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Effectiveness of *Eucalyptus camaldulensis* Leaves Oil in Upregulating *exoU* expression in *Pseudomonas aeruginosa.*

Nihad T. M. Jaddoa[1], Mahmood A. H. AL-Sheikhly[2], Israa A.M. Aldobaissi[1],and Harith J. F. Al-Mathkhury[1]

[1]*Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq, 10071*
[2]*Laboratory Department, Directorate of Technical matters, Ministry of Health, Baghdad, Iraq*

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ABSTRACT

Results of the current study demonstratedthat out of eighty-three isolatesof *Pseudomonas aeruginosa*,only twenty-five isolateswere resistant to five different antibiotics (of different classes) that were consequentlyconsideredmultidrug resistant isolates.These isolates developed variable susceptibility toward *Eucalyptuscamaldulensis*leavesoil (ECO). GC-MS analysis of ECOrevealed that the aromatic oil eugenol is the major constituent.However, the most frequent MIC was 0.39 µg/ml, while the lowest frequent MIC was 3.125 µg/ml.Moreover, this oil at ½ MIC (0.195µg/ml) increased the gene expression of *exoU*. Itis concluded from the outcomes of the studythat ECOmay cause severe damagewhen used to treat infections caused by *P. aeruginosa*.

Keywords: *Eucalyptuscamaldulensis*, *Pseudomonas aeruginosa*, *exoU*, Leaves oil

فعالية زيت اوراق *Eucalyptus camaldulensis* في زيادة التعبير الجيني لجين *exoU* في بكتريا *Pseudomonas aeruginosa*

نهاد طه محمد جدوع[1] ،محمود عبد الرزاق الشيخلي[2]، اسراء عبد الرزاق الدبيسي[1]، حارث جبار المذخوري[1]

[1] قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

[2] وزارة الصحة، دائرة الامور الفنية، قسم المختبرات، مدير الشعبة الحكومية

الخلاصة

أظهرت نتائج الدراسة الحالية انه من مجموع ثلاثة وثمانون عزلة من بكتريا الزائفة الزنجارية، خمسة وعشرون عزلة فقط قاومت خمسة أنواع مختلفة من المضادات الحياتية والتي اعتبرت عزلات متعددة المقاومة للمضادات وقد تم اختبار هذه العزلات تحت تاثير زيت أوراق اليوكالبتوس(ECO)***Eucalyptuscamaldulensis***. أظهرت النتائج حساسيه متغيره تجاه الزيت المستعمل ، وكشفت تحليلات GC–MS لـEcoأن المكون الرئيسي للزيت العطري هو االيوجينول. واظهرت نتائج التركيز المثبط الادنى ان قيمة التركيز المثبط الادنى الأكثر تكرارا بلغت0.39 ميكروغرام / مل. بينما كانت قيمة التركيز المثبط الادنى الاقل تكرارا 3.125ميكروغرام / مل. علاوة على ذلك، فإن هذاالزيت عند التركيز تحت المثبط الادنى (0.195 ميكروغرام / مل)قد زاد من التعبير الجيني لجين *exoU* .يستنتج من خلال هذه الدراسة، ان زيت اليوكالبتوس قد يتسبب في اضرار شديدة عند استعماله لعلاج الاخماج الناجمة عن بكتريا *P. aeruginosa*.

*Email: israa.aldobaissi@sc.uobaghdad.edu.iq

1. Introduction

Pseudomonas aeruginosa, an aerobic, motile, gram-negative rod like bacterial isolate, is widely distributed in varioushabitats[1]. Moreover, since multidrug-resistant *P. aeruginosa* is also emphasised as a significant concern bythe World Health Organization due to it beingcarbapenem-resistant,priority of the highest level for the need to develop novel antibioticsalso requires great attention[2].

