity determined against *C. albicans* DSMZ 1386, *E. aerogenes* ATCC 13048, *E. faecalis* ATCC 29212, *L.*

monocytogenes ATCC 7644, *E. coli* (MDR), *K. pneumonia* (MDR), *S. haemolyticus* (CI), *S. lugdunensis* (CI), *S. boydi* (CI), *A. boumanii* (CI), *S. flexneri* (CI) and *C. tropicalis* (CI).

When compare to gram negative and gram positive bacteria, gram negative bacteria have more resistance than gram positive bacteria against bioactive component.[25] Therefore, much greater activity was obtained against gram positive bacteria in related research. Our experiment has similar results and most of the activity was observed against gram positive bacteria.

Saeedi and Morteza-Semnani[26] found that essential oil of *H. europaeum* has antimicrobial activity against *B. subtilis* by disk diffusion method. However it showed weak activity against *E. coli* and *no activity against S. aureus and C. albicans*. The results are in parallel with our results except for *S. aureus*.

S. aureus is known one of the common nosocomial infections in medical intensive care units.[27] Several researchers study antimicrobial activity of some plant extracts on *S. aureus* strains. In our study, we observed 9 mm zone against standart and MRSA, 8 mm zone against MRSA+MDR and food isolated, and 7 mm zone against clinic isolated *S. aureus* strains. *H. europaeum* is active against *S. aureus* when compared to some other higher plants.[28]

A. baumannii has progressively been implicated in serious nosocomial infections, including bloodstream infection (BSI), nosocomial and ventilator-related pneumonia, and meningitis. These infections are particularly common in critically ill patients, with mortalities as high as 40-64% for pneumonia and 17-46% for BSI.[29] The extensive use of broad-spectrum antibiotic agents within hospitals has led to the rapid emergence of multi drug resistant (MDR) *A. baumannii* strains. Only a few antimicrobial agents are active against MDR *A. baumannii* infections.[29] In our study, we observed 7 mm zone against *A. baumannii* (MDR).

E. faecium has long been thought of as a harmless commensal of the mammalian GI tract. However, *E. faecium* has become an important cause of nosocomial infections. These infections are often difficult to treat owing to the resistance of *E. faecium* to a large number of antibiotics.[30] Mojab et al.[31] identified that methanol extract of *Thymus daenensis* caused 8 mm of inhibition zone against *E. faecium* whereas Ilhan et al.[32] identified that methanol extract of *Palustriella commutata* observed no activity. In our study, we observed 8 mm zone against *E. faecium* (FI).

IV. CONCLUSION

As a result, it can be concluded that there is clear antimicrobial activity of *H. europaeum* against 28 of the strains tested. The results of our study clearly presents that *H. europaeum* could have a possible medicinal uses specially against *E. coli*, *E. faecium*, *P. fluorescens*, *S. epidermidis*, and all strains of *S. aureus.*

But further researches are needed to be conducted in order to analyse the active substances and their activity mechanisms in details.

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