This opportunistic pathogen can cause numerous acute and chronic infections which can result in high mortality rates in a variety of hosts and organs due to its extensive repertoire of virulence factors and complex regulatory network of intra- and inter-cellular signals enabling it to adapt, flourish, and evade host defences. A key predictor of virulence has been discovered as the Type-III secretion systems (T3SS) which is responsible for injecting*ExoS, ExoT, ExoU,* and *ExoY*exotoxins inside the host cell. Nevertheless, ExoU is indicated as the most toxic as well as being the most important exotoxin that is related to epithelial injury in variousinfections. The release of ExoU leads to the fast destruction of the host cell cytoplasmic membrane [3].

Remarkably, there is a global apprehension that the extensive use of antibioticshas resulted in the establishment and dissemination of organisms with multiple antibiotic resistance that has further complicated the therapeutic strategies. Such worrisome discovery has expaneds the horizons for using medicinal plants as natural sources for new antimicrobial agents [4]. Many people all around the world have used plants for medicinal purposes to cure or prevent infections, although large number of these herbal medications have not been tested for their safety and efficiency. Additionally, the purity and potency of such herbal products are not controlled, therefore, most of inacceptable effects might be brought on by contamination or batch-to-batch variations. Meanwhile, the potential for several negative consequences may be increased by the potency of such herbal products [5].

Of interest, *E. camaldulensis*a well-known plantin Australia, belongs totheMyrtaceae family and is commonly known as river red gum[6].Many medicinal benefits have been implicated in*E. camaldulensis*leaves.The phytochemical investigationhas revealed the presence of severaldifferent secondary metabolites with antimicrobial activityin *E. camaldulensis*leaves oil [7].

Taking that into consideration, the present study aimed to inspect theimpact ofECO on the gene expression of *exoU* in *P. aeruginosa*.

2.Materials and Methods

1. Plant Description and Phytochemical Analysis

- Plant Description

The main macromorphological properties were described to simplify the identification of studied tree as proposed by Al- Masoudi & Al-Dobaissi.[8].

An amount of 100 g of dried *E. camaldulensis*leaves were crushed and then hydro distilledfor 4 hr. in Clevenger apparatus to extract ECO which was later stored in glass vials at 4-5°C until required [9, 10].

- Gas Chromatograph-Mass Spectrometry (GC-MS)Analysis

An AOC-20i auto-injector-equipped GC-MS Shimadzu QP 2010 plus was used to analyse the oil from *E. camaldulensis* leaves (Shimadzu, Japan). The GC temperature was set to be 80°C for the column, 280°C for the injection, helium as the carrier gas, split injection mode, and pressure flow control mode. The column flow was 1.46 ml/min with total flow being 11.8

mL/min, the linear velocity was 44.5 cm/sec, the purge flow was 3.0 mL/min, the split ratio was 5.0, and 100.0 kPa pressure. GCMS-QP 2010 plus program was used and the conditions were: Detector Gain of 0.70 kV, interface temperature of 280°C, and ion source temperature of 200 °C. Scan speed was increased from 50 m/z to 800 m/z [11]. By matching the spectra data with a known molecule that was stored in the NIST library, the sample components were identified [12] which were confirmed according to peak area, retention time and molecular formula.

2. Pseudomonas aeruginosa Isolates

A total of 83 isolates of *P. aeruginosa* were obtained from the Microbiology lab at the Department of Biology, College of Science,University of Baghdad. These isolates were originally isolated from different specimens including blood, wound swabs, sputum, and mid-stream urine. All these isolates were previously identifiedby amplifying16SrRNA.

3. Antimicrobial Susceptibility Test

The Kirby-Bauer method was performed to detect the antibiotic susceptibility of *P. aeruginosa* isolates towards five distinct therapeutically significant antibiotics: Ciprofloxacin (5µg/disk), amikacin(30µg/disk), gentamicin (10µg/disk), imipenem(10µg/disk) and ceftazidime(30µg/disk).Clinical and Laboratory Standards Institute guidelines (CLSI) were applied to categorize an isolate if it was sensitive, intermediate and resistant [13].

4. Assay for Minimal Inhibitory Concentration

CLSIapproved microtiter broth dilution method was used to determine the minimal inhibitory concentration (MIC) of ECO [13]. The MIC is the lowest ECO concentration at which there was no visiblegrowth was detected. The ECO was serially double-diluted in Mueller-Hinton broth from 12.5 to 0.024µg/ml. which were prepared in a 96-well plate (100 µl per well). Wells without ECO wereregarded as positive growth control. A final concentration of 5×10^5 CFU/ml was achieved by adding a diluted bacterial sample to each well which was verified by viable counts.Bacteria-freewellsserved as negative growth control. The plates were kept in the incubator for 24 hours at 37°C [14].

5. Gene Expression

The effects of ECO at ½MIC on the expression levels of *exoU* gene was assessed in four isolates with similar MIC (0.195µg/ml) using quantitative reverse transcription PCR (qRT-PCR). *P. aeruginosa* isolates were cultured in fresh tryptic soy broth with or without ECOat ½MIC for a duration of 24 hours at 37°C.The sample's RNA was extracted using the TRIzol™ Reagent.As directed by the manufacturer, qRT-PCR was carried out using the GoTaq 1-Step RT-qPCR System (Promega,USA). As previously mentioned, qRT-PCR was performed[15] using the following primers: *ExoURT*for (5′-AATTGCGCGAGCAAACCGTTG-3′) and *ExoURTrev*(5′-TTCTGTTGAGCAACACTGGTGAGC-3), which amplify *exoU*. Samples were normalized to the *fbp* transcript using primers PA5110for 5′-CCTACCTGTTGGTCTTCGACCCG-3′ and PA5110 revat 5′-GCTGATGTTGTCGTGGGTGAGG-3′. The reaction conditions for *P. aeruginosaexoU* amplification consisted of transcriptase activation at 37°C for 15 min, initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 20 sec, 56°C for 20 sec,and 72°C for 20 sec. With a temperature increase of 1°C every second, a melting curve was produced at temperatures ranging from 60-95°C. Expression levels were measured through relative quantification. In order to compare the treated groups to the calibrator, the cycle thresholds (Ct) and fold changes were examined [16]. Fold change less than two-fold was regarded asinsignificant [17,18].

3.Results and Discussion

1. Plant Description

Eucalyptus camaldulensis is a tall tree up to 20 meter high with deciduous bark. It is characterized by pendulous branches and the leaves are linear to lanceolate green to slightly glaucous. The flowers are white, 5-7 in umbels. The fruits are hemispherical, disk prominent, presented with 3-4 incurved valves.

2. E. camaldulensisLeaves Oil GC-MS Analysis

GC-MS analysis of ECOrevealed 12 peaks (Figure 1). The major constituent of plant oil was the aromatic oileugenolwhichembraced82.22% of total constituents.

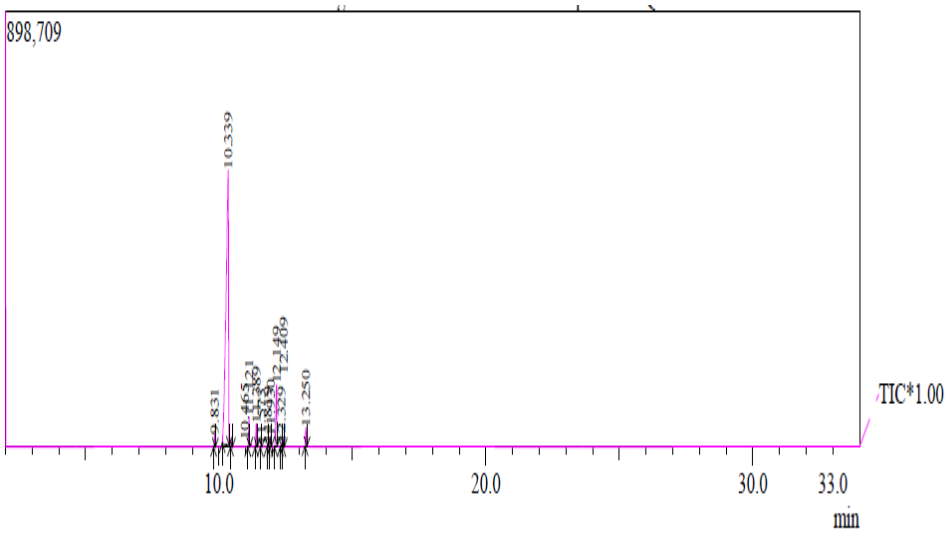

Figure1: Chromatogram of *Eucalyptus camaldulensis* leaves oil.

This finding is in line with what was documented byCaputo *et al*. [19] and Saxena, *et al*. [20]. Other phytochemical groups were sesquiterpene, which hadaromadendrene(7.745%) and spathulenol(1.67%), as well as other compounds from the same group such as copaene, zingiberene, laurenone, andcadinene with low percentage (Table 1).

Table1: *E. camaldulensis*leaves oil compounds detected by GC-MS.

Peak No.	Retention Time	Area %	Name	Formula
1	9.831	0.98	Santolinatriene	C10H16
2	10.339	82.22	Eugenol (Caryophyllic acid)	C10H12O2
3	10.465	0.52	Copaene	C15H24
4	11.121	2.64	Aromadendrene	C15H24
5	11.389	2.13	Aromadendrene	C15H24
6	11.573	0.10	Neopentylpyridine	C10H15N
7	11.819	0.20	3-methoxy-2-butanone	C6H12O2
8	11.930	0.91	Zingiberene	C15H24
9	12.149	7.74	Aromadendrene	C15H24
10	12.329	0.29	Laurenone	C15H24O2
11	12.409	0.59	Delta-Cadinene	C15H24
12	13.250	1.67	spathulenol	C15H24O

3. Susceptibility of Pseudomonas aeruginosa Isolatesto Antibiotics

The isolates of *P. aeruginosa* were examined for antibiotic susceptibility in the current investigation. Upon identifying isolates that are multi-drug resistant (MDR), the result revealed that out of eighty-three, only twenty-five isolates were resistant to all five classes of

antibiotics used, and that these isolatesalso reflected as MDR depending on breakpoints of CLSI 2023 [13].These isolates were chosen to test the effect of *Eucalyptus camaldulensis* leaves oil (ECO).

4. Estimation of Minimal Inhibitory Concentration of ECO

The MDRisolates(n= 25) developed variable susceptibility towards ECO. Figure 2 depicts that the most frequent MIC (0.39 µg/ml)was developed by the isolates P13, P19, P20, P21, P22, and P23 towards*P. aeruginosa*; while the lowest frequent MIC (3.125 µg/ml) was developed by the isolatesP1 and P7.

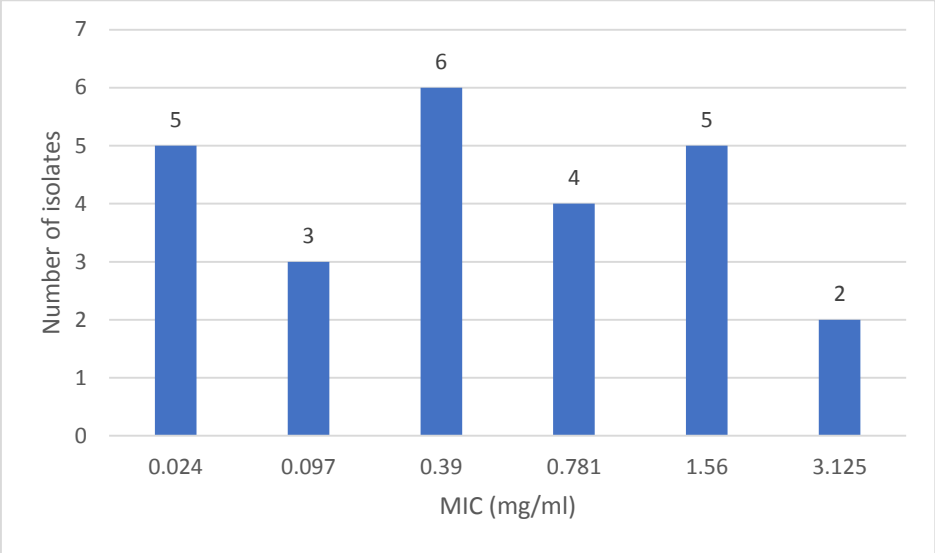

Figure 2: Frequency of *E. camaldulensis*leaves oil MIC towards MDR *P. aeruginosa* isolates.

Yang*et al.* [21] reported thatin addition to delaying the formation of biofilms, *Eucalyptus* oil had a lower MIC and a better bactericidal impact on *P. aeruginosa*. Ghalem and Mohamed[22], on the other hand,showed that *E. camaldulensis* oil prevented the growth of *Staphylococcus aureus* and *E.coli*.

Apart of the antibacterial activity of ECO, our results showed that this oil at ½ MIC (0.195µg/ml) increased the gene expression of *exoU* by 9.4, 2.6, and 26.7-fold in isolates P13, P18, and P19 respectively. Nonetheless *exoU* was downregulated in the isolate P20 (Figure 3).Given that *exoU* is known to upgrade the virulence of *P. aeruginosa* and increase its antibiotic resistance [23] andby evading immune regulation at critical signaling checkpoints within the neutrophil, it leads to heightened neutrophil recruitment, thereby exacerbating the process [24].Thus, when ECO upregulates the gene expression of *exoU*, it will inevitably result in undesired side effects.

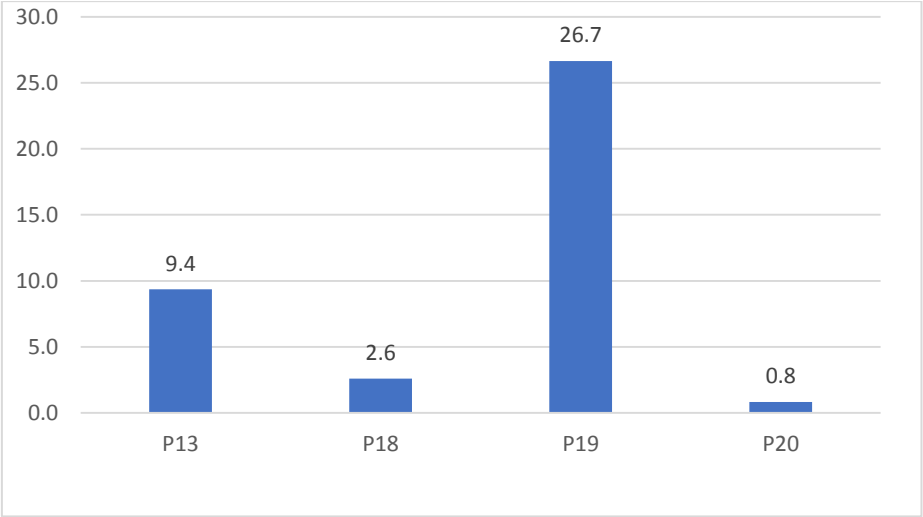

Figure 3: Fold change of *exoU* expression in *Pseudomonas aeruginosa* treated with *E. camaldulensis*leavesoil.

4. Conclusion

By virtue of the present findings that revealed an upregulation of *exoU* expression due to treatment with *E. camaldulensis* leaf oil, it can be concluded that this oilis unsafe to employ asantipseudomonal medication. However, much work is needed to elucidate the mechanism that leads to such effects.

